Antibodies Specific for the High-Molecular-Weight Adhesion Proteins of Nontypeable *Haemophilus influenzae* Are Opsonophagocytic for both Homologous and Heterologous Strains

Linda E. Winter and Stephen J. Barenkamp*

Department of Pediatrics, Saint Louis University School of Medicine, and the Pediatric Research Institute, Cardinal Glennon Children’s Hospital, Saint Louis, Missouri 63104

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The HMW1/HMW2-like adhesion proteins of nontypeable *Haemophilus influenzae* (NTHI) are expressed by 75% of NTHI strains. Antibodies directed against these proteins are opsonophagocytic in vitro and are protective in an animal model of infection. The objective of the present study was to determine the opsonophagocytic activity of high-titer anti-HMW1/HMW2 immune sera against both homologous and heterologous NTHI strains. Chinchillas were immunized with purified HMW1/HMW2-like proteins from five prototype NTHI strains. Serum opsonophagocytic activity was monitored in an assay that uses a human promyelocytic cell line, HL-60, as the source of phagocytic cells. Preimmune sera did not demonstrate opsonophagocytic killing of any strains. In contrast, the immune sera demonstrated killing of the five homologous NTHI strains at titers ranging from 1:320 to 1:640. The immune sera also demonstrated killing of eight heterologous NTHI strains that express HMW1/HMW2-like proteins at titers ranging from 0 to 1:640. Killing of heterologous strains sometimes demonstrated a prozone phenomenon. None of the immune sera killed NTHI strains that did not express HMW1/HMW2-like proteins. Adsorption of immune sera with HMW1/HMW2-like proteins purified from either homologous or heterologous NTHI strains eliminated opsonophagocytic killing of homologous strains in most cases. These data demonstrate that antibodies produced following immunization with the HMW1/HMW2-like proteins are opsonophagocytic for both homologous and heterologous NTHI and strongly suggest that common epitopes recognized by functionally active antibodies exist on the HMW1/HMW2-like proteins of unrelated NTHI strains. The results argue for the continued investigation of the HMW1/HMW2-like proteins as potential vaccine candidates for the prevention of NTHI disease.

Otitis media remains a significant health problem for children in the United States and elsewhere in the world (13, 14, 47). Most children in the United States have had at least one episode of otitis by their third birthday, and one-third of these children have had three or more episodes (47, 59). In addition to the short-term morbidity and costs of this illness, a subject of considerable concern is the potential for delay or disruption of normal speech and language development in children with persistent middle ear effusions (45, 46, 58). Otitis media experts have strongly recommended that efforts be made to develop safe and effective vaccines for the prevention of otitis media in young children (28, 36). Although total prevention of the disease will be a difficult goal to achieve, prevention of even a portion of cases would be beneficial given the magnitude and costs of the problem.

Bacteria, usually in pure culture, can be isolated from middle ear exudates in approximately two-thirds of cases of acute otitis media (20, 33, 61). *Streptococcus pneumoniae* is the most common bacterial pathogen recovered in all age groups, with isolation rates usually ranging from 35% to 40% (33, 48, 61). Nontypeable *Haemophilus influenzae* is the second most commonly recovered bacterium and accounts for 20% to 30% of cases of acute otitis media and a larger percentage of cases of chronic and recurrent disease (20, 38, 39). Interestingly, since the introduction of the pneumococcal conjugate vaccine as part of the regular childhood vaccine schedule, nontypeable *Haemophilus influenzae* has become an even more common cause of acute and recurrent middle ear disease, often surpassing *Streptococcus pneumoniae* in its frequency of recovery from middle ear specimens (3, 19).

Host immunity is thought to play an important role in the prevention of middle ear disease (26, 27, 53, 55). During middle ear infection, immunoglobulins (Igs), complement components, and phagocytic cells are all found within the middle ear space. In addition, serum and middle ear fluid antibodies directed against infecting organisms develop during the course of otitis media (15, 17, 26, 27, 54). In cases of nontypeable *Haemophilus influenzae* disease, the presence of these antibodies is associated with both decreased numbers of bacteria in the middle ear fluid (26) and more rapid resolution of infection (17, 53). These data suggest that it should be possible to impact the incidence or severity of nontypeable *Haemophilus* otitis media by vaccination of susceptible individuals (28). However, at present, it is unclear which bacterial components should be included in a nontypeable *Haemophilus influenzae* vaccine.

