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Antigenic Heterogeneity of Outer Membrane Proteins of Nontypable *Haemophilus influenzae* is a Basis for a Serotyping System

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A serotyping system for nontypable *Haemophilus influenzae* (NTHI) was developed by using isolated outer membrane protein (OMP) preparations and rabbit antisera. OMPs of 23 strains were isolated by molecular sieve chromatography of outer membranes in 1.5% sodium deoxycholate buffer. These OMP preparations were relatively free of lipopolysaccharide as determined by silver staining of sodium dodecyl sulfate gels and by dot assay with a monoclonal antibody which is specific for the lipid A of *H. influenzae*. Three antisera raised to whole organisms were used to serotype 21 of 23 strains with a kinetic enzyme-linked immunosorbent assay. Digestion of OMP preparations with proteinase K removed >90% of the antigenic reactivity, indicating that the system is based on OMP antigens. Marked antigenic heterogeneity of OMPs exists among strains of NTHI. By determining the pattern of serological reactivity of OMPs with the three antisera, isolates were divided into groups based on antigenic differences. Six serotypes were identified. This OMP serotyping system is based on multiple antigenic determinants. Future studies will focus on identifying serotype-specific epitopes to further refine this serological classification scheme for NTHI.

Nontypable *Haemophilus influenzae* (NTHI) is now well established as an important pathogen in adults and children. This bacterium causes pneumonia, bacteremia, meningitis, post-partum sepsis, and acute febrile tracheobronchitis in adults (4, 8, 23, 28, 33, 34). In addition, NTHI causes neonatal sepsis and is a frequent etiologic agent in acute otitis media in infants and children (15, 33). With the recognition of NTHI as a human pathogen, studies are currently under way to understand the pathogenesis and epidemiology of infection due to this bacterium. Serological classification schemes have greatly aided the understanding of the epidemiology and pathogenesis of infections due to other bacteria, including *Neisseria meningitidis*, streptococci, *Escherichia coli*, *Salmonella* spp., and others.

Studies from our laboratory and others have demonstrated differences in outer membrane protein (OMP) composition among strains of NTHI by sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis (3, 18, 21). We have previously defined eight OMP subtypes of NTHI based on the molecular weights of the two major OMPs (21). In the current study, we used rabbit antiserum raised against prototype strains of previously defined subtypes. The purpose of this study was to characterize the antigenic heterogeneity of OMPs of NTHI and to develop a serotyping system based on this antigenic heterogeneity.

MATERIALS AND METHODS

Bacteria. A total of 23 strains of NTHI were obtained from our own collection. Of these strains, 17 were recovered from clinical specimens (15 sputum isolates, 1 throat isolate, and 1 blood isolate) from adults at Erie County Medical Center and the Buffalo Veterans Administration Medical Center. The remaining six strains were isolated from infants or children at the Buffalo Children's Hospital.

The identity of strains of *H. influenzae* was confirmed by colonial morphology and growth requirement for hemin and NAD. Capsular serotypes were determined by counterimmunoelectrophoresis with reference strains and antiserum obtained from the Centers for Disease Control (21). Isolates were stored in Mueller-Hinton broth plus 10% glycerol at −70°C.

Isolation of OMPs. The method described by Rice and Kasper (24, 25) for isolating OMPs of *Neisseria gonorrhoeae* was modified to isolate OMPs of NTHI. The method is similar to that recently described by Gnehm et al. (10). Bacteria were grown on chocolate agar overnight at 37°C under 5% CO_2. The bacteria were scraped from the plates and suspended in EDTA buffer (0.05 M Na_2PO_4, 0.15 M NaCl, 0.01 M EDTA [pH 7.4]). This suspension was then incubated at 56°C for 30 min; bacteria release some of their lipopolysaccharide (LPS) under these conditions (17). Cells were then disrupted by sonication (Branson Sonic Power, Danbury, Conn.) on ice with four 15-s periods of sonication at 100 W. Unbroken cells and debris were removed by centrifugation at 10,000 × *g* for 20 min at 4°C. The supernatant was saved, and the resulting pellet was suspended in EDTA buffer and sonicated as described above. Unbroken cells and debris were again removed by centrifugation at 10,000 × *g* for 20 min at 4°C. The supernatants were pooled and centrifuged at 80,000 × *g* for 2 h at 4°C. The small, clear, gel-like pellets were suspended in distilled water and lyophilized. The resulting powder is referred to as outer membrane complex, which consists of OMPs and LPS (10, 24, 25).

