Antibodies to the HMW1/HMW2 and Hia Adhesins of Nontypeable *Haemophilus influenzae* Mediate Broad-Based Opsonophagocytic Killing of Homologous and Heterologous Strains

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The HMW1/HMW2 and Hia proteins are highly immunogenic surface adhesins of nontypeable *Haemophilus influenzae* (NTHi). Approximately 75% of NTHi strains express HMW1/HMW2 adhesins, and most of the remaining 25% express an Hia adhesin. Our objective in this study was to assess the ability of antisera raised against purified HMW1/HMW2 proteins or recombinant Hia proteins to mediate opsonophagocytic killing of a large panel of unrelated NTHi strains. Native HMW1/HMW2 proteins were purified from three HMW1/HMW2-expressing NTHi strains. Recombinant fusion proteins expressing surface-exposed segments of either of two prototype Hia proteins were purified from *Escherichia coli* transformants. Immune sera raised in guinea pigs were assessed for their ability to mediate killing of NTHi in an opsonophagocytic assay with the HL-60 phagocytic cell line. The three HMW1/HMW2 antisera mediated killing of 22 of 65, 43 of 65, and 28 of 65 unrelated HMW1/HMW2-expressing NTHi strains, respectively. As a group, the three sera mediated killing of 48 of 65 HMW1/HMW2-expressing strains. The two Hia immune sera mediated killing of 12 of 24 and 13 of 24 unrelated Hia-expressing NTHi strains, respectively. Together, they mediated killing of 13 of 24 Hia-expressing strains. Neither the HMW1/HMW2 nor the Hia antisera mediated killing of NTHi expressing the alternative adhesin type. Antibodies directed against native HMW1/HMW2 proteins and recombinant Hia proteins are capable of mediating broad-based opsonophagocytic killing of homologous and heterologous NTHi strains. A vaccine formulated with a limited number of HMW1/HMW2 and Hia proteins might provide protection against disease caused by most NTHi strains.

Nontypeable *Haemophilus influenzae* (NTHi) strains are small Gram-negative bacteria that colonize the upper respiratory tract of humans beginning at a very early age (1). Although these organisms are normally commensals, when host defenses are compromised by underlying medical conditions, such as malnutrition, immunodeficiency, chronic lung disease, or acute viral infection, disease caused by NTHi may develop (2, 3). Among children in the developed world, NTHi strains are currently responsible for an estimated 40 to 50% of the cases of acute otitis media and an even higher percentage of cases of chronic and recurrent disease (4, 5). Among adults, particularly among patients with chronic obstructive pulmonary disease, NTHi strains are a major cause of illness, particularly during the acute exacerbations that often characterize this disease (6). A vaccine capable of preventing disease caused by these organisms would offer substantial benefit to the adult and pediatric populations alike.

NTHi vaccine development efforts are ongoing in a number of laboratories. Published studies suggest that NTHi outer membrane proteins are the principal targets of bactericidal and protective antibodies (7–9). Several protein antigens have been the subject of detailed investigation as potential vaccine candidates (10–12). The proteins known as P2 and P6 have been studied in great detail. Each is a target of human bactericidal antibody (13–15), and each has demonstrated partial protection against infection in animal models (16, 17). Another leading vaccine candidate, the so-called P5-fimbrin adhesin (18, 19), has also demonstrated protection in the chinchilla otitis model (18, 20, 21). Other proteins still under active investigation as possible vaccine candidates include protein D (22), protein E (23), type IV pili (24, 25), and OMP 26 (21, 26). Even lipoooligosaccharide, in the form of detoxified conjugate preparations, has been investigated as a potential vaccine candidate (27–29). A recent human clinical trial in which children were immunized with a protein D-pneumococcal polysaccharide conjugate vaccine reported protection against pneumococcal and NTHi otitis media (30, 31), but protection against NTHi disease was quite modest and did not correlate with serum anti-protein D antibody levels. A follow-up study of the same vaccine in a younger population demonstrated only marginal protection against NTHi otitis media (32).

Despite work by many groups, it remains unclear which, if any, of the many NTHi vaccine candidates under study is best suited for inclusion in a human protective vaccine. The strain heterogeneity known to be present among NTHi is a challenge that must be overcome for any vaccine development effort to succeed (33–35). Some in the NTHi vaccine development community have suggested that only highly conserved proteins should be investigated as potential vaccine candidates (36), but it is questionable whether any conserved proteins exist that are capable, by themselves, of inducing a broad-based protective immune response. Many in the field have speculated that only by formulating a vaccine with mul-
tiple protective antigens will we be successful in developing a vaccine capable of protecting young children and adults against disease (12).