Many different *Haemophilus* antigens have been suggested as possible vaccine candidates (1, 2, 4, 5, 7, 12, 24, 29, 40, 42, 43, 62, 67). Nontypeable *Haemophilus influenzae* outer membrane proteins appear to be the principal targets of bactericidal and protective antibody (7, 29, 35), and as a group, they have...
been the major focus of vaccine development efforts. *Haemophilus* proteins P2 and P6 have each been demonstrated to be specific targets of human bactericidal antibodies (42, 43), and immunization of animals with the P6 protein has been demonstrated to modify the course of experimental disease (22). Immunization of animals with the F5-fimbria adhesion protein, another leading vaccine candidate, or with peptides derived from the protein has also been demonstrated to modify the course of experimental infection in chinchillas and rats (4, 5, 37). Other proteins demonstrating protection against nontypeable *Haemophilus* disease following immunization in various experimental systems include recombinant HtrA (40), transferrin receptor (62), OMP26 (24, 37), and lipoprotein D (1, 2, 49). It is notable that a recent clinical trial reported that immunization of children with a protein D-pneumococcal polysaccharide conjugate vaccine was partially protective against both pneumococcal and *Haemophilus* otitis media (49). *Haemophilus* lipo polysaccharide has also been investigated as a vaccine candidate, and antibodies generated against detoxified lipo polysaccharide conjugated to several different carrier proteins have demonstrated in vitro and in vivo functional activity against *Haemophilus influenzae* (32, 66, 67). However, despite many excellent preclinical studies, it remains unclear which of the many vaccine candidates still being investigated are most appropriate to include in a broadly protective human nontypeable *Haemophilus influenzae* vaccine (28).

In previously reported work, we identified a family of high-molecular-weight (HMW) proteins that are major targets of serum antibody in children who have recovered from *Haemophilus influenzae* otitis media (8). We cloned and sequenced the genes encoding two such immunogenic high-molecular-weight proteins from a prototype strain (9) and demonstrated that the proteins encoded by these genes were critical for the attachment of nontypeable *Haemophilus influenzae* to human epithelial cells in vitro (56). The prototypic proteins were designated HMW1 and HMW2, and we demonstrated that approximately 75% of unrelated nontypeable *Haemophilus* organisms express such proteins (9, 57). Subsequently, we reported that immunization of experimental animals with the HMW1 and HMW2 proteins was protective in the chinchilla model of otitis media (6). More recently, we demonstrated that naturally occurring human antibodies specific for these proteins were opsonophagocytic for nontypeable *Haemophilus influenzae* in vitro (65). The objective of the present study was to expand upon these recent observations and assess the opsonophagocytic activity of high-titer immune sera generated against purified HMW1/HMW2-like proteins when those sera were assayed against a panel of homologous and heterologous nontypeable *Haemophilus influenzae* strains.

**MATERIALS AND METHODS**

**Bacterial strains.** Strains 5, 12, 14, 15, 16, 17, 18, and 64 are nontypeable *Haemophilus influenzae* strains that express HMW1/HMW2-like proteins. Each of these strains expresses two distinct high-molecular-weight HMW1/HMW2-like proteins (8, 9). Strain 11 is a nontypeable *Haemophilus* strain that expresses the Hia high-molecular-weight adhesion protein but not the HMW1/HMW2-like proteins (11). Each of the strains was isolated in pure culture from a middle ear fluid specimen of a child with acute otitis media. Each strain was identified as *H. influenzae* by standard methods and was classified as nontypeable by its failure to agglutinate with a panel of typing antisera for *H. influenzae* types a to f (Burroughs Wellcome Co., Research Triangle Park, N.C.) and by its failure to show lines of precipitation with these antisera in counterimmunoelectrophoresis assays. All organisms used in this work were stored at −70°C in skim milk within two or three subpassages of the initial clinical isolation.

**Purification of HMW adhesion proteins.** The native HMW1/HMW2-like proteins were purified from the five prototype nontypeable *Haemophilus* strains as previously described (6). In brief, a frozen bacterial stock culture of each strain was streaked onto a chocolate agar plate and allowed to grow overnight at 37°C in an atmosphere of 5% CO2. The following day, a 50-ml starter culture of brain heart infusion broth supplemented with hemin and NAD was inoculated with 5 to 10 colonies and shaken at 37°C in a rotary incubator at 250 rpm until the optical density reached an A600 of 0.6 to 0.8. Six 500-ml flasks of supplemented brain heart infusion broth were inoculated with 8 to 10 ml of the bacterial suspension from the starter culture and allowed to grow to an optical density of 1.2 to 1.5. Bacterial cells were pelleted by centrifugation at 12,000 × g for 10 min at 4°C and frozen overnight at −20°C in preparation for protein purification.

The following day, the bacterial pellets were resuspended and combined in 250 ml of extraction solution (0.5 M NaCl, 0.01 M Na2EDTA, 0.01 M Tris, and 50 μM 1,10-phenanthroline, pH 7.5). The cells were not sonicated or otherwise mechanically disrupted but were simply resuspended and incubated on ice for 60 min. The suspensions were centrifuged at 12,000 × g for 10 min at 4°C to remove the majority of intact cells and cellular debris. The supernatant, containing the water-soluble HMW1/HMW2-like proteins, was centrifuged at 100,000 × g for 60 min at 4°C to remove membrane fragments and other debris. The final supernatant was dialyzed overnight at 4°C against 0.1 M Na phosphate, pH 6.0.