OMPs were separated from LPS by chromatography over a Bio-Gel P-100 column. A 10-mg amount of outer membrane complex was added to 1.5 ml of NaD buffer (0.05 M glycine, 0.001 M EDTA, 1.5% sodium deoxycholate [pH 9.0]). After 5 drops of 1 N NaOH was added, the solution was agitated vigorously and centrifuged for 3 min in a Beckman microfuge to remove any undissolved particles. The solution was then applied to a Bio-Gel P-100 column (ca. 1.5 by ca. 85 cm) which was equilibrated with NaD buffer. Fractions of 1.5 ml were collected. OMPs elute from the column at the void volume, and LPS elutes after the void volume since LPS disaggregates in the presence of 1.5% deoxycholate. The

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fractions containing LPS were determined by dot assay with monoclonal antibodies to LPS. The major protein peak was identified by optical density at 280 nm (OD_{280}) measurements, and these OMP fractions were pooled and dialyzed for 48 h against water adjusted to pH 9.0 with 1 N NaOH to remove deoxycholate. To displace some of the deoxycholate bound to the protein, ethanol was then added to the OMP sample (10% [vol/vol]), and it was dialyzed for another 48 h against 0.025 M glycine plus 0.15 M NaCl [pH 9.0]. These OMP samples were stored at 4°C. The protein concentration was determined by measurements of the OD_{280} with bovine serum albumin to construct a standard curve.

SDS-PAGE. Outer membrane complex and purified OMPs of selected strains were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on modified Laemmli gels by using the Mighty Small vertical slab gel unit to characterize these outer membrane preparations (16). The separating gel contained 12% acrylamide, 0.32% bis-acrylamide, 1 M urea, 0.375 Tris hydrochloride, and 0.1% SDS. The stacking gel was similar except that it contained 6.0% acrylamide and 0.16% bis-acrylamide. Electrophoresis was performed at 100 V (constant voltage).

Gels were subjected to silver staining after being fixed overnight in 40% ethanol and 5% acetic acid. Glass dishes were washed with concentrated nitric acid before use, and the gel was subjected to three 10-min washes with distilled water between each step. All steps were performed with shaking. The gel was oxidized for 5 min in 0.7% periodic acid in 40% ethanol–5% acetic acid. After being washed, the gel was placed in a freshly prepared staining reagent for 10 min. The staining reagent contained 28 ml of 0.1 N NaOH, 2 ml of concentrated ammonium hydroxide, 5 ml of 20% silver nitrate, and 115 ml of distilled water. After being washed, the gel was then placed in a developing solution which contained 50 mg of citric acid and 0.5 ml of 37% formaldehyde in 1 liter of distilled water. When the gel was appropriately stained, it was placed in water. This method of silver staining results in staining of LPS and most protein.

Succinic dehydrogenase assay. To determine whether cytoplasmic membrane antigens were present in the outer membrane complex preparations, we performed succinic dehydrogenase assays. The method used was that described by Johnston and Gotschlich (14). For the purpose of this assay, outer membrane complex was prepared as described above, except that the cells were incubated in EDTA buffer for 30 min at room temperature rather than at 56°C.

Development of antisera. New Zealand white rabbits were immunized with the eight prototype strains of NTHI based on OMP subtypes (21). Each animal was immunized with a single strain. Bacteria were grown overnight on chocolate agar and suspended in sterile phosphate-buffered saline (PBS) to an OD_{660} of 0.2. Rabbits were given 0.5 ml intravenously on day 0 and 0.2 ml intravenously on day 28. They were bled on day 45, and the sera were stored at −20°C until use.

A mouse monoclonal antibody designated 3D2 was prepared to NTHI strain 3524 by the methods previously described (2). This antibody recognizes an epitope on the lipid A of NTHI.