In our early work, we demonstrated that development of bacterial antibody in the sera of children recovered from acute NTHi otitis media was associated with the appearance of serum antibodies directed against highly immunogenic high-molecular-weight proteins (37). This work led subsequently to the identification and characterization of the HMW1/HMW2 family of proteins (38). The HMW1/HMW2 proteins were subsequently shown to be major adherins of NTHi (39, 40), as well as targets of opsonophagocytic (41, 42) and protective antibodies in the chinchilla otitis model (43). The HMW1/HMW2-like proteins are expressed by approximately 75% of NTHi strains (38, 44). The 25% of NTHi strains that do not express HMW1/HMW2 proteins also express other immunogenic high-molecular-weight proteins that are recognized by human convalescent-phase serum antibodies (37). Almost all HMW1/HMW2-negative strains were subsequently shown to express a second distinct class of adhesin known as Hia (45). The Hia proteins are members of a large family of bacterial proteins, known as autotransporters, that are found in many Gram-negative bacteria (46–48). These proteins have also been shown recently to serve as targets for antibodies mediating opsonophagocytosis (49). Nearly all NTHi strains that lack HMW1/HMW2 proteins contain an hia gene and express an Hia protein; conversely, strains that express HMW1/HMW2 proteins lack an hia gene (44, 45).

The objective of the present study was to extend our earlier studies and examine the ability of antibodies directed against the HMW1/HMW2 and Hia proteins to mediate opsonophagocytic killing of a much larger and more diverse collection of NTHi than we studied in earlier work. The potential usefulness of any NTHi protein as a vaccine component is dependent upon its ability to generate antibodies that mediate relatively broad-based killing of homologous and heterologous NTHi. This study was intended to address how well the HMW1/HMW2 and Hia proteins satisfy this requirement.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture conditions.** The 89 NTHi bacterial strains studied in this work are listed in Tables 1 and 2. These isolates were collected during episodes of clinical disease from children and a few adults between 1973 and 2011. The source patients were drawn from a wide geographic region, with isolates included from Alabama, Michigan, Missouri, New York, Ohio, and Pennsylvania. Of these isolates, 44 were cultured from middle ear effusions collected by tympanocentesis or directly from middle ear drainage, 21 were isolated from blood cultures, 13 were cultured from respiratory tract secretions of patients with acute infection, 7 were cultured from eye drainage of patients with acute conjunctivitis, and 4 were cultured from cerebrospinal fluid. Many of these strains have been described previously in work from our laboratory and those of our collaborators, and the strains are known to represent a very heterogeneous population of organisms (42, 44, 49–51).

The NTHi strains were identified as such by standard methods, and each was classified as nontypeable by clinical appearance of the bacterial colonies on chocolate agar plates and by failure to agglutinate with a panel of typing antisera for *Haemophilus influenzae* types a to f (Burroughs Wellcome Co., Research Triangle Park, NC) (52). Each of the 25 strains recovered from blood or cerebrospinal fluid was also examined for the presence of capsular genes by PCR using methods described by Satola and coworkers (53). None of these invasive isolates demonstrated the presence of capsular genes. All NTHi strains were stored at −70°C in skim milk