The next day, the sample was centrifuged at 12,000 × g for 10 min at 4°C to remove any insoluble debris. The resulting supernatant was passed over a 10-ml CM Sepharose column (Sigma Chemical Co., St. Louis, MO) preequilibrated with 1.5 M Na phosphate (pH 6.0) followed by washes with a solution containing 0.01 M Na phosphate. The column-bound proteins were eluted using a 0 to 0.5 M KCl gradient. Column fractions were analyzed on Coomassie blue-stained gels to identify fractions containing high-molecular-weight proteins. Column fractions containing the HMW1/HMW2-like proteins were pooled and concentrated to a volume of 1 to 3 ml and maintained overnight on ice. The next day, the sample was applied to a Sepharose CL-6B (Sigma) gel filtration column equilibrated with phosphate-buffered saline (PBS, pH 7.5). Column fractions containing high-molecular-weight proteins free of contamination by lower-molecular-weight species were identified by analysis on Coomassie blue-stained gels. The relevant fractions were pooled and stored at −70°C in preparation for animal immunization, enzyme-linked immunosorbent assay (ELISA), or adsorption studies.

**Western immunoblot assay with purified HMW proteins.** Five micrograms of each purified HMW1/HMW2-like protein preparation was solubilized in electrophoresis sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% acrylamide gels, and transferred onto nitrocellulose with a Genie electrophoretic blotter (Idea Scientific Company, Corvallis, Oreg.) for 45 min at 24 V. After transfer, the nitrocellulose sheet was blocked with a 3% gelatin solution in Tris-buffered saline (TBS) (pH 7.5). Column fractions containing high-molecular-weight proteins free of contamination by lower-molecular-weight species were identified by analysis on Coomassie blue-stained gels. The relevant fractions were pooled and stored at −70°C in preparation for animal immunization, enzyme-linked immunosorbent assay (ELISA), or adsorption studies.

**Chinchilla immunization protocol for generation of immune sera.** Chinchillas were immunized with HMW1/HMW2-like proteins purified from five prototype nontypeable *Haemophilus influenzae* strains: strains 5, 12, 15, 16, and 17. Two experimental animals received each protein preparation. The immunization series consisted of five subcutaneous injections with 100 μg of the respective protein preparations administered every 4 to 6 weeks. The first dose of each preparation was mixed with Freund’s complete adjuvant, and subsequent doses were mixed with incomplete Freund’s adjuvant. Serum antibody responses were monitored by ELISA, and the antibody responses of the two animals that received each protein preparation were compared with one another. Only the sera from the animal in each pair with the higher of the two ELISA antibody responses were examined subsequently in the opsonophagocytic assay.

**ELISA measurement of antibodies directed against purified high-molecular-weight proteins.** Chinchilla serum antibodies recognizing the HMW1/HMW2-like proteins were measured by ELISA in an assay modified slightly from one described previously (6). In brief, 96-well flat-bottomed enzyme immunoassay microtitration plates (Lmbro/Titertek; Flow Laboratories, Inc., McLean, Va.) were coated overnight at 4°C with HMW1/HMW2-like proteins purified from one of the five prototype strains. The proteins were diluted in NaCO3 buffer, pH 9.6, at a concentration of 10 μg per ml. The following day, the plates were blocked with PBS–0.5% bovine serum albumin (BSA) at room temperature for 1 h and washed with PBS–0.5% BSA–0.05% Tween 20 prior to the addition of...
the chinchilla serum specimens. The chinchilla sera were diluted in PBS-0.5% BSA-0.05% Tween 20 and incubated for 1 h at room temperature. After additional washes, the wells were incubated with a 1:50,000 dilution of rabbit anti-chinchilla IgG antiserum for 1 h at room temperature. Following additional washes, the wells were incubated with a 1:3,000 dilution of IgG-specific goat anti-rabbit IgG. The plates were washed three additional times, the wells were incubated with a 1:3,000 dilution of IgG-specific goat anti-rabbit IgG, and the plates were washed five additional times. Optical density readings were recorded with a Titertek Multiscan spectrophotometer (Flow Laboratories). Preimmune serum and immune serum in all assays gave absorbance readings of 0.1. The titer for each sample was defined as the highest dilution with an absorbance that was at least twice that of control wells after a 60-min incubation.

Adsortion of anti-HMW antisera with homologous and heterologous high-molecular-weight proteins. Aliquots of chinchilla antisera raised against HMW1/HMW2-like proteins purified from the five prototype strains were each individually adsorbed with the respective homologous and four heterologous strains. In brief, 0.5 ml of each immune serum was incubated overnight with rocking at 4°C with 0.5 ml of a 1-mg/ml solution of purified HMW1/HMW2-like proteins from the homologous or heterologous strains. The following morning, insoluble debris was removed from the solution by centrifugation at 14,000 × g for 10 min at 4°C. The resulting supernatant was saved and used in the opsonophagocytic assay as described below. As negative controls, immune sera were also adsorbed with a 1-mg/ml solution of bovine serum albumin or an irrelevant soluble recombinant protein.

Growth conditions of bacteria for opsonophagocytosis assay. Bacteria were recovered from skim milk stocks by transfer of a loopful of thawed organisms to a chocolate agar plate and incubation for 16 h at 37°C in a 5% CO2 atmosphere. The next day, 5 to 10 colonies were isolated with a sterile loop and used to inoculate 50 ml of brain heart infusion broth supplemented with NAD and hemin, each at 10 µg per ml. Growth proceeded for 4 to 6 h at 37°C in 250-ml Erlenmeyer flasks with a shaker-incubator (model G25; New Brunswick Scientific Co., Inc., Edison, NJ). Bacteria in mid-log phase with an A600 of 0.5 to 0.6 were harvested by centrifugation at 12,000 × g for 4°C and washed twice with Veronal-buffered saline with 0.5% BSA and with CaCl2 and MgCl2 at final concentrations of 0.15 mM and 0.5 mM, respectively. In preliminary experiments, bacteria demonstrated no loss of viability when maintained in this buffer for up to 4 h at 0 or 37°C. The washed bacterial cells were maintained at 0°C for less than 1 h before being used in the opsonophagocytosis assay described below.