Quantitative, single-tube K-ELISA. Kinetic-dependent enzyme-linked immunosorbent assays (K-ELISAs) (32) were performed by using ultra-UV disposable polystyrene cuvettes (Fisher Scientific Co., Pittsburgh, Pa.) which were washed with ethanol. One-ml volumes were used for all steps. Cuvettes were coated with purified OMPs dissolved in sensitization buffer (0.05 M Tris, 0.002 M EDTA, 0.3 M KCl [pH 8.0]) for 1 h at 37°C, then overnight at room temperature. The cuvettes were then washed three times with 0.3% Tween 20 in PBS (PBS-Tween) and rabbit antiserum (1:50 dilution in PBS-Tween) was added to each cuvette. The antiserum was incubated for 1 h at 37°C. After being washed, a 1:3,000 dilution of protein A-peroxidase conjugate (Zymed Laboratories) in PBS-Tween was added and incubated for 1 h at 37°C. Each cuvette was then washed and placed in a Varian 634 spectrophotometer with the analog output connected through an A-D converter to an Apple IIe computer which was programmed to calculate the rate of change per minute of absorbance at 460 nM. A 1-ml amount of substrate was added to the cuvette, the OD_{460} was measured for 3 min, and the change in OD_{460} per minute was calculated. The substrate was prepared by dissolving 10 mg of O-dianisidine · 2HCl (Sigma Chemical Co., St. Louis, Mo.) in 1 ml of methanol plus 1 ml of substrate buffer (0.05 M $NaHCO_3$ adjusted to pH 5.0 with glacial acetic acid). Next, 1.67 ml of this was added to 0.1 ml of 3% hydrogen peroxide and substrate buffer was added to a final volume of 100 ml.

Two controls were run with each set of K-ELISAs. One cuvette was "coated" with sensitization buffer only (no antigen), followed by antiserum, conjugate, and substrate as described above. In the other control cuvette, PBS-Tween was used in place of antiserum (no antibody), and the other steps were performed as described above.

Digestion of OMPs with proteinase K. To determine whether antibody in the rabbit antiserum was recognizing OMPs in K-ELISA, OMPs were digested with proteinase K (Boehringer GmbH, Mannheim, West Germany). This enzyme hydrolyzes proteins but has no effect on LPS (7, 13). OMPs were incubated with proteinase K for 30 min at 37°C at a concentration identical to the concentration of the OMPs in the sample. These samples were tested in K-ELISA.

RESULTS

Characterization of OMP samples. Outer membrane complex was prepared from 23 strains of NTHI, and OMPs were isolated by molecular sieve chromatography over a Bio-Gel P100 column in deoxycholate buffer. Figure 1 shows the results of one such procedure with NTHI strain 7891. A 10-mg amount of outer membrane complex of 7891 was run over a P100 column, and the OD_{280} was measured for each 1.5-ml fraction. Figure 1A shows a graph of the OD_{280} for each fraction. The large protein peak corresponds approximately to the void volume of the column. Figure 1B shows an immunodot assay in which a 10-μl dot of each fraction was placed on a nitrocellulose sheet. LPS was detected by using mouse monoclonal antibody 3D2, which recognizes an epitope on the lipid A portion of *H. influenzae* LPS. The immunodot assay demonstrates that the majority of the LPS was in fraction numbers 26 to 36, which are separate from the major protein peak which contains OMPs. LPS is present in the OMP peak as indicated by the presence of visible dots in fraction numbers 16 to 23. It should be noted that the outer membrane complex which was chromatographed on the column contained only approximately half of the LPS from the bacterial strain since it was incubated in EDTA during preparation of the outer membrane complex (17).

Figure 2 is a photograph of an SDS gel which was silver stained to detect protein and LPS. Lanes A, C, and E contain outer membrane complex of strains 1479, 4971, and 7891, respectively. Lanes B, D, and F contain the purified OMPs of the same strains, 1479, 4971, and 7891. LPS is represented by the prominent dark staining material at the bottom of the gel in lanes A, C, and E. This gel shows that