| Strain no. | Sourceb | Yr isolated | Location | Killing by anti-HMW1/HMW2 antiseraa |
|------------|----------|-------------|----------|------------------------------------|
| 5          | MEF      | 1985        | MO       | <1:10                              |
| 7          | MEF      | 1985        | MO       | <1:10                              |
| 12         | MEF      | 1985        | OH       | <1:10                              |
| 14         | MEF      | 1986        | OH       | <1:10                              |
| 16         | MEF      | 1986        | OH       | <1:10                              |
| 17         | MEF      | 1986        | OH       | <1:10                              |
| 18         | MEF      | 1986        | OH       | <1:10                              |
| 24         | MEF      | 1982        | OH       | <1:10                              |
| 28         | MEF      | 1982        | OH       | <1:10                              |
| 30         | MEF      | 1983        | OH       | <1:10                              |
| 44         | Blood    | 1993        | MO       | <1:10                              |
| 45         | CSF      | 1994        | MO       | <1:10                              |
| 47         | Blood    | 1994        | MO       | <1:10                              |
| 49         | Blood    | 1995        | MO       | <1:10                              |
| 51         | Blood    | 1996        | MO       | <1:10                              |
| 53         | Blood    | 1997        | NY       | <1:10                              |
| 57         | Sputum   | 1999        | MO       | <1:10                              |
| 58         | Sputum   | 1999        | MO       | <1:10                              |
| 63         | Sputum   | 1999        | MO       | <1:10                              |
| 65         | Sputum   | 1999        | MO       | <1:10                              |
| 67         | Sputum   | 1999        | MO       | <1:10                              |
| 68         | Conjunctiva | 1999    | MO       | <1:10                              |
| 69         | BAL      | 2003        | MO       | <1:10                              |
| 70         | Sputum   | 2000        | MO       | <1:10                              |
| 71         | Conjunctiva | 2000    | MO       | <1:10                              |
| 72         | Blood    | 2003        | MO       | <1:10                              |
| 75         | Blood    | 2003        | MO       | <1:10                              |
| 77         | Conjunctiva | 2004    | MO       | <1:10                              |
| 79         | Blood    | 2006        | MO       | <1:10                              |
| 82         | Blood    | 2008        | MO       | <1:10                              |
| 83         | Conjunctiva | 2008    | MO       | In                                  |
| 85         | Blood    | 2009        | MO       | In                                  |
| 86         | Nasal    | 2010        | MO       | <1:10                              |
| 87         | Conjunctiva | 2010    | MO       | <1:10                              |
| 88         | Nasal    | 2010        | MO       | <1:10                              |
| 89         | Blood    | 2011        | MO       | <1:10                              |
| 1161       | Blood    | 1982        | MO       | <1:10                              |
| 1276       | Blood    | 1982        | MO       | <1:10                              |
| 1396A       | CSF      | 1983        | MI       | <1:10                              |
| 1309       | MEF      | 1986        | PA       | <1:10                              |
| 1311       | MEF      | 1986        | PA       | <1:10                              |
| 1635       | Blood    | 1983        | MO       | In                                  |
| 1667       | Blood    | 1983        | MO       | <1:10                              |
| 1674A       | Blood    | 1983        | MO       | <1:10                              |
| 1688A       | Conjunctiva | 1983    | MO       | <1:10                              |
| 1732A       | Blood    | 1983        | MO       | <1:10                              |
| 1853B       | Blood    | 1983        | MO       | <1:10                              |
| 1856A       | Blood    | 1983        | MO       | In                                  |
| 1863A       | Conjunctiva | 1983    | MO       | In                                  |
| 3179A       | MEF      | 1975        | MA       | <1:10                              |
| 3181A       | MEF      | 1977        | MA       | <1:10                              |
| 3181B       | MEF      | 1978        | MA       | In                                  |
| 3183A       | MEF      | 1976        | MA       | <1:10                              |
| 3184A       | MEF      | 1975        | MA       | <1:10                              |
| 3185A       | MEF      | 1976        | MA       | <1:10                              |
| 3222A       | MEF      | 1976        | AL       | <1:10                              |
| 3245A       | MEF      | 1983        | OH       | <1:10                              |
| 3247A       | MEF      | 1983        | OH       | <1:10                              |
| 3655A       | MEF      | 1984        | MO       | <1:10                              |
| 3837A       | MEF      | 1984        | OH       | <1:10                              |
| 3839A       | MEF      | 1984        | OH       | <1:10                              |

a Assays in which opsonophagocytic killing was observed are highlighted. In, indeterminate result because the preimmune serum also mediated killing of the indicated strains.

b MEF, middle ear fluid; CSF, cerebrospinal fluid; BAL, bronchoalveolar lavage specimen.
TABLE 2 Opsonophagocytic killing of individual Hia-expressing NTHi strains mediated by two different anti-Hia antisera

| Strain no. | Source | Yr isolated | Location | Killing by antisera to: |
|-----------|--------|-------------|----------|-----------------------|
| 11        | MEF    | 1985        | MO       | >1:40 1:10           |
| 25        | MEF    | 1982        | OH       | >1:40 1:10           |
| 27        | MEF    | 1982        | OH       | <1:10 <1:10          |
| 32        | MEF    | 1984        | OH       | <1:10 <1:10          |
| 33        | MEF    | 1985        | OH       | >1:40 1:40           |
| 35A       | MEF    | 1983        | OH       | >1:40 1:40           |
| 35B       | MEF    | 1983        | OH       | >1:40 1:40           |
| 38A       | MEF    | 1984        | OH       | <1:10 <1:10          |
| 38B       | MEF    | 1984        | OH       | >1:40 1:40           |
| 46        | Sputum | 1994        | MO       | <1:10 <1:10          |
| 48        | CSF    | 1994        | MO       | <1:10 <1:10          |
| 50        | Blood  | 1995        | MO       | >1:40 1:10           |
| 52        | Blood  | 1997        | NY       | <1:10 <1:10          |
| 56        | BAL    | 1999        | MO       | >1:40 1:40           |
| 61        | BAL    | 1999        | MO       | <1:10 <1:10          |
| 62        | CSF    | 1999        | MO       | <1:10 <1:10          |
| 78        | MEF    | 2005        | MO       | In                 |
| 81        | Blood  | 2007        | MO       | >1:40 1:40           |
| 1175      | MEF    | 1975        | AL       | <1:10 <1:10          |
| 1177      | MEF    | 1975        | AL       | <1:10 1:20           |
| 1182      | MEF    | 1975        | AL       | <1:10 <1:10          |
| 1512A     | MEF    | 1986        | PA       | >1:40 1:10           |
| 1512B     | MEF    | 1986        | PA       | >1:40 1:10           |
| 3248A     | MEF    | 1983        | OH       | 1:20 1:40           |

* Assays in which opsonophagocytic killing was observed are highlighted. In, indeterminate result because the preimmune serum also mediated killing of the indicated strains.