Growth and differentiation of HL-60 cells. The methods used for growth and differentiation of the HL-60 cells have been described previously (52, 65). In brief, the human promyelocytic tissue culture cell line HL-60 (CCL 240; American Type Culture Collection, Rockville, MD) was used as the source of effector cells. A frozen stock passage obtained from the ATCC was diluted 1:20 and expanded in tissue culture flasks (Costar T-75; Corning, Corning, NY) to a cell density of 6 × 10⁵ cells per ml in 85% RPMI 1640 medium containing 1% t-glutamine (Life Technologies, Grand Island, NY), 15% fetal bovine serum (Life Technologies), and antibiotics (1× penicillin-streptomycin solution; Life Technologies). Cells were grown in suspension to approximately 10⁶ cells per ml at 37°C in a 5% CO2 atmosphere.

Dilution of HL-60 cells was carried out in T-150 culture flasks containing RPMI 1640 medium with 1% t-glutamine, 15% fetal bovine serum, and 100 mM N,N-dimethylformamide (DMF) (99.8% purity; Sigma). First, 2 × 10⁶ cells from the undifferentiated cell stock were recovered by centrifugation (150 × g for 8 min at room temperature) and resuspended in 150 ml of differentiation medium without DMF. Next, 1.5 ml of DMF was added to a 30-ml aliquot of culture medium, mixed thoroughly, and added to the 150-ml cell suspension. Cultures were incubated in a horizontal position at 37°C in a 5% CO2 atmosphere for 5 to 7 days. Granulocytic differentiation was monitored by visual examination of the cell cultures for characteristic morphological changes with an inverted microscope and by microscopic examination of Giemsa-stained smears.

Opsonophagocytic assay with HL-60 cells. Differentiated HL-60 cells were differentiated in T-250 culture flasks containing RPMI 1640 medium with 1% t-glutamine, 15% fetal bovine serum, and 100 mM N,N-dimethylformamide (DMF) (99.8% purity; Sigma). First, 2 × 10⁶ cells from the undifferentiated cell stock were recovered by centrifugation (150 × g for 8 min at room temperature). The volume of differentiated cell culture required per microtiter plate was determined by the viable cell count of the culture (always ≥90% for the cells to be considered acceptable for use) and by the number of microtiter wells being used in a given day's assay. The appropriate volume was centrifuged as described above, and the supernatant was discarded, removing any excess medium. The cell pellet was resuspended in Hanks’ buffer without Ca2+ and Mg2+ (Life Technologies) using 5 ml per 50 ml of centrifuged cell culture. The resuspended cells were kept at 37°C in a 5% CO2 atmosphere until immediately before use in the functional assay. At this point, the cell suspension was centrifuged as described above, and the supernatant was discarded. The cell pellet was gently resuspended in opsonophagocytosis buffer (3 ml of Veronal-buffered saline with Ca2+ and Mg2+ and 0.5% BSA) (8) and used immediately in the functional assay.

To activate intrinsic complement activity, all serum samples were heat inactivated for 30 min at 56°C prior to use in the assay. Each serum sample to be studied was assayed in duplicate on the same 96-well microtiter plate (Costar, Cambridge, MA). Twenty microliters of serum was added to the first well in each dilution series, and samples were twofold serially diluted in opsonophagocytosis buffer. Once all of the serum samples were prepared, 20 µl of the bacterial suspension diluted in opsonophagocytosis buffer (approximately 5 × 10⁷ CFU) was added to each well. The bacterial suspension was prepared by dilution of freshly grown and harvested log-phase bacteria as described above. The microtiter plate was allowed to incubate at 37°C in a 5% CO2 atmosphere for 15 min. Following the incubation period, 1.5 µl of complement source (sterile guinea pig serum [Invitrogen Life Technologies, Carlsbad, CA, or Rockland Inc., Gilbertsville, PA]) was added to each well. Guinea pig serum was maintained frozen at −70°C in 0.5-ml aliquots until use. Immediately after the addition of complement, 60 µl of washed, differentiated HL-60 cells (approximately 5 × 10⁵ cells) was added to each well. The assay plate was then incubated in room air at 37°C for 90 min with horizontal shaking (220 rpm) to promote the phagocytic process. At the end of the incubation period, a 10-µl aliquot from each well was plated onto a chocolate agar plate and spread for subsequent counting. Culture plates were incubated overnight at 37°C, and viable colony counts were performed the following day. Each serum sample had two heat-inactivated complement control wells included on the same plate. Active complement control wells included all of the test reagents except the serum samples. The percent killing at each serum dilution was calculated by determining the ratio of the bacterial colony count at each dilution to that of the respective heat-inactivated complement control. The means and standard deviations were calculated using data from the serum samples run in duplicate on each plate. Statistical analyses were performed using the NCSS 2000 software package (NCSS, Kaysville, UT). Opsonophagocytosis titers were defined as the reciprocal of the serum or antibody dilution that resulted in ≥50% killing of the bacterial inoculum compared to growth in the heat-inactivated complement control wells.