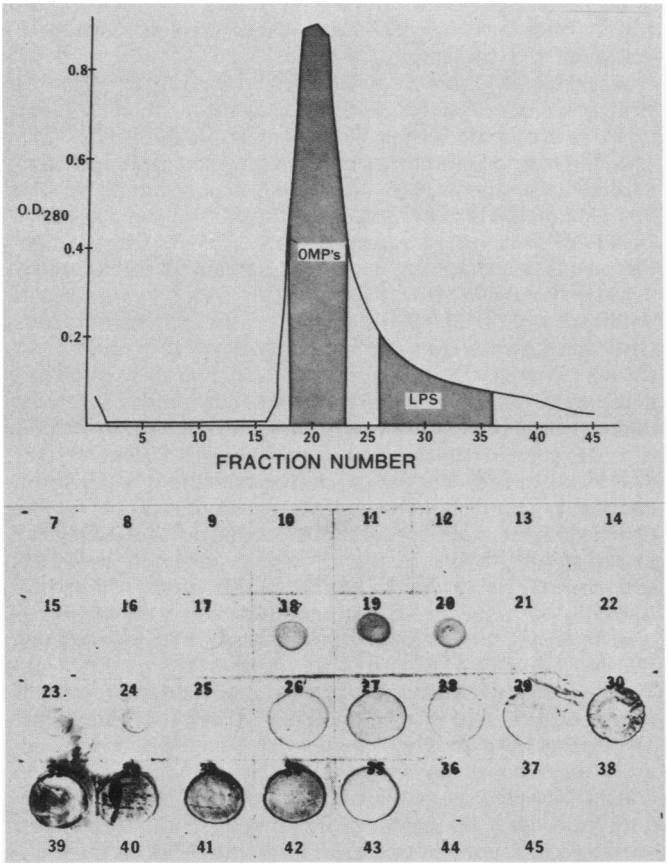

FIG. 1. Elution profile of outer membranes of NTHI 7891 on a Bio-Gel P100 column equilibrated with 0.05 M glycine–0.001 M EDTA–1.5% sodium deoxycholate [pH 9] buffer. (A) The OD_{280} (y axis) relative to each 1.5-ml fraction (x axis). (B) Dot assay in which 10 μl of each fraction was placed on a nitrocellulose sheet and LPS was detected with monoclonal antibody 3D2, protein A-peroxidase, and BioRad HRP color developer.

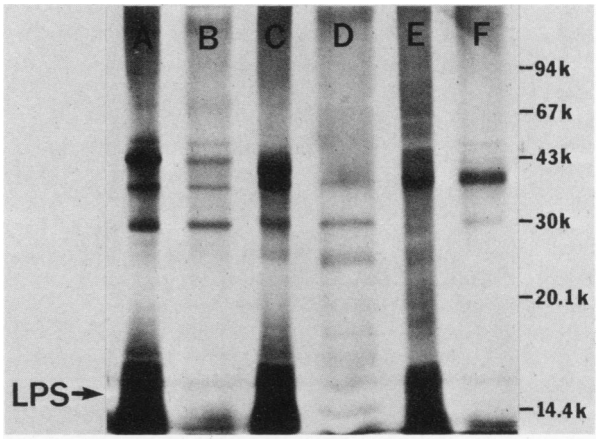

FIG. 2. SDS-PAGE of outer membrane complex and isolated OMPs of three strains of NTHI. Lanes: A, 1479 outer membrane complex; B, 1479 OMPs; C, 4971 outer membrane complex; D, 4971 OMPs; E, 7891 outer membrane complex; F, 7891 OMPs. The gel is silver stained and molecular weights are noted in thousands on the right. The dark staining material at the bottom of the gel in lanes A, C, and E is LPS.

LPS which was present in the outer membrane complex was almost entirely removed when OMPs were purified by chromatography over a P100 column in deoxycholate buffer.

Assays for succinic dehydrogenase were performed on one strain to determine whether cytoplasmic membrane antigens were present in the outer membrane complex preparations. For the purpose of the enzyme assays, outer membrane complex of 1479 was prepared without heating to avoid denaturing the enzyme. Assays were performed on a whole-cell sonicate and on three fractions obtained during preparation of the outer membrane complex. These included (i) the pellet after slow-speed centrifugation (containing largely unbroken cells), (ii) the supernatant after high-speed centrifugation of the outer membrane complex, and (iii) the outer membrane complex itself. The results were expressed in micromolar succinate converted per minute per milligram of protein. Protein concentrations were determined by the method of Lowry (19).

The succinic dehydrogenase activity in a whole-cell sonicate of 1479 was 0.021 μM/min per mg. The pellet from the slow-speed centrifugation had an activity of 0.040 μM/min per mg, and the supernatant from the high-speed centrifugation had an activity of 0.082 μM/min per mg. The outer membrane complex had no detectable succinic dehydrogenase activity. These data indicate that the cytoplasmic membrane was solubilized in EDTA buffer and that the outer membrane complexes were free of cytoplasmic membrane antigens.

K-ELISA. Rabbit antiserum was tested for the presence of antibody to the purified OMPs by using K-ELISA. The optimal dilutions of antiserum and optimal concentrations of OMPs to be used in K-ELISA were determined for each individual antiserum. Figure 3 shows the results of an experiment with purified OMPs of strain 1479 with a 1:50 dilution of antiserum raised to 1479 (upper curve). This graph demonstrates that at concentrations of 1479 OMPs