### TABLE 3 Bacterial strains and plasmids

| Strain or plasmid | Relevant genotype or description | Reference or source |
|-------------------|----------------------------------|---------------------|
| **E. coli strains** |                                  |                     |
| DH5×              | q80lacZΔM15 recA1 endA1 gyrAB thi-1 hidR17 (rK m-') supE44 relA1 deoR Δ(lacZYA-argF)U169 phoA | Invitrogen Life Technologies |
| JM101             | supE thi Δ(lac-proAB) F' (traD36 proAB lacP2ZΔM15) | New England Biolabs |
| **Plasmids**      |                                  |                     |
| pGEMEX-1          | T7 expression plasmid for construction of in-frame T7 gene 10 fusions, Amp’ | Promega 49 |
| pGEMEX-11 Hia, BstEII del | pGEMEX-2 derivative with an in-frame fusion of T7 gene 10 and the portion of the strain 11 hia gene encoding amino acids 200 to 788 of the Hia protein | This study 54 |
| p3248A Hia        | pT7-7 derivative containing the full-length hia gene from NTHi strain 3248A | This study |
| pGEMEX-3248 Hia   | pGEMEX-1 derivative with an in-frame fusion of T7 gene 10 and the portion of the 3248A hia gene encoding amino acids 260 to 671 of the 3248A Hia protein | This study |

Within two or three subpassages of initial clinical isolation, NTHi strains were grown on chocolate agar or in brain heart infusion broth supplemented with hemin and NAD, as previously described (42, 49).

The Escherichia coli strains and bacterial plasmids used in this work are summarized in Table 3. E. coli strains were grown on Luria-Bertani (LB) agar or in LB broth and were stored at -70°C in LB broth with 10% glycerol. Strain DH5× was used for preparation of the plasmid constructs described below, and strain JM101 was used for generation of recombinant proteins. Ampicillin (100 μg/ml) was used for selection of strains containing the plasmids whose construction is described below.

### Construction of recombinant expression plasmids

The construction of the recombinant plasmid pGEMEX-Hia, which expresses a translational fusion protein comprising of T7 gene 10 derived from the pGEMEX-2 vector and the truncated strain 11 hia gene, has been described previously (49). A similar strategy was used to construct pGEMEX-3248 Hia, a plasmid which contains a 5' truncated hia gene derived from NTHi strain 3248A which is fused in frame with T7 gene 10. To generate the latter construct, we first cloned the strain 3248A hia gene into plasmid pT7-7 as described in an earlier work and designated that plasmid p3248A Hia (54). The pGEMEX-3248 Hia expression plasmid was constructed by first excising a 4.0-kbp SspI fragment from p3248 Hia and ligating the fragment into Hincl-digested pGEMEX-1. This SspI fragment encodes amino acids 260 to 1002 of the strain 3248A Hia protein and includes the 3' terminus of the 3248A hia gene as well as additional downstream DNA. The plasmid pGEMEX-3248A Hia PstI deletion contains the truncated hia gene just described with an additional 3' gene deletion. The latter plasmid was constructed by digesting pGEMEX-3248 Hia with PstI, recovering the larger 6.2-kbp fragment, and religating the free ends. Plasmid pGEMEX-3248A Hia PstI deleted encodes amino acids 260 to 671 of the strain 3248A Hia protein. The fidelity of the plasmid constructs was determined by selection of constructs with the expected restriction endonuclease gel profile and by detection of immunologically anti-Hia-reactive recombinant proteins of the expected molecular weight. E. coli strain DH5× was used for all of the cloning and subcloning work just described.