RESULTS

High-molecular-weight proteins purified from prototype strains. High-molecular-weight HMW1/HMW2-like proteins were purified from five unrelated nontypeable Haemophilus influenzae strains. Almost all nontypeable Haemophilus influenzae strains express two closely related but distinct HMW1/HMW2-like proteins that copurify with our standard purification protocol (9, 57). Figure 1A shows a Coomassie blue-stained SDS-PAGE gel demonstrating the HMW1/HMW2-like proteins purified from three of the strains used in the studies described below. The protein bands present in the bracketed regions represent the major protein species expressed by each strain. Several notable findings are apparent in the figure. First, the proteins purified from the three strains demonstrate significant size heterogeneity. Their apparent molecular masses range from approximately 110 kDa to 150 kDa. This variability is typical of the heterogeneity that we observed previously when the HMW1/HMW2-like proteins from other nontypeable Haemophilus influenzae strains were examined by Western immunoblot analyses (9, 10). The size heterogeneity likely reflects known differences in the sizes of the respective structural genes (9, 18) but may also reflect variation in the degree and type of glycosylation present on each protein (31). Second, as seen with the strain 5 proteins, the proteins ex-
pressed by a given strain sometimes comigrate with one another, giving the impression that only a single protein is being expressed. However, in the case of strain 5, the expression of two distinct HMW1/HMW2-like proteins was demonstrated definitively in previous studies in which we selectively inactivated one or the other structural gene and demonstrated continued protein expression from the remaining intact locus (56). Finally, as can be appreciated most notably by examination of the strain 5 and strain 12 proteins, even when one uses a careful preparative technique, some degree of proteolysis is not infrequently observed, a property that the HMW1/HMW2-like proteins share with other related bacterial secretory proteins (34, 50).

Figure 1B shows a companion Western immunoblot in which the same protein preparations examined in Fig. 1A were assessed for their reactivity with two HMW1/HMW2-specific monoclonal antibodies (10). One can easily appreciate the prominent reactivity of the monoclonal antibodies with the major protein bands in each strain. In addition, one can appreciate the many immunoreactive lower-molecular-weight bands that represent degradation products of the mature HMW1/HMW2-like proteins. These fragments range in size from just slightly smaller than the respective mature proteins to as small as 45 kDa. These immunoreactive bands correspond to similar but more faintly staining bands seen in the Coomassie blue-stained gel. Also of note in the bracketed regions that highlight the major protein species of strain 5 and strain 12 are the three closely spaced but distinct immunoreactive bands. In previous studies, we demonstrated by N-terminal sequence analysis that the upper band in the strain 12 three-band cluster represents the mature HMW1 protein, while the two lower bands are both HMW2 derivatives (6). The three major bands in the strain 5 preparation likely represent similar derivatives of one or the other of the two HMW1/HMW2-like proteins expressed by strain 5. It is notable that the immunoblot is capable of distinguishing these three different major protein bands in the strain 5 preparation, whereas the Coomassie blue-stained gel showed only a single homogeneous band (compare Fig. 1A and B).

### Opsonophagocytic activity of anti-HMW1/HMW2 immune sera against homologous nontypeable Haemophilus influenzae

To assess the opsonophagocytic activities of antibodies directed against the HMW1/HMW2-like proteins, we measured the activity of the anti-HMW1/HMW2 immune sera in an assay system that we described previously (65). Early in this project, we observed that the ability of antibodies to mediate opsonophagocytic activity in our system was species dependent. When guinea pig serum was used as the complement source, the standard for our assay, rabbit antibodies were incapable of mediating opsonophagocytic killing. In contrast, both guinea pig and chinchilla antibodies could mediate opsonophagocytic killing in the system. The differences between the species presumably relate to the ability, or lack thereof, of antibodies from a given species to activate complement components in guinea pig serum and to bind to Fc receptors present on the HL-60 cells (52). Because we could obtain larger quantities of serum from immunized chinchillas than from guinea pigs, chinchillas were used to generate the immune sera for our studies.

Prior to their examination in the opsonophagocytic assay, the chinchilla immune sera were monitored for ELISA antibody activity against purified homologous and heterologous HMW1/HMW2-like proteins (Table 1). The proteins used to

### TABLE 1. ELISA antibody titers of chinchilla HMW1/HMW2 antisera assayed against homologous and heterologous HMW1/HMW2-like proteins

| Antiserum | Titer of HMW1/HMW2-like proteins |
|-----------|----------------------------------|
|           | 5 HMW | 12 HMW | 15 HMW | 16 HMW | 17 HMW |
| α-5 HMW   | ≥64,000 | ≥64,000 | 32,000 | ≥64,000 | ≥64,000 |
| α-12 HMW  | 16,000 | ≥64,000 | ≥64,000 | ≥64,000 | 32,000 |
| α-15 HMW  | 8,000 | 32,000 | 32,000 | 4,000 | 16,000 |
| α-16 HMW  | 16,000 | 64,000 | 32,000 | ≥64,000 | 16,000 |
| α-17 HMW  | ≥64,000 | ≥64,000 | ≥64,000 | ≥64,000 | 16,000 |

*α*-5 HMW, antibody to strain 5 HMW proteins.