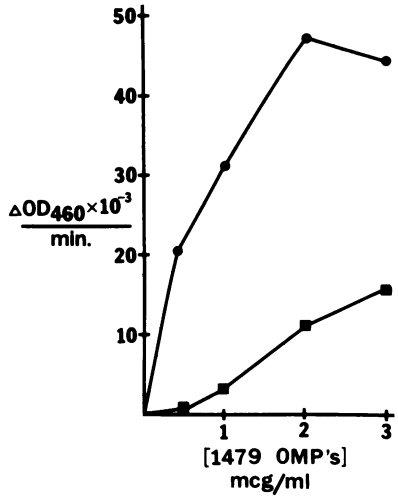

FIG. 3. Effect of OMP concentration on the ΔOD_{460} per minute as measured in K-ELISA by using antiserum 1479a at a 1:50 dilution. Each point represents the mean of duplicate assays. The upper curve (●) represents 1479 OMPs as the antigen coated on the cuvette. The relationship of ΔOD_{460} per minute to OMP concentration is approximately linear at concentrations below 2 μg/ml. The lower curve (■) represents proteinase K-digested 1479 OMPs as the antigen coated on the cuvette. Digestion of OMPs with this enzyme reduced the ΔOD_{460} per minute to less than 10% of the undigested protein at OMP concentrations of 1 μg/ml or less.

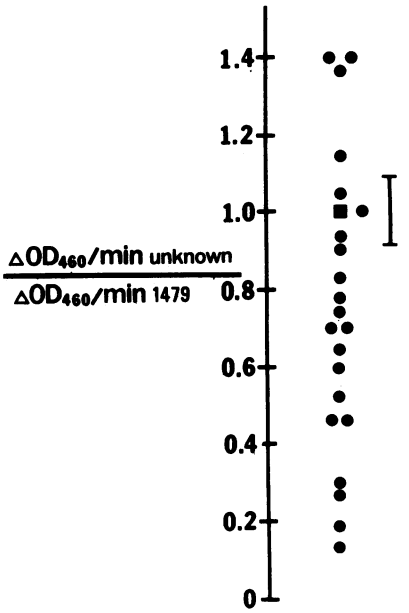

FIG. 4. Ratio of the ΔOD_{460} per minute of 23 strains with antiserum 1479a to the ΔOD_{460} per minute of the homologous 1479 OMPs. Each point (●) represents results of OMPs from a separate strain. Homologous OMPs, ■. The bar to the right of the points represents the mean plus or minus two standard deviations of 1479 OMPs assayed in quadruplicate.

below 2.0 μg/ml, the curve approximates a straight line. This indicates that at these concentrations, the OMP antigen is the limiting element in the assay. Conditions were adjusted in this way so that antibody was in excess and antigen was limiting; as a result, changes in the amount of antigen were reflected by a change in the ΔOD_{460} per minute. For example, at concentrations above 2.0 μg/ml, 1479 OMPs were not the limiting element, since a change in concentration (i.e., from 2.0 to 2.5 μg/ml) did not result in a change in ΔOD_{460} per minute (Fig. 3).

Figure 3 also demonstrates the results of testing 1479 OMPs which were digested with proteinase K (lower curve). Proteinase K digests proteins but leaves LPS intact (7, 13). Proteinase K reduced antigenicity of 1479 OMPs by more than 90% at concentrations of 0.5 and 1.0 μg/ml (Fig. 3). This observation indicates that at these concentrations, OMPs accounted for greater than 90% of the reactivity of 1479 antiserum to purified OMPs of 1479. Testing 1479 OMPs in K-ELISA against monoclonal antibody 3D2, which recognizes an epitope on lipid A of *H. influenzae* LPS, results in a curve which closely parallels the proteinase K curve of Fig. 3 (data not shown).

Based on the above experiments, 1479a antiserum was used at a dilution of 1:50, and OMPs were tested at a concentration of 1.0 μg/ml. As outlined above, under these conditions, the OMP antigen concentration was the limiting element and OMPs accounted for greater than 90% of the immunologic reactivity detected. Similar experiments were carried out for each antiserum to determine the optimal concentration of OMPs and the optimal dilution of antiserum. With all antisera, proteinase K digestion of OMPs reduced the curve to less than 10% of the undigested OMPs of the homologous strain. All antisera were used at a 1:50 dilution and with all except one, the optimal concentration of OMPs was 1.0 μg/ml. OMPs were used at a concentration of 1.5 μg/ml with antiserum 7502a.