### Generation and purification of the GEMEX-Hia recombinant fusion proteins

In brief, each of the recombinant expression plasmids was first purified from DH5× transformed cells, and the resulting purified plasmids were used to transform E. coli strain JM101. To prepare the recombinant T7 gene 10-Hia fusion proteins, JM101/pGEMEX-11 Hia BstEII del or JM101/pGEMEX-3248 Hia PstI del was first recovered from a frozen stock culture and grown overnight at 37°C on LB agar with ampicillin. Several isolated colonies from the overnight growth were then used to inoculate a flask of LB broth containing 50 μg/l of ampicillin per ml, and the cells were allowed to grow to an A600 of 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) then was added to 1 mM, mGPI-2, an M13 phage phasing the T7 RNA polymerase gene, was added at a multiplicity of infection of 10, and 2 to 3 h later, cells were harvested. The recombinant proteins exist as inclusion bodies when generated using this method. The cell pellet recovered from each of the induced cell cultures was lysed in a Tris-HCl, NaCl, EDTA solution containing lysozyme and 1% Triton X-100, and DNase and RNase were added to digest DNA and RNA, respectively. The remaining pellets were washed repeatedly with a...
Triton X-100 solution prior to solubilization in 3 M guanidine. Refolding and resolubilization of recombinant proteins were achieved by slowly dialyzing the solution at 4°C in decreasing concentrations of guanidine in phosphate-buffered saline (PBS). Insoluble debris was removed from the solution by centrifugation, and protein purity was assessed by examination of fractions on polyacrylamide gels. Lipopolysaccharide content of the rHia fusion protein preparations was determined with a Limulus amebocyte lysate QCL-1000 kit (BioWhittaker, Walkersville, MD) according to the manufacturer’s instructions.

**Generation and purification of HMW1/HMW2 proteins.** The HMW1/HMW2 high-molecular-weight adhesion proteins were purified from NTHi strains 5, 12, and 15 using previously described methods (42). 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 each bacterial strain was grown in bulk to late log phase in six 500-ml flasks of brain heart infusion broth supplemented with NAD and hemin. The bacteria were then pelleted and frozen overnight at −20°C. The following day the bacterial pellets were resuspended in extraction solution (0.5 M NaCl, 0.01 M Na2EDTA, 0.01 M Tris, 50 μM 1, 10 phenanthroline, pH 7.5) on ice for 1 h, followed by centrifugation at 12,000 × g to remove the intact cells and cellular debris. The supernatants containing the water-soluble HMW1/HMW2 proteins from each strain were then centrifuged at 100,000 × g for 1 h to remove membrane fragments and other residual debris, and the final supernatants were dialyzed overnight against 0.01 M Na phosphate, pH 6.0. The following day, the pH-equilibrated supernatants were passed over 10-ml CM Sepharose columns that captured the HMW1/HMW2 proteins. The bound proteins were then eluted using a 0 to 0.5 M KCl gradient. Column fractions were analyzed on Coomassie blue-stained gels to identify fractions containing HMW1/HMW2 proteins. Fractions containing the HMW1/HMW2 proteins were then pooled and concentrated prior to passage over a Sepharose CL-6B (Sigma) gel filtration column. Column fractions containing HMW1/HMW2 proteins were again identified on Coomassie blue-stained gels, and the relevant fractions were pooled and stored at −70°C in preparation for animal immunization studies. The lipooligosaccharide content of the HMW1/HMW2 protein preparations was also determined with the Limulus amebocyte lysate QCL-1000 kit.

**Generation of anti-HMW1/HMW2 and anti-Hia antisera in guinea pigs.** Guinea pigs were immunized with the purified HMW1/HMW2 proteins from strain 5, 12, or 15 or recombinant GEMEX-Hia protein from strain 11 or 3248A, each prepared as described above. Each animal received five subcutaneous injections with 100 μg of each protein preparation administered every 4 weeks. The first dose was admixed with Freund’s complete adjuvant, and subsequent doses were admixed with incomplete Freund’s. A total volume of 1 ml of each preparation was divided between five or six subcutaneous injection sites. Serum antibody responses were monitored by enzyme-linked immunosorbent assay (ELISA) and by Western immunoblot assays with purified HMW1/ HMW2 and Hia proteins to document antibody responses. Once high-titer antibody responses were demonstrated, immune sera were assessed for their ability to mediate opsonophagocytic killing in the assay described below. All animal experiments complied with federal and institutional guidelines and were approved by the local Institutional Animal Care and Use Committee.

**Opsonophagocytic killing assay with NTHi target strains.** In brief, the growth conditions of the bacteria, the growth and differentiation of the HL-60 cells, and the opsonophagocytic assay itself were performed as described previously, with the exception that we used guinea pig immune sera in this study, in contrast to the chinchilla immune sera used in earlier work (41, 42, 49). The opsonophagocytic assay was performed in 5-ml capped polystyrene tubes (Sarstedt, Newton, NC). The complement source was human serum collected from a single healthy adult that was adsorbed to remove serum IgG by passing aliquots repeatedly over a protein G affinity column at 4°C. This treatment removed >95% of the serum IgG, as determined by laser nephelometry, yet maintained functional complement activity, as assessed in a standard total hemolytic complement assay. 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 complement control. Opsonophagocytic 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 complement control reaction tubes. The immune sera were screened at dilutions from 1:10 to 1:320 for the five homologous NTHi strains. The immune sera were screened at dilutions of 1:10 to 1:40 for the heterologous NTHi strains.

