INFECTION AND IMMUNITY, Aug. 1996, p. 3032–3037 Vol. 64, No. 8
0019-9567/96/$04.00+0

Identification of Surface-Exposed B-Cell Epitopes on High-Molecular-Weight Adhesion Proteins of Nontypeable *Haemophilus influenzae*

STEPHEN J. BARENKAMP[1]* AND JOSEPH W. ST. GEME III[2]

Department of Pediatrics, St. Louis University School of Medicine, and The Pediatric Research Institute, Cardinal Glennon Children's Hospital,[1] and Edward Mallinckrodt Department of Pediatrics and Department of Molecular Microbiology, Washington University School of Medicine,[2] St. Louis, Missouri

Received 28 February 1996/Returned for modification 17 May 1996/Accepted 29 May 1996

We previously reported that two surface-exposed high-molecular-weight proteins, HMW1 and HMW2, expressed by a prototypic strain of nontypeable *Haemophilus influenzae* (NTHI) mediate attachment to human epithelial cells. These proteins are members of a family of highly immunogenic proteins common to most nontypeable *Haemophilus* strains. We also reported that immunization with an HMW1-HMW2 mixture modified the course of disease in an animal model of otitis media, suggesting the potential usefulness of these proteins as NTHI vaccine components. Identification of surface-accessible B-cell epitopes could be important to efforts to develop recombinant or synthetic peptide vaccines based upon these high-molecular-weight proteins. Thus, the purpose of the present study was to identify surface-accessible epitopes on the HMW1 and HMW2 proteins by using monoclonal antibodies (MAbs) and to determine the prevalence of these epitopes among the high-molecular-weight proteins expressed by heterologous nontypeable *Haemophilus* strains. MAbs were generated by immunizing mice with high-molecular-weight proteins purified from prototype strains and were screened by immunoelectron microscopy (IEM) for the ability to recognize surface epitopes. Two MAbs, designated AD6 and 10C5, that recognized surface epitopes by IEM were recovered. In order to map the epitopes recognized by these two MAbs, we constructed a set of HMW1 and HMW2 recombinant fusion proteins using the pGEMEX vectors and examined the reactivity of the MAbs with these fusion proteins. MAb AD6 recognized an epitope in both HMW1 and HMW2 which mapped to the last 75 amino acids at the carboxy termini of the two proteins. When examined for reactivity with heterologous strains, MAb AD6 recognized high-molecular-weight proteins in 75% of 125 unrelated nontypeable *Haemophilus* strains and, in addition, reacted with three of three such strains when examined by IEM. MAb 10C5 recognized an epitope that mapped to a 155-amino-acid segment near the carboxy terminus of HMW1. This epitope was adjacent to but distinct from the AD6 epitope and was absent from HMW2. The 10C5 epitope was expressed by 40% of the AD6 reactive strains. Identification of shared surface-exposed epitopes on the high-molecular-weight adhesion proteins suggests the possibility of developing recombinant or synthetic peptide-based vaccines protective against disease caused by the majority of NTHI strains.

Nontypeable *Haemophilus influenzae* (NTHI) organisms are nonencapsulated gram-negative organisms which are common inhabitants of the human upper respiratory tract (18). These organisms cause a variety of common mucosal surface infections such as otitis media, sinusitis, conjunctivitis, and chronic bronchitis (19). A critical first step in the pathogenesis of these infections is colonization of the respiratory tract mucosa. The bacterial molecules that mediate colonization have yet to be fully characterized, but in previous works, we identified a family of high-molecular-weight proteins that are important in attachment of NTHI to human epithelial cells (4, 26). The two closely related adhesion proteins expressed by our prototype strain were designated HMW1 and HMW2.