Screening of OMPs by K-ELISA. Purified OMPs from 23 strains were tested by K-ELISA against rabbit antisera raised to prototype strains of the various OMP subtypes (21). The ΔOD_{460} per minute was determined for each and standardized by dividing by the ΔOD_{460} per minute of the homologous OMPs. For example, the ΔOD_{460} per minute of 1479 OMPs against 1479a antiserum was used to standardize the results for all strains tested against 1479a antiserum by dividing the result of OMPs of each strain by the result obtained with 1479 OMPs. In this way, the homologous OMP is defined as 1.0. OMPs which had half the reactivity of the homologous OMPs were said to be 0.5. OMPs which had more reactivity than homologous OMPs had a result greater than 1.0. Figure 4 shows the results of K-ELISAs of OMPs from 23 strains assayed at 1.0 μg/ml against 1479a antiserum. The Y axis represents the ΔOD_{460} per minute of each strain divided by the ΔOD_{460} per minute of OMPs of 1479. This figure demonstrates that marked antigenic heterogeneity exists among OMPs of the 23 strains detected by 1479a antiserum. The OMPs from 23 strains were assayed in K-ELISA with the rabbit antisera and results were standardized by using the ΔOD_{460} per minute of the homologous OMPs.

Development of serotyping system. Based on the results of testing OMPs from 23 strains, three antisera (1479a, 3198a, and 7502a) were chosen to be used in developing a serotyping system based on antigenic differences in OMPs. The OMPs showed a range of immunologic reactivity for these antisera. Figure 4 demonstrates that the 23 strains showed a spectrum of immunologic reactivity with 1479a antiserum.

To determine the reproducibility of the assay, several strains with various degrees of reactivity were run in quadruplicate or quintuplicate. The mean of the values was determined as well as the standard deviation. In the experiment depicted in Fig. 4, 1479 OMPs were run in quadruplicate. The mean and standard deviation were determined for the four values. The mean plus or minus two standard deviations yields a range of 0.91 to 1.09 on the graph in Fig. 4. This is indicated by the bar to the right of the points. Testing of other strains in quintuplicate gave very similar-sized ranges. These results indicated that the assay was highly reproducible.

For the purpose of developing a serotyping system, OMPs of strains with reactivity close to or greater than the homologous OMPs were defined as reactive (positive) and strains with less reactivity were defined as nonreactive (negative). A level of reactivity which was approximately three-fourths that of the homologous OMPs was chosen. In view of the spectrum of immunologic reactivity among OMPs of different strains, we used a semiquantitative method to determine the "cutoff point" to define whether a strain was positive or negative with a particular antiserum. The 23 strains were assayed in the same run, and the homologous OMPs were assayed in quintuplicate. The mean ($\bar{X}$ hom) and standard deviation (SD) of the five values for the homologous OMPs were determined. The cutoff point was defined as: ($\bar{X}$ hom − 2 SD) × 0.8. OMPs of strains which had ΔOD_{460} per minute greater than or equal to this value were defined as positive, and those with results less than this value were defined as negative. Under such a system, strains which were near the cutoff point varied from run to run with regard to testing as positive or negative. However, the majority of strains (greater than 80%) were consistently positive or negative when tested this way. Even when

TABLE 1. Serotypes of 21 strains of nontypable *H. influenzae* based on reactivity of OMPs with three antisera[a]

Strains with the following serotype and serologic pattern:					
I, +++	II, +−+	III, −++	IV, +−−	V, −+−	VI, −−+
C7961	C8057	5657	1479	7891	2019
4971		3122		3198	7502
C9607		1574			3524
6243		8130			4449
C9623					
C1093					
1831					
4213					
C8024					

[a] +++ represents reactivity with antisera 1479a, 3198a, and 7502a, respectively; − indicates nonreactivity.

assayed on different days and several months apart, strains which were not near the cutoff point were consistent and reproducible.

Three antisera (1479a, 3198a, and 7502a) were used to serotype 23 strains. Each strain was defined as positive or negative with each antiserum using this method. The strains were grouped according to their patterns of reactivity with the three antisera (Table 1). A total of 21 strains were distributed among six serotypes. Strains 1735 and 2030 were nonreactive to all three antisera by the criteria defined. However, 1735 was more reactive with 1479a than with 3198a and 7502a, so it resembled serotype IV. While 1735 OMPs did not meet the criteria for this serotype, OMPs of that strain clearly shared antigenic determinants with OMPs of 1479. Similarly, strain 2030 resembled the pattern of serotype II even though it did not meet the criteria for that serotype.

Table 2 lists the 23 strains by serotype in relation to OMP subtype (21), biotype, patient age, site of isolation, and disease state. Although the numbers were small, it is of interest that five of the six pediatric isolates were serotype I. Large numbers of strains will need to be tested to determine whether serotype is related to any of the characteristics listed in Table 2.