*5* HMW, strain 5 HMW proteins.
sensitize the ELISA plates were not subjected to detergent treatment or other harsh conditions prior to or during the sensitization process. Thus, one would predict that any conformational epitopes would be well preserved and that antibodies detected in the assay would include those recognizing such epitopes. None of the preimmune serum samples had measurable antibody against the HMW1/HMW2-like proteins of any strain. In contrast, the immune sera raised against each of the HMW1/HMW2-like protein preparations demonstrated very high ELISA antibody titers (Table 1). Measured titers were $\geq 64,000$ against all but one of the homologous proteins and against many of the heterologous proteins. The antisera raised against the strain 15 proteins demonstrated the lowest titers of the group, but even it had substantial ELISA activity raised against the strain 16 proteins demonstrated the lowest titers ranging from 80 to 640 when the five different immune sera were assayed against this strain (Table 2).

A more detailed examination of data from individual opsonophagocytic assays was also quite informative. Figures 3 and 4 present composite data in which the opsonophagocytic killing mediated by immune serum raised against either the strain 12 proteins (Fig. 3) or the strain 16 proteins (Fig. 4) was assayed against seven nontypeable *Haemophilus* strains. As can be seen, for most of the strains against which the antisera demonstrated opsonophagocytic killing, typical concentration-dependent decreases in activity were observed as the preparations were serially diluted. However, a few of the immune sera consistently demonstrated prozone phenomena when assayed against certain heterologous strains. An example of this can be seen in Fig. 3, where the antisera raised against the strain 12 proteins killed strain 16 only modestly at the 1:10 dilution but killing of those strains. However, for some strains, immune sera raised against heterologous proteins were nearly as active. For example, the immune serum raised against the strain 16 HMW1/HMW2-like proteins was as active against strain 17 as the immune serum raised against the strain 17 proteins themselves, and vice versa. Clear differences among the individual strains in their susceptibilities to opsonophagocytic killing were also observed. For example, nontypeable *Haemophilus* strain 14 was not killed by any of the five immune sera, yet it clearly expressed two distinct high-molecular-weight proteins when examined in Western blot assays (9). In contrast, strain 17 was the most susceptible strain examined, with opsonophagocytic titers ranging from 80 to 640 when the five different immune sera were assayed against this strain (Table 2).

Effect of adsorption with homologous and heterologous HMW1/HMW2-like proteins on opsonophagocytic activities of individual immune sera. The previous set of experiments strongly suggested that epitopes are present on the HMW1/HMW2-like proteins that are recognized by cross-reactive op-

### Table 2. Opsonophagocytic activities of a panel of HMW1/HMW2 antisera assayed against eight HMW1/HMW2-expressing nontypeable *Haemophilus influenzae* strains

| Antiserum* | Titer for bacterial strain |
|------------|---------------------------|
|            | 5 | 12 | 14 | 15 | 16 | 17 | 18 | 64 |
| $\alpha$-5 HMW | 640 | 40 | <10 | <10 | 40 | 80 | 40 | 80 |
| $\alpha$-12 HMW | <10 | 640 | <10 | 160 | 40 | 160 | 20 | 320 |
| $\alpha$-15 HMW | 160 | 80 | <10 | 320 | <10 | 160 | 20 | 160 |
| $\alpha$-16 HMW | 20 | <10 | <10 | <10 | 320 | 640 | 80 | 40 |
| $\alpha$-17 HMW | 20 | <10 | <10 | <10 | 40 | 320 | 640 | 160 |

* $\alpha$-5 HMW, antibody to strain 5 HMW proteins.

![FIG. 2. Opsonophagocytic activities of chinchilla antisera raised against strain 12 or strain 16 HMW1/HMW2-like proteins assayed against the respective homologous nontypeable *Haemophilus influenzae* strain.](image-url)
sonophagocytic antibodies. This is an important finding from the standpoint of the possible use of these proteins as components of a nontypeable Haemophilus influenzae vaccine. To further explore and validate this observation, we performed a series of adsorption experiments in which each immune serum was adsorbed with HMW1/HMW2-like proteins from all five of our prototype strains. The resulting adsorbed sera were then assayed for residual opsonophagocytic activity. If functionally active antibodies that recognize cross-reactive epitopes do exist, one would predict that the adsorption of immune sera with HMW1/HMW2-like proteins purified from both homologous and heterologous strains would be capable of removing such activity, at least to a degree.