**RESULTS**

High-molecular-weight proteins purified from prototype strains and generation of immune sera. High-molecular-weight HMW1/ HMW2 proteins were purified from three prototype NTHi strains in preparation for generation of immune sera (Fig. 1). As noted in our earlier publications, almost all NTHi strains that express HMW1/HMW2 proteins express two closely related but distinct proteins that copurify with our standard purification protocol (38, 42). The protein preparations shown in Fig. 1 are mixtures of the respective HMW1 and HMW2 proteins from each of the three strains. As can be appreciated, the HMW1/HMW2 proteins from the three strains demonstrate notable differences in apparent molecular weight, likely reflecting the known differences in the sizes of the respective structural genes (38, 51), and also possibly in the degree and types of glycosylation present (55). As we noted in an earlier publication (42), the proteins are also subject to some degree of proteolysis, as reflected in the lower-molecular-weight species seen below the major proteins bands.

The proteins shown in Fig. 1 represent the fully processed mature forms of the respective HMW1 and HMW2 proteins expressed by each strain (51). As was reported in our earlier studies, the predicted amino acid sequences of the six HMW1/HMW2 proteins from these three prototype strains demonstrate between 52 and 62% identity and 63 and 72% similarity when pair-wise comparisons of the sequences are performed (see Table 2 and Fig. 7 in reference 51). The sequence variation among these six proteins is typical of that present among the HMW1/HMW2 proteins of the entire population of NTHi organisms whose sequences are currently available in public databases (56). The lipooligosaccharide content of each of the protein preparations was less than 0.05% as determined by the chromogenic Limulus amebocyte lysate assay.

Once the HMW1/HMW2 proteins of strains 5, 12, and 15 were purified, they were used to immunize guinea pigs for generation of high-titer antisera. In our earlier studies, we had generated high-titer anti-HMW1/HMW2 immune sera for use in opsonophagocytic assays in chinchillas (42, 49). However, we found that preimmune sera from a significant number of chinchillas had preexisting antibody that compromised the interpretation of results with immune sera.
prepared from the same animals. Guinea pig immune sera appeared to be equally active in the opsonophagocytic assay, but only infrequently was there preexisting antibody present.

**Opsonophagocytic activity mediated by anti-HMW1/HMW2 immune sera with HMW1/HMW2-expressing NTHi.**

Sixty-five disease-associated NTHi strains that expressed HMW1/HMW2-like proteins were examined in the opsonophagocytic assay (Table 1). Each strain was isolated from a child or adult with one of several different clinical illnesses caused by NTHi that included otitis media, bacteremia with sepsis, sinusitis, pneumonia, conjunctivitis, or meningitis. The strains were collected from a wide geographic area over a long time frame; thus, they represent a very diverse selection of isolates. Each strain was assessed for susceptibility to opsonophagocytic killing mediated by preimmune and immune sera raised against purified HMW1/HMW2 proteins from each of the three prototype NTHi strains, and all assays were run in duplicate. A very few strains were susceptible to opsonophagocytic killing mediated by the preimmune sera, and they are indicated in Table 1 by the designation In, for indeterminate, but most strains were not susceptible to killing mediated by any of the preimmune sera.

The three anti-HMW1/HMW2 immune sera mediated killing of 22 of 65, 43 of 65, and 28 of 65 unrelated HMW1/HMW2-expressing NTHi strains, respectively (Tables 1 and 4). As a group, the three sera mediated killing of 48 of the 65 HMW1/HMW2-expressing NTHi strains in the collection. None of the HMW1/HMW2 immune sera mediated killing of representative anti-Hia expressing NTHi strains.

The immune sera were also analyzed with respect to their ability to mediate opsonophagocytic killing in relation to the source of the bacterial isolates. As a group, the three HMW1/HMW2 immune sera mediated opsonophagocytic killing of 22 of 28 middle ear isolates, 8 of 10 respiratory tract isolates, 12 of 18 blood isolates, 6 of 7 conjunctival isolates, and 0 of 2 cerebrospinal fluid isolates. There was no obvious relationship between the source of the isolates and their susceptibility or resistance to opsonophagocytic killing, although the study did not have a sufficient number of strains in all categories to rigorously address the issue.

**Construction of Hia recombinant plasmids and generation of recombinant T7 gene 10-Hia fusion proteins.** Hia recombinant proteins were prepared from two unrelated NTHi strains in preparation for generation of the Hia immune sera. The construction of the strain 11 Hia recombinant plasmid and generation and purification of the strain 11 recombinant Hia protein were described in detail in our earlier work (49). We constructed a second Hia recombinant expression plasmid using the hia gene from NTHi strain 3248A (54). The strain 3248A hia gene was selected for study, because the Hia protein it encodes was known to be relatively distant from that of the strain 11 Hia protein when the Hia proteins of the nine hia genes that we had sequenced were phylogenetically compared (54).