DISCUSSION

These studies indicate the feasibility of a serotyping system for NTHI based on OMPs. An ELISA system was used because it is a sensitive method for detecting differences in antigenicity and because it is a relatively simple method. A K-ELISA was chosen because it offers advantages over conventional endpoint ELISA (32). First, K-ELISA is based on enzyme rate kinetics and yields quantitative, linear data on antigen or antibody concentration. When antibody is present in excess, the results of K-ELISA are linear with regard to antigen concentration (Fig. 3). In endpoint ELISA, enzyme concentration is linear with respect to activity only during the brief initial phase of the reaction and then only when the enzyme concentration is lower than that of substrate (32). A second advantage of K-ELISA is that less reagents are necessary because multiple dilutions are not required and lower concentrations of antigens are used. Finally, in our hands, the K-ELISA shows better day-to-day reproducibility than endpoint ELISA.

One must consider the possibility that LPS is present in the OMP samples and contributing to some of the observed

TABLE 2. Characteristics of the 23 strains of NTHI

Strain	Serotype	OMP subtype[a]	Biotype	Patient age (yr)	Site of isolation	Diagnosis[b]
C7961	I	4	II	9	Sputum	Pneumonia
4971	I	5	II	56	Sputum	COPD in exacerbation
C9607	I	4	II	8 mo	Throat	Otitis media
6243	I	6	II	87	Sputum	COPD in exacerbation
C9623	I	4	II	8 mo	Middle ear	Otitis media
C1093	I	3	V	7	Throat	NA[c]
1831	I	ND[d]	ND	76	Blood	Pneumonia with bacteremia
4213	I	7	IV	72	Sputum	Pneumonia, COPD
C8024	I	2	III	7	Middle ear	Otitis media
C8057	II	1	I	Child	Adenoid	NA
5657	III	7	II	61	Sputum	COPD in exacerbation
3122	III	7	II	23	Sputum	No infection
1574	III	6	II	29	Throat	No infection
8130	III	1	V	67	Sputum	Stable COPD
1479	IV	1	NB[e]	66	Sputum	COPD in exacerbation
7891	V	6	NB	80	Sputum	Pneumonia
3198	V	8	III	67	Sputum	COPD in exacerbation
2019	VI	2	III	59	Sputum	No infection
7502	VI	3	II	67	Sputum	Pneumonia, COPD
3524	VI	2	ND	Adult	Sputum	COPD in exacerbation
4449	VI	3	ND	Adult	Sputum	NA
1735	NS[f]	5	V	65	Sputum	Pneumonia, COPD
2030	NS	8	III	65	Sputum	Stable COPD

[a] OMP subtype determined by SDS-PAGE as described in reference 21.
[b] Diagnoses determined by definitions described in reference 20. COPD, Chronic obstructive pulmonary disease.
[c] NA, Information not available.
[d] ND, Not done.
[e] NB, Not biotypable.
[f] NS, Not serotypable.

antigenic differences. Several lines of evidence indicate that the OMP preparations are relatively free of LPS. First, silver staining detects little or no LPS in the OMP samples in polyacrylamide gels. Since this method detects nanogram quantities of LPS (31), and in Fig. 2 there are about 10 μg of protein in each lane, protein is present in greater than 1,000 times the concentration of LPS in the OMP samples. Second, immunodot assay of OMP and LPS fractions with a monoclonal antibody 3D2, which has specificity for an epitope on the lipid A of *H. influenzae* LPS, indicates that the majority of the LPS has been removed from the OMP fraction by chromatography over a P100 column in the presence of deoxycholate (Fig. 1). Third, OMP samples were digested with proteinase K, an enzyme which hydrolyzes proteins but not LPS (7, 13). Digestion of OMPs with proteinase K removes greater than 90% of the reactivity detected in K-ELISA (Fig. 3). These observations indicate that the proposed serotyping system is indeed based on OMPs.

In our proposed serotyping system, the strains do not separate into discrete groups because the serotypes are based on multiple antigenic determinants. A similar observation was made by Sandstrom and Danielsson (27) in their gonococcal system based on multiple antigenic determinants. They graded positive reactions as 1+, 2+, or 3+ based on the degree of agglutination. Our system standardizes each assay relative to the homologous strain and uses a semiquantitative method for determining positive reactions among various strains. This minimizes the variability in classifying strains. Despite some variability in strains which are near the cutoff point of positive and negative, our studies demonstrate that definite antigenic heterogeneity exists among OMPs of NTHI and that it is possible to develop groups of strains based on these antigenic differences. The present studies can be viewed as a basis for the development of a more widely applicable serotyping system. In the system described here, the specificity is in the purity of the OMPs; the antiserum was raised to whole organisms. Monoclonal antibodies might be developed to serotype-specific antigens based on antigenic differences defined in this study. In this way, large numbers of strains can then be conveniently serotyped, and the overlap among strains will be reduced.