Table 3 shows the results of the opsonophagocytic assays in which the immune sera and their respective HMW1/HMW2-adsorbed samples were assayed against the respective homologous strains. As can be seen, adsorption of the immune sera with HMW1/HMW2-like proteins purified from the respective homologous strains completely eliminated opsonophagocytic killing of those strains. In addition, adsorption of these same immune sera with HMW1/HMW2-like proteins purified from heterologous strains also eliminated opsonophagocytic killing of the respective homologous strains in most instances. For example, with the immune serum raised against the HMW1/HMW2-like proteins of either strain 16 or strain 17, adsorption with proteins purified from each of the five strains completely eliminated killing of the respective homologous strains, namely, strains 16 and 17. Similar findings were observed with the immune sera raised against the strain 12 and strain 15 proteins. Here again, adsorption with most but not all heterologous proteins led to a loss of killing activity against the respective homologous strains. The most notable exception to the above-described results was the experiment with the strain 5 immune serum. Here, only adsorption with the homologous strain 5 proteins and the heterologous strain 12 proteins led to significant decreases in the opsonophagocytic killing activity against strain 5. Control experiments in which each of the immune sera was adsorbed with either albumin or an unrelated Haemophilus recombinant protein led to no decrease in killing.

![Figure 3](image3.png)

**FIG. 3.** Opsonophagocytic activity of chinchilla antiserum raised against strain 12 HMW1/HMW2-like proteins assayed against a panel of prototype nontypeable Haemophilus influenzae strains.

![Figure 4](image4.png)

**FIG. 4.** Opsonophagocytic activity of chinchilla antiserum raised against strain 16 HMW1/HMW2-like proteins assayed against a panel of prototype nontypeable Haemophilus influenzae strains.

| Antiserum | Titer after protein adsorption |
|-----------|-------------------------------|
|          | Unadsorbed | 5 HMW | 12 HMW | 15 HMW | 16 HMW | 17 HMW | Albumin |
| α-5 HMW  | 640       | <20   | <20   | 320    | 320    | 640    |
| α-12 HMW | 640       | <20   | <20   | 40     | <20    | 640    |
| α-15 HMW | 320       | 160   | <20   | <20    | 160    | 320    |
| α-16 HMW | 320       | <20   | <20   | <20    | <20    | 320    |
| α-17 HMW | 640       | <20   | <20   | <20    | <20    | 640    |

*α-5 HMW, antibody to strain 5 HMW proteins.

*5 HMW, strain 5 HMW proteins.

![Figure 5](image5.png)

**FIG. 5.** Opsonophagocytic activity of chinchilla antiserum raised against strain 12 HMW1/HMW2-like proteins pre- and postadsorption with HMW1-HMW2-like proteins purified from a panel of prototype nontypeable Haemophilus influenzae strains. Samples are being assayed against strain 12. Ads, adsorbed.
activity, reassuring us that the loss of activity was not a non-specific phenomenon.

To provide some additional detail, graphs depicting individual opsonophagocytic assays in which the immune serum against the strain 12 HMW1/HMW2 proteins or strain 16 HMW1/HMW2-like proteins was assayed pre- and postadsorption against the respective homologous strain are shown in Fig. 5 and 6. As was summarized above and as can be seen here, adsorption of each of these immune sera with any of the HMW1/HMW2-like protein preparations led to marked decreases in opsonophagocytic killing of the homologous strain at all dilutions tested. In contrast, adsorption with albumin led to no demonstrable change in activity.

DISCUSSION

Host immunity against nontypeable *Haemophilus influenzae* is mediated by both the relatively nonspecific components of the innate immune system (12, 30, 41, 63, 64) and specific antibodies that induce bacteriolyis of the infecting organisms (8, 42, 43) or facilitate opsonophagocytosis in concert with host leukocytes and complement (44, 65). In previous work, we characterized human opsonophagocytic antibodies recognizing nontypeable *Haemophilus influenzae* by using an assay modified from one developed for the measurement of antibodies against *Streptococcus pneumoniae* (51, 52). In that earlier work, we demonstrated that antibodies directed against the HMW1/HMW2-like proteins were major contributors to the opsonophagocytic activity mediated by naturally acquired human antibodies (65). Furthermore, in a limited fashion, we demonstrated that antibodies specific for the HMW1/HMW2-like proteins could mediate opsonophagocytic activity not only against homologous but also against heterologous nontypeable *Haemophilus influenzae* strains (65). In the current study, we were able to expand upon these previous observations. We demonstrated that high-titer immune sera raised against a panel of purified HMW1/HMW2-like proteins were frequently opsonophagocytic for both homologous and heterologous non-typeable *Haemophilus influenzae* strains.

Several important observations were made in the course of this work. As might have been predicted, the opsonophagocytic activity of a given immune serum was in all instances as active or more active against the respective homologous strain than were any of the other immune sera (Table 2). However, despite the known variability in the predicted amino acid sequences of the HMW1/HMW2-like proteins from different nontypeable *Haemophilus influenzae* strains (9, 18, 23), the immune sera were frequently able to mediate opsonophagocytic killing of heterologous strains. As a case in point, the predicted amino acid sequences of the HMW1/HMW2-like proteins of strains 12 and 15 demonstrated between 65 and 81% sequence identity when the four sequences were aligned and compared (18, 60). Even so, the strain 12 immune serum was capable of killing strain 15 at a moderately high titer of 1:160, while the strain 15 immune serum killed strain 12 at a titer of 1:80. Similarly, the predicted amino acid sequences of the two HMW1/HMW2-like proteins from strain 5 are between 67 and 74% identical with the sequences of the two strain 15 proteins (18, 60), yet the strain 15 immune serum mediated killing of strain 5 at a titer of 1:160. Thus, these data suggest that a simple comparison of the predicted amino acid sequences of the HMW1/HMW2-like proteins from different strains may not accurately predict the likelihood that cross-protective antibodies directed against these proteins may be generated following immunization.