Bacterial surface molecules that mediate adherence are of particular interest as possible vaccine candidates (6). We recently reported that immunization with an HMW1-HMW2 mixture modified the course of disease in an animal model of otitis media (2), suggesting the potential usefulness of these proteins as components of a vaccine against NTHI. Knowledge of surface-accessible B-cell epitopes on these high-molecular-weight proteins could be important to efforts to develop recombinant or synthetic peptide vaccines based upon the proteins (24). Thus, the purpose of the present study was to identify surface-accessible epitopes on HMW1 and HMW2 by using monoclonal antibodies (MAbs) and to determine the percentage of heterologous NTHI strains that express such epitopes on their high-molecular-weight proteins.

* Corresponding author. Mailing address: Department of Pediatrics, Cardinal Glennon Children's Hospital, 1465 South Grand Blvd., St. Louis, MO 63104-1095. Phone: (314) 577-5644. Fax: (314) 268-6411. Electronic mail address: barenksj@sluvca.slu.edu.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The NTHI strains used in these studies have been described previously (3, 4). The majority of organisms were isolated in pure culture from middle-ear fluid specimens obtained from children with acute otitis media. Each strain was identified as *H. influenzae* by standard methods (17) 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 failure to show lines of precipitation with these antisera in counterimmunoelectrophoresis assays (28). Strains 5, 12, and 15 were the prototypic strains used in this investigation for purification of the high-molecular-weight adhesion proteins and for immunoelectron microscopy studies (3). The strain 12 mutants that are deficient in expression of the high-molecular-weight adhesion proteins have been described previously (26). All organisms were stored at −70°C in skim milk within two or three subpassages of the initial clinical isolation.

The T7 expression vectors pGEMEX-1 and pGEMEX-2 were obtained from Promega Corporation (Madison, Wis.). The M13 phage mGP1-2 (containing the bacteriophage T7 RNA polymerase gene) was a gift of Stanley Tabor (27).

Purification of native high-molecular-weight adhesion proteins. Native high-molecular-weight proteins were purified from prototypic nontypeable *Haemophilus* strain 5 and strain 12 as described elsewhere (2). In brief, a frozen bacterial stock culture was streaked onto a chocolate plate and allowed to grow overnight at 37°C. The following day, 5 to 10 colonies from the plate were used to inoculate a 50-ml starter culture of brain heart infusion broth supplemented with hemin and NAD, each at 10 μg/ml. The starter culture was shaken at 37°C in a rotary incubator at 250 rpm until the optical density of the culture reached an A_{600} of 0.6 to 0.8. Six 500 ml flasks of supplemented brain heart infusion broth were then 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 from the six flasks were pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C and frozen overnight at −20°C in preparation for purification of the proteins. The following day, the bacterial pellets from the six flasks were gently resuspended, combined, and incubated at 0°C for 60 min in 250 ml of extraction solution (0.5 M NaCl, 0.01 M Na_2–EDTA, 0.01 M Tris, 50 μM 1,10-phenanthroline [pH 7.5]). The bacterial cells were not sonicated or otherwise mechanically disrupted. The bacterial suspensions were then 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 soluble high-molecular-weight adhesion proteins, was then subjected to centrifugation at 100,000 × *g* for 60 min at 4°C to remove additional debris and membrane fragments. The supernatant from this ultracentrifugation step, which contained the water-soluble high-molecular-weight proteins, was dialyzed overnight at 4°C against 0.01 M sodium phosphate, pH 6.0.

The following day, the sample was centrifuged at 12,000 × *g* for 10 min at 4°C to remove insoluble debris that precipitated from the solution during dialysis. The resulting supernatant was then applied to a 10-ml carboxymethyl Sepharose column (Sigma Chemical Co., St. Louis, Mo.) which had been preequilibrated with 0.01 M sodium phosphate, pH 6.0. Following application of the protein-containing sample, the column was washed with 2 column volumes of 0.01 M sodium phosphate and the proteins were eluted with a 0 to 0.5 M KCl gradient. Column fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to identify fractions containing high-molecular-weight proteins. The column fractions that contained the high-molecular-weight proteins were pooled and concentrated to a volume of 1 to 3 ml and maintained at 0°C overnight. The following 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 gels. The relevant fractions were pooled and stored at −70°C in preparation for mouse immunizations.