To generate the 3248A recombinant protein used in this work, we constructed the pGEMEX-3248 Hia PstI del recombinant plasmid described in Materials and Methods. pGEMEX-3248 Hia PstI del encodes a large fragment of the Hia passenger domain from the mature 3248A Hia protein that is fused in frame with the T7 gene 10 derived from the pGEMEX-1 expression plasmid. The hia gene fragment cloned into pGEMEX-3248 Hia PstI del encodes amino acids 260 to 671 of the mature 3248A Hia protein. This particular fragment was selected for cloning and recombinant protein expression because it possessed useful restriction sites at the 5’ and 3’ termini, it encoded a relevant portion of the mature 3248A Hia protein, and we were able to generate abundant amounts of soluble recombinant protein from E. coli transformed with pGEMEX-3248 Hia PstI del.

Shown in Fig. 2A and B are schematic diagrams demonstrating and comparing the full-length strain 11 and 3248A Hia proteins and the respective fragments expressed by the recombinant plasmids used for generation of the strain 11 and strain 3248A Hia recombinant fusion proteins. Shown in Fig. 3 is an SDS-PAGE gel demonstrating the purified soluble strain 11 and strain 3248A recombinant Hia proteins expressed by the respective expression plasmids. A few minor lower-molecular-weight degradation products are present in each preparation, but they comprise a very small percentage of the overall protein present in each of the preparations. The lipopolysaccharide content of the two recombinant Hia protein preparations was less...
specific antibodies that induce bacteriolysis of the infecting organism and purify HMW1/HMW2 proteins could mediate variable opsonophagocytic killing of a small number of homologous and heterologous NTHi strains (42). In later similar work with the Hia-like proteins, we demonstrated that high-titer immune sera raised against a single prototype recombinant Hia protein could mediate opsonophagocytic killing of a small panel of heterologous Hia-expressing strains (49). The conclusions one could draw from those earlier studies concerning the cross-reactivity of antibodies specific for the HMW1/HMW2 and Hia proteins were limited by the relatively small number of strains examined, particularly in light of the known strain heterogeneity of NTHi in general (35, 50) and of the HMW1/HMW2 and Hia proteins in particular (38, 45, 51, 54).

The strength of the current work lies in the diversity and number of individual NTHi strains examined in the opsonophagocytic assay. While our data suggest that any single HMW1/HMW2 or Hia protein used in a vaccine would be unlikely to generate antibodies that mediate killing of most or all NTHi strains, the degree of cross-reactive opsonophagocytic killing was substantial and much greater than one might have predicted based upon the known differences in the amino acid sequences of the respective proteins (38, 45, 51, 54). As shown in Tables 1 and 4, a single HMW1/HMW2 immune serum mediated killing of over half of the HMW1/HMW2-expressing strains, and the three preparations together mediated killing of almost three-quarters of the HMW1/HMW2-expressing isolates. Similarly, each of the two recombinant Hia immune sera mediated killing of half of the Hia-expressing strains examined in this work, and together they mediated the killing of almost two-thirds of the Hia-expressing strains examined. These data suggest that a vaccine formulated with a limited number of HMW1/HMW2-like and Hia-like proteins could provide protection against disease caused by the great majority of NTHi strains.

At this time, there is no recognized correlate of protection for disease caused by NTHi (12). Our studies have focused on the ability of antibodies directed against the HMW1/HMW2 and Hia proteins to mediate opsonophagocytic killing. Whether this type of functional antibody activity is a good predictor of the potential value of these proteins as vaccine candidates is unknown. However, it should be noted that our assay was adapted from a previously described opsonophagocytic assay used extensively in the Streptococcus pneumoniae field (61, 62). The latter assay has been extremely valuable in assessing the functional antibody responses of recipients of several different pneumococcal vaccines and in predicting protection against disease in those individuals based upon the results of the opsonophagocytic assay (63–65). It is quite possible that the opsonophagocytic activity of the antibodies we are measuring in the NTHi assay would also be predictive of the functional activity of these antibodies in vivo and also could be of value in predicting the efficacy of future vaccines.

Although the data presented concerning the ability of anti-HMW1/HMW2 antibodies to mediate killing of homologous and heterologous strains are encouraging, other properties of these proteins could present challenges in terms of their utility as vaccine components. Expression of the HMW1/HMW2 proteins is subject to phase variation mediated by alterations in the number of 7-bp tandem repeats in the regions upstream of the hmw1A and hmw2A structural genes (66). In animal models of otitis media caused by NTHi (43) and in adults with chronic obstructive pulmonary disease associated with NTHi (67), it appears that immune pressure can select for phase variants with decreased levels of HMW1/HMW2 protein expression relatively quickly.