This system is based on antigenic differences in purified OMPs as a whole. It is possible that the differences detected in K-ELISA are due to quantitative differences among strains of a single immunodominant antigen. Future studies will address this possibility as well as the issue of which specific proteins are responsible for serotype specificity. In addition, future studies will address the issue of which serotype-specific OMPs are surface exposed and which ones are not. This is potentially important because intact organisms could be used in a serotyping system based on surface-exposed determinants, whereas a system which uses unexposed determinants would likely require disruption of the bacterial cells, for example, by boiling with SDS.

The observation that marked antigenic heterogeneity exists in the OMPs of isolates of NTHI is not surprising. Immunologic studies of isolates of *H. influenzae* type b have documented evidence of antigenic differences in somatic antigens (OMPs and LPS) (1, 5, 9, 11, 20, 29). Erwin and Kenny (9) studied 50 strains of *H. influenzae* type b with six rabbit sera by immunoblot assay. On the basis of antigenic variation in an OMP with a molecular weight of 49,000 to 51,000, they divided the isolates into 13 groups. Anderson et al. (1) studied the bactericidal activity of human sera which had been depleted of antibody to capsular polysaccharide and found that strains of *H. influenzae* type b differed in susceptibility to these sera; this suggests that antigenic differences exist in noncapsular antigens. Similarly, Stull et al. (29) showed that different isolates of *H. influenzae* type b varied widely in resistance to killing by human serum. Finally, animal studies have shown that antibodies to membrane antigens confer protection against infection with the same strain of *H. influenzae* type b but not necessarily other strains (5, 20).

Previous work from our laboratory and others has demonstrated differences in OMP composition among strains of NTHI by SDS-PAGE analysis (3, 18, 21). More recent work has provided evidence of antigenic differences among OMPs of NTHI. Musher et al. (22) showed varying bactericidal activity among different strains of NTHI and different normal human serum samples. Adsorption of normal human serum with NTHI removed bactericidal activity against the adsorbing isolate but not necessarily against others, suggesting antigenic diversity in surface structures. Hansen et al. (12) assayed serum samples from patients with NTHI infections for the presence of antibody to OMPs. They found marked differences among patient sera when studied with their homologous isolates, indicating antigenic diversity among OMPs of NTHI. The present study also demonstrates antigenic diversity among OMPs of NTHI; in addition, this study shows that this antigenic diversity can be used as a basis for a serotyping system for NTHI.

The serotyping system for NTHI proposed in this study is analogous to that developed for *Neisseria gonorrhoeae* by Sandstrom and Danielsson (6, 26, 27). They prepared selectively absorbed rabbit hyperimmune serum against gonococcal major outer membrane protein serotype strains. Three antigen classes, termed W, J, and M, were identified. Class W and J antigens were, in part, sensitive to proteolytic enzymes, and class M antigens were sensitive to periodate but resistant to proteolytic enzymes. Therefore, this system is based on multiple antigenic determinants, including OMPs and LPS. The classification scheme proposed for NTHI in the present study is similar in that both schemes are based on multiple antigenic determinants. The NTHI system is somewhat simpler than the gonococcal scheme since we used unabsorbed antisera and our system is based solely on OMPs. In addition, since a kinetic ELISA assay was used in the current study there is a semiquantitative basis for the determination of serotypes.

The scheme of Sandstrom and Danielsson which is based on cross-absorbed polyclonal rabbit antisera laid the groundwork for the development of a serological classification scheme for *N. gonorrhoeae* based on 16 monoclonal antibodies against the gonococcal principal OMP (30). This has been further refined to a system with monoclonal antibodies to well-characterized OMP antigens (E. Sandstrom, 1985, Serological classification as a tool for the study of the evolution of *Neisseria gonorrhoeae*, *In* G. K. Schoolnik [ed.], *Proceedings of the Aseloma Conference on Pathogenic Neisseria*, in press). In an analogous fashion, the serotyping system for NTHI described in this study might form the foundation for strain selection for a more refined classification scheme for NTHI in the future.

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