Generation of hybridomas producing MAbs that recognize *Haemophilus* high-molecular-weight adhesion proteins. MAbs were generated by standard techniques (9). In brief, female BALB/c mice (4 to 6 weeks old) were immunized by intraperitoneal injection with high-molecular-weight proteins purified from nontypeable *Haemophilus* strain 5 or strain 12. The first injection of 40 to 50 μg of protein was administered with Freund's complete adjuvant, and the second dose, given 4 to 5 weeks after the first, was administered with PBS. Three days following the second injection, the mice were sacrificed and splenic lymphocytes were fused with SP2/O-Ag14 plasmacytoma cells.

Two weeks following fusion, hybridoma supernatants were screened for the presence of high-molecular-weight protein-specific antibodies by a dot blot assay. Purified high-molecular-weight proteins at a concentration of 10 μg/ml in Tris-buffered saline (TBS) were used to sensitize nitrocellulose sheets (Bio-Rad Laboratories, Richmond, Calif.) by soaking for 20 min. Following a blocking step with TBS–3% gelatin, the nitrocellulose was incubated for 60 min at room temperature with individual hybridoma supernatants, diluted 1:5 in TBS–0.1% Tween, by using a 96-well Bio-Dot microfiltration apparatus (Bio-Rad). After being washed, the sheets were incubated for 1 h with alkaline-phosphatase-conjugated affinity-isolated goat anti-mouse immunoglobulin G (IgG) and IgM antibodies (Tago, Inc., Burlingame, Calif.). Following additional washes, positive supernatants were identified by incubation of the nitrocellulose sheet in alkaline phosphatase buffer (0.10 M Tris, 0.10 M NaCl, 0.005 M $MgCl_2$) containing nitroblue tetrazolium (0.1 mg/ml) and 5-bromo-4-chloro-3-indolylphosphate (0.05 mg/ml).

For antibody isotyping and immunoelectron microscopy studies, the MAbs were purified from hybridoma supernatants. The antibodies we recovered in this study were of the IgG class. To purify the MAbs, the hybridoma supernatants were first subjected to ammonium sulfate precipitation (50% final concentration at 0°C). Following overnight incubation, the precipitate was recovered by centrifugation and resolubilized in PBS. The solution was then dialyzed overnight against 0.01 M sodium phosphate buffer, pH 6.0. The following day the sample was applied to a DEAE-Sephacel column preequilibrated with the same phosphate buffer, and the proteins were subsequently eluted with a KCl gradient. Column fractions containing the MAbs were identified by examination of samples on polyacrylamide gels for protein bands typical of light and heavy chains.

Antibody isotyping. The isotype of each MAb was determined by immunodiffusion using the Ouchterlony method. Immunodiffusion plates were prepared on glass slides with 10 ml of 1% DNA-grade agarose (FMC Bioproducts, Rockland, Maine) in PBS. After the agarose solidified, 5-mm wells were punched into the agarose in a circular pattern. The center well contained a concentrated preparation of the MAb being evaluated, and the surrounding wells contained goat anti-mouse subclass-specific antibodies (Tago). The plates were incubated for 48 h in a humid chamber at 4°C and then examined for white lines of immunoprecipitation.

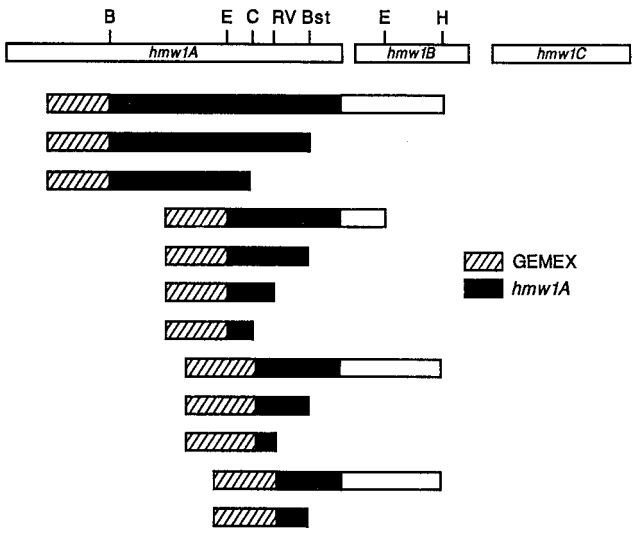

FIG. 1. Schematic diagram of pGEMEX-*hmw1* recombinant plasmids. The methods used for construction of the plasmids are described in detail in Materials and Methods. The hatched bars correspond to pGEMEX sequences, while the black bars represent segments of the *hmw1A* structural gene. A stop codon representing the termination codon of the *hmw1A* gene is present at the junction of the black and white segments. The locations of restriction enzyme recognition sequences used in construction of the plasmids are as indicated in the figure. Restriction enzymes are represented by the following abbreviations: B, *Bam*HI; E, *Eco*RI; C, *Cla*I; RV, *Eco*RV; Bst, *Bst*EII; and H, *Hin*dIII. The *hmw1A* gene is predicted to encode a protein of 1,536 amino acids. The restriction sites in the *hmw1A* gene encode amino acids at the junction sites corresponding to the following amino acid residues of the HMW1 protein: B, residue 466; E, residue 1020; C, residue 1122; RV, residue 1233; and Bst, residue 1388.

Western blot (immunoblot) assays with murine MAbs. Hybridoma supernatants that were reactive in the dot blot assay described above were examined by Western blot analysis, both to confirm the reactivity with the high-molecular-weight proteins of the homologous nontypeable *Haemophilus* strain and to examine the cross-reactivity with related proteins in heterologous strains. NTHI cell sonicates containing 100 μg of total protein were solubilized in electrophoresis sample buffer, subjected to SDS-PAGE on 7.5% acrylamide gels, and transferred to nitrocellulose with a Genie electrophoretic blotter (Idea Scientific Company, Corvallis, Oreg.) for 45 min at 24 V. After the transfer, the nitrocellulose sheet was blocked and then probed sequentially with the hybridoma supernatant and with alkaline phosphatase-conjugated goat anti-mouse IgG and IgM secondary antibody. Bound antibodies were detected by incubation with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate solution. This same assay was employed to examine the reactivity of the MAbs with recombinant fusion proteins expressed in *Escherichia coli* (see below).

Immunoelectron microscopy. Bacteria were grown overnight on chocolate agar supplemented with 1% IsoVitaleX, and several colonies were suspended in PBS. A 20-μl drop of this bacterial suspension was then applied to a carbon-coated, Formvar-strengthened grid and incubated for 2 min. Excess fluid was removed, and the specimen was rinsed twice with PBS. Next the sample was incubated for 30 min with PBS containing 1% bovine serum albumin and then for 1 h with the MAb being analyzed. Following removal of excess fluid and three rinses with PBS, the specimen was incubated for 30 min with goat anti-mouse IgG conjugated to 10-nm-diameter colloidal gold particles (Sigma). Finally, specimens were rinsed sequentially with PBS and distilled water and stained for 1 min with 0.5% uranyl acetate. After drying, they were examined in a Zeiss 10A electron microscope.

Construction of pGEMEX-*hmw1* and pGEMEX-*hmw2* recombinant plasmids. To map epitopes recognized by the MAbs, we examined their reactivity with a panel of recombinant fusion proteins expressed by pGEMEX recombinant plasmids. These plasmids were constructed by cloning various segments of the *hmw1A* or *hmw2A* structural genes (4, 5) into one of the pGEMEX vectors. Figures 1 and 2 depict the segments derived from the *hmw1* and *hmw2* gene clusters cloned into the pGEMEX expression plasmids. These segments were inserted such that in-frame fusions were created at each junction site. Thus, these plasmids would encode recombinant fusion proteins containing pGEMEX-encoded T7 gene 10 amino acids and *hmw1A*- or *hmw2A*-encoded amino acids.

Four discrete restriction sites within the *hmw1A* structural gene (*Bam*HI,

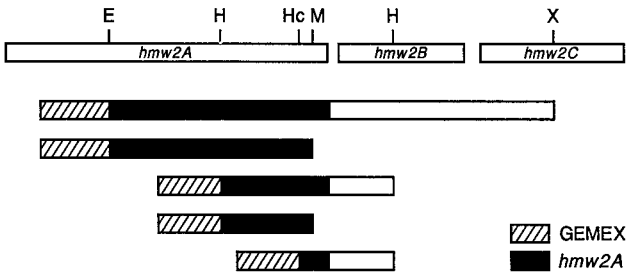

FIG. 2. Schematic diagram of pGEMEX-*hmw2* recombinant plasmids. The methods used for construction of the plasmids are described in detail in Materials and Methods. The hatched bars correspond to pGEMEX sequences, while the black bars represent segments of the *hmw2A* structural gene. A stop codon representing the termination codon of the *hmw2A* gene is present at the junction of the black and white segments. The locations of restriction enzyme recognition sequences used in construction of the plasmids are as indicated in the figure. Restriction enzymes are represented by the following abbreviations: E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; M, *Mlu*I; and X, *Xho*I. The *hmw2A* gene is predicted to encode a protein of 1,477 amino acids. The restriction sites in the *hmw2A* gene encode amino acids at the junction sites corresponding to the following amino acid residues of the HMW2 protein: E, residue 463; H, residue 973; Hc, residue 1336; and M, residue 1402.

*Eco*RI, *Cla*I, and *Eco*RV) were selected as the 5′ ends of the *hmw1* inserts (Fig. 1). For each 5′ end, a series of progressively smaller inserts was created by taking advantage of convenient downstream restriction sites. Three discrete sites within the *hmw2A* structural gene (*Eco*RI, *Hin*dIII, and *Hin*cII) were selected as the 5′ ends of the *hmw2* inserts (Fig. 2). A downstream *Mlu*I site was used to make truncated *hmw2* constructs.

Each of the recombinant plasmids was used to transform *E. coli* JM101. The resulting transformants were used to generate the recombinant fusion proteins employed in the mapping studies. To prepare recombinant proteins, the transformed *E. coli* strains were grown to an A_{600} of 0.5 in L broth containing 50 μg of ampicillin per ml. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added to 1 mM, and mGP1-2, the M13 phage containing the T7 RNA polymerase gene, was added at a multiplicity of infection of 10. One hour later, cells were harvested, and a sonicate of the cells was prepared. The protein concentrations of the samples were determined, and cell sonicates containing 100 μg of total protein were solubilized in electrophoresis sample buffer, subjected to SDS-PAGE, and stained with Coomassie blue to assess the expression level of recombinant fusion proteins. Once high levels of expression of the recombinant fusion proteins were confirmed, the cell sonicates were used in the Western blot analyses described above.

RESULTS

Generation of hybridomas producing MAbs against high-molecular-weight adhesion proteins of NTHI. We recovered 14 different hybridomas that produced MAbs reactive with the purified HMW1 and HMW2 proteins of nontypeable *Haemophilus* strain 12 in the immunoblot screening assay. Of the MAbs screened by immunoelectron microscopy, two were demonstrated to bind surface epitopes on prototype strain 12. These two MAbs, designated AD6 and 10C5, were both of the IgG1 subclass. The remainder of this communication details the studies carried out with these two MAbs.

Shown in Fig. 3 is an electron micrograph demonstrating surface binding of MAb AD6 to representative NTHI strains. In the upper left panel of the figure is nontypeable *Haemophilus* strain 12, and in the upper right panel is a strain 12 derivative that no longer expresses HMW1 or HMW2 (26). As can be seen, colloidal gold particles decorate the surface of strain 12, indicating bound AD6 antibody on the surface. In contrast, no gold particles are evident on the surface of the strain 12 mutant that no longer expresses HMW1 and HMW2. These results indicate that MAb AD6 is recognizing a surface-exposed epitope on the high-molecular-weight proteins of strain 12. Analogous studies with MAb 10C5 demonstrating that it too bound to surface-accessible epitopes on the high-molecular-weight proteins of strain 12 were performed.

Mapping of epitopes recognized by MAbs AD6 and 10C5. Having identified two surface-binding MAbs, we proceeded to map the epitope recognized by each of these antibodies. To localize the epitope recognized by MAb AD6, we examined the pattern of reactivity of this MAb with our large set of recombinant fusion proteins. Figure 4 shows a Western blot that demonstrates the pattern of reactivity of MAb AD6 with five recombinant fusion proteins, an informative subset of the larger number originally examined. On the basis of analysis of the pattern of reactivity of MAb AD6 with this set of proteins, the epitope it recognizes maps to a very short segment of the HMW1 and HMW2 proteins. A brief summary of this analysis follows. For reference, the relevant portions of the *hmw1A* or *hmw2A* structural genes that were expressed in the recombinant proteins being analyzed are indicated in the diagram at the top of Fig. 4.

As shown in lane 1, MAb AD6 recognizes an epitope encoded by fragment 1, a fragment that encompasses the 3′ one-fifth of the *hmw1A* gene and encodes 303 amino acids. Reactivity is lost when only the portion of the gene comprising fragment 2 is expressed. This fragment encodes the same 155 amino acids that are present in the proximal half of the fragment 1 peptide. Assuming that MAb AD6 is recognizing a linear epitope, this result localizes the epitope somewhere within the last 160 amino acids at the carboxy terminus of the HMW1 protein. MAb AD6 also recognizes an epitope encoded by fragment 3, a fragment that was derived from the *hmw2A* structural gene. This rather large fragment encompasses approximately one-third of the gene and encodes a 504-amino-acid peptide. Reactivity is lost when fragment 4 is expressed. The only difference between fragments 3 and 4 is that fragment 4 lacks 225 bp at the 3′ end of the *hmw2A* structural gene which encode residues 1402 through 1477 of the HMW2 protein. Again, assuming that AD6 is recognizing a linear epitope, this result suggests that the AD6 epitope is encoded by this short terminal segment of the *hmw2A* gene. Consistent with this hypothesis is the observation that MAb AD6 binds to the recombinant protein encoded by fragment 5, a fragment encompassing the distal 1/10 of the *hmw2A* structural gene which encodes residues 1336 through 1477 of the HMW2 protein. Taken together, these data identify the AD6 epitope as common to both the HMW1 and HMW2 proteins and strongly suggest that it lies somewhere within the 75-amino-acid segments at the carboxy termini of the HMW1 and HMW2 proteins. The amino acid sequences of the two proteins are 76% identical over this region (4).

Figure 5 shows a Western blot demonstrating the pattern of reactivity of MAb 10C5 with the same five recombinant fusion proteins examined in Fig. 4. As shown in lane 1, MAb 10C5 recognizes an epitope encoded by fragment 1. In contrast to MAb AD6, MAb 10C5 also recognizes an epitope encoded by fragment 2. Also in contrast to MAb AD6, MAb 10C5 does not recognize any of the *hmw2A*-derived recombinant fusion proteins. Thus, these data demonstrated that the 10C5 epitope is unique to the HMW1 protein and is encoded by fragment 2. This fragment corresponds to a 155-amino-acid segment of the HMW1 protein (4).

Expression of epitopes recognized by MAbs AD6 and 10C5 in heterologous NTHI strains. Having identified the approximate locations of the epitopes on HMW1 and HMW2 recognized by the two MAbs, we next determined the extent to which these epitopes are shared by the high-molecular-weight proteins of heterologous NTHI strains. In Western blot assays with bacterial cell sonicates, MAb AD6 reacted with the high-

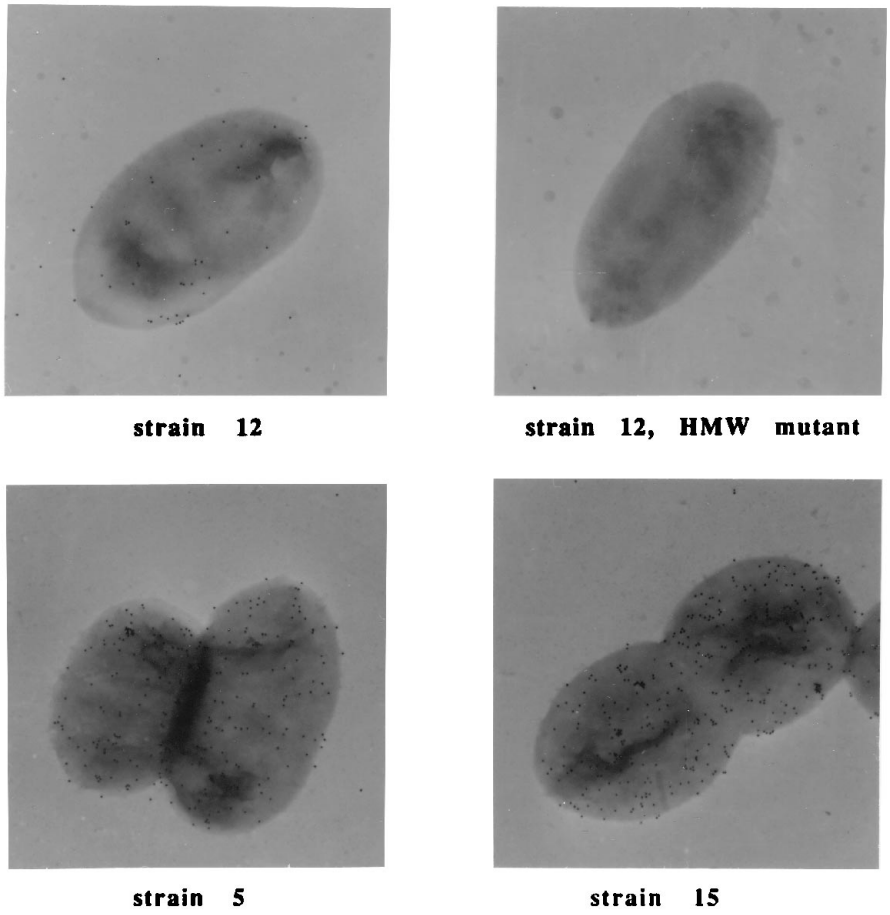

FIG. 3. Immunoelectron micrographs of representative NTHI strains after incubation with MAb AD6 followed by incubation with goat anti-mouse IgG conjugated with 10-nm-diameter colloidal gold particles. Strains are as labeled, with the HMW mutant being a strain 12 mutant deficient in expression of the high-molecular-weight proteins.

molecular-weight proteins of 75% of our collection of more than 125 nontypeable *Haemophilus* strains. Of note, this MAb recognized high-molecular-weight proteins in virtually all strains that we previously identified as expressing HMW1-HMW2-like proteins (4). Figure 6 is an example of a Western blot demonstrating the reactivity of MAb AD6 with a representative panel of such heterologous strains. As can be seen, the MAb recognizes one or two bands in the 100- to 150-kDa range in each of these strains.

In contrast to the broad cross-reactivity observed with MAb AD6, MAb 10C5 was much more limited in its ability to recognize high-molecular-weight proteins in heterologous strains. MAb 10C5 recognized high-molecular-weight proteins in approximately 40% of the strains that expressed HMW1-HMW2-like proteins. Neither MAb AD6 nor MAb 10C5 recognized proteins in any of the nontypeable *Haemophilus* strains which did not express HMW1-HMW2-like proteins.

Reactivity of MAb AD6 with surface-accessible epitopes on heterologous NTHI strains. In a limited fashion, we have examined the reactivity of MAb AD6 with surface-exposed epitopes on the heterologous strains. In the bottom two panels of Fig. 3 are electron micrographs demonstrating the reactivity of MAb AD6 with surface-accessible epitopes on nontypeable *Haemophilus* strains 5 and 15. As can be seen, abundant colloidal gold particles are present on the surface of each of these two strains, confirming their surface expression of the AD6

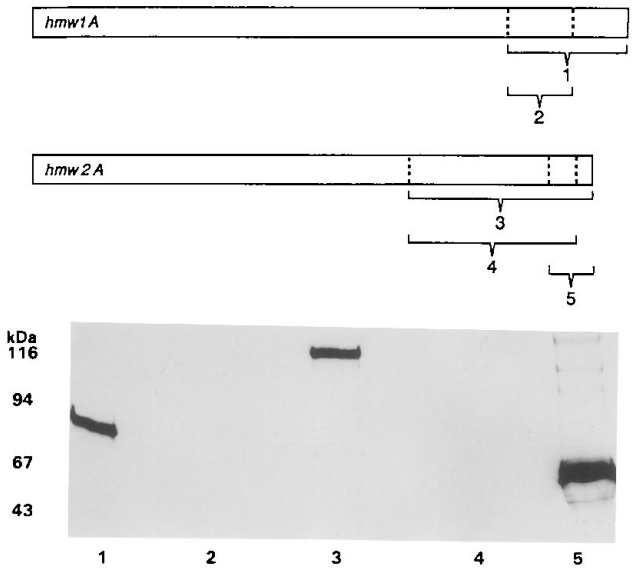

FIG. 4. Western immunoblot assay with MAb AD6 and HMW1 or HMW2 recombinant proteins. The upper panel indicates the segments of the *hmw1A* or *hmw2A* structural genes that are being expressed in the recombinant proteins. The lane numbers correspond to the indicated segments. Segments 1 and 2 encode amino acid residues 1233 to 1536 and 1233 to 1388, respectively, of the HMW1 protein; Segments 3 to 5 encode residues 973 to 1477, 973 to 1402, and 1336 to 1477, respectively, of the HMW2 protein.

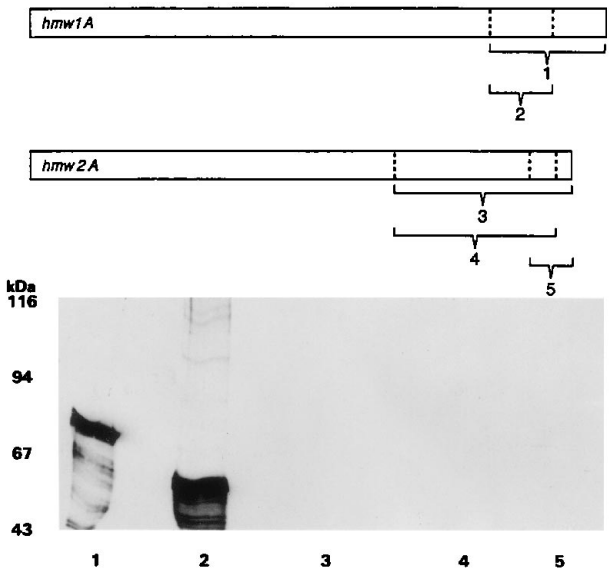

FIG. 5. Western immunoblot assay with MAb 10C5 and HMW1 or HMW2 recombinant proteins. The upper panel indicates the segments of the *hmw1A* or *hmw2A* structural genes that are being expressed in the recombinant proteins. The lane numbers correspond to the indicated segments. The segments being expressed are the same as those indicated in the legend to Fig. 4.

epitope. Although limited in scope, these data suggest that the AD6 epitope may be a common surface-accessible epitope on the high-molecular-weight adhesion proteins of most NTHI strains that express HMW1-HMW2-like proteins.

DISCUSSION

The components of the protective host immune response to NTHI infection have yet to be fully defined. Bacterial outer membrane proteins have been demonstrated experimentally to be potentially important protective antigens (1–3, 8, 16, 23, 25). Specifically