### DISCUSSION

Host immunity against NTHi is mediated both by relatively nonspecific components of the innate immune system (56–59) and by specific antibodies that induce bacteriolysis of the infecting organisms (13, 14, 37) or facilitate opsonophagocytosis in concert with host leukocytes and complement (41, 49, 60). In our early work, we demonstrated that antibodies directed against the HMW1/HMW2 proteins were major contributors to the opsonophagocytic activity mediated by naturally acquired human antibodies present in a human intravenous immunoglobulin preparation (41). In subsequent work, we demonstrated that high-titer immune sera raised in chinchillas against a representative panel of purified HMW1/HMW2 proteins could mediate variable opsonophagocytic killing of a small number of homologous and heterologous NTHi strains (42). In later similar work with the Hia-like proteins, we demonstrated that high-titer immune sera raised against a single prototype recombinant Hia protein could mediate opsonophagocytic killing of a small panel of heterologous Hia-expressing strains (49). The conclusions one could draw from those earlier studies concerning the cross-reactivity of antibodies specific for the HMW1/HMW2 and Hia proteins were limited by the relatively small number of strains examined, particularly in light of the known strain heterogeneity of NTHi in general (35, 50) and of the HMW1/HMW2 and Hia proteins in particular (38, 45, 51, 54).

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### TABLE 5 Opsonophagocytic killing of Hia-expressing NTHi mediated by anti-Hia antisera

| Antiserum | No. of strains tested | No. (%) of strains killed |
|-----------|-----------------------|--------------------------|
| 11 rHia   | 24                    | 12 (50)                  |
| 3248A rHia| 24                    | 13 (54)                  |
| Either    | 24                    | 15 (63)                  |

than 0.1% as determined by the chromogenic Limulus amebocyte lysate assay. Once the two recombinant Hia proteins were expressed and purified, each was used to prepare high-titer immune sera in guinea pigs in preparation for analysis in the opsonophagocytic assays.

Opsonophagocytic activity mediated by anti-Hia immune sera against Hia-expressing NTHi. Twenty-four disease-associated NTHi strains that expressed Hia-like proteins were examined in the opsonophagocytic assay (Table 2). As with the HMW1/HMW2-expressing strains, each was isolated from a child or adult with one of several different clinical illnesses caused by NTHi. Each strain was assessed for susceptibility to opsonophagocytic killing mediated by preimmune and immune sera raised against the purified recombinant Hia proteins from each of the two Hia-expressing NTHi strains. As with the HMW1/HMW2-expressing strains, a few test strains were susceptible to opsonophagocytic killing mediated by the preimmune sera. They were removed from the analysis completely if they were killed by both preimmune sera. If only one of the two preimmune sera mediated killing of a given strain, such is indicated in Table 2 by the designation In, for indeterminate.

The two anti-Hia immune sera mediated killing of 12 of 24 and 13 of 24 Hia-expressing NTHi strains, respectively (Tables 2 and 5). Together, the two sera mediated killing of 15 of the 24 Hia-expressing NTHi strains in the collection. None of the Hia immune sera mediated killing of representative HMW1/HMW2-expressing strains. As we did with the HMW1/HMW2-expressing strains, we also examined the Hia immune sera for their ability to mediate opsonophagocytic killing in relation to the source of the bacterial isolates. Together, the two Hia immune sera mediated opsonophagocytic killing of 11 of 16 middle ear isolates, 2 of 3 respiratory tract isolates, 2 of 3 blood isolates, and 0 of 2 cerebrospinal fluid isolates. Again, there was no obvious relationship between the source of the isolates and their susceptibility or resistance to opsonophagocytic killing.
from in vitro experiments with prototype Hia-expressing NTHi suggest that expression of the Hia proteins also is subject to phase variation (our unpublished observations). We have demonstrated experimentally that the susceptibility of prototype Hia-expressing strains to killing in the osonophagocytic assay is dependent upon the level of Hia protein expression (49), and similar relationships may exist with HMW1/HMW2-expressing strains. These sorts of data suggest that to be optimally effective in a vaccine, the HMW1/HMW2 and Hia proteins may need to be combined with one or more additional Haemophilus influenzae antigens in a multicomponent vaccine to prevent the emergence of variants with decreased levels of adhesin protein expression (12).

To conclude, in the current study we demonstrated that immune sera specific for the HMW1/HMW2 or Hia high-molecular-weight adhesins of NTHi could mediate broad-based osonophagocytic killing of bacteria expressing the corresponding adhesin type. While no single HMW1/HMW2 or Hia protein will likely be identified that could serve as a universal vaccine, our data suggest that a vaccine formulated with a limited number of HMW1/HMW2 and Hia proteins, perhaps in combination with other Haemophilus influenzae surface antigens, would be capable of providing protection against most NTHi strains. These data argue strongly for the continued investigation of the HMW1/HMW2 and Hia proteins as vaccine candidates for prevention of NTHi disease.

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