Reciprocal cross-reactivity of opsonophagocytic killing was frequently observed with the various antiserum-bacterial strain pairs shown in Table 2. For example, the antisera raised against the HMW1/HMW2-like proteins of strains 16 and 17 very effectively killed both the homologous and heterologous strains in this pair but had relatively limited activity against the other strains in the panel. Similarly, antisera raised against the proteins purified from either strain 12 or strain 15 effectively killed each of these latter two strains but had more modest activity when tested against several of other strains in the panel. Even so, reciprocal cross-reactivity was not always observed. For example, with strain 5 and strain 15, the strain 15 antisera effectively killed strain 5, yet the strain 5 antisera had no apparent killing activity against strain 15. How can one explain such an apparent discrepancy? Recall that we used outbred chinchillas for the generation of the immune sera. Each animal’s response in terms of antibody titer, antibody affinity, and epitope recognition may be quite variable and unpredictable. Although one might hope to see consistent evidence of reciprocal cross-reactivity, as we did with the antisera raised against several proteins, it is not surprising that this was not observed with all of the antiserum-bacterial strain pairs.

Additional evidence for the existence of surface epitopes capable of eliciting cross-reactive opsonophagocytic antibodies was provided by the adsorption experiments. As summarized in Table 3 and depicted in more detail in Fig. 5 and 6, adsorption of immune sera with HMW1/HMW2-like proteins purified from either homologous or heterologous strains substantially decreased or eliminated opsonophagocytic killing of the homologous strains in most cases. In fact, the degree to which absorption of a given immune serum by heterologous proteins could eliminate killing of the homologous strain was greater...
than might have been predicted from the opsonophagocytic data shown in Table 2. How can one explain the inability of an antisem to kill a given heterologous strain in the face of adsorption data demonstrating that HMW1/HMW2-like proteins purified from that heterologous strain efficiently remove opsonophagocytic antibodies? We can only speculate at this point, but a likely explanation is that the antibodies capable of opsonizing bacteria and supporting their phagocytic killing must be of high specificity and affinity. The anti-HMW1/HMW2 antibodies generated against the proteins of a particular strain may recognize and bind the proteins of heterologous strains, but if the binding affinity for those proteins is not sufficiently high, opsonophagocytosis may not occur, or if it does occur, it may be less efficient. In contrast, in the adsorption experiments that we performed, the HMW1/HMW2-like proteins were present in excess. These conditions may allow antibodies with even relatively low affinity for the heterologous proteins to be quite efficiently removed from solution. These “low-affinity” antibodies may have much higher affinity and specificity for the homologous proteins and may be critical for the efficient opsonophagocytosis of the respective homologous nontypeable Haemophilus strains. Thus, when the adsorbed sera are monitored for their opsonophagocytic killing ability, little residual activity may remain. This question will be very important to address experimentally in future work.

Clear differences were apparent among our eight prototype nontypeable Haemophilus strains in their susceptibilities to opsonophagocytic killing (Table 2), a result similar to those reported previously in our studies of human antibodies directed against the HMW1/HMW2-like proteins (65). For example, strain 17 was susceptible to opsonophagocytic killing by each of the immune sera at relatively high titers. In contrast, strain 5 was completely resistant to killing by two of the heterologous immune sera and was susceptible only to killing by a third immune serum at a low titer. At the extreme was nontypeable Haemophilus strain 14, a strain resistant to killing by all of the immune sera that we had prepared. While strain-to-strain variation in the amino acid sequences of the HMW1/HMW2-like proteins undoubtedly accounts for some of the differences observed (9, 18, 23), other explanations also deserve consideration. The bacterial surface of nontypeable Haemophilus influenzae is known to be a very dynamic structure. A number of surface molecules have been demonstrated to influence the interactions of these bacteria with the host immune system and also to undergo antigenic and phase variation (16, 21, 25, 63, 64). In the case of the HMW1/HMW2-like proteins, variation in their levels of expression may directly influence the ability of the immune system to clear infection in vivo (6). Similar differences in protein expression levels in vitro likely influence the ability of antibodies directed against these proteins to mediate opsonophagocytic activity. At the present time, we have no information on differences that may exist in the lipooligosaccharide phenotypes or in the relative levels of expression of the HMW1/HMW2-like proteins for our panel of prototype strains, but these will also be important areas to investigate in future studies.

To conclude, in our previous work, we demonstrated that the HMW1/HMW2-like proteins are major targets of naturally acquired human opsonophagocytic antibodies (65). In the current study, we demonstrated that shared surface-exposed epitopes recognized by opsonophagocytic antibodies are present on the HMW1/HMW2-like proteins of unrelated nontypeable Haemophilus influenzae strains. Together, the findings from these two studies provide a strong rationale for continued investigation of the HMW1/HMW2-like proteins as possible components in a vaccine for prevention of nontypeable Haemophilus influenzae disease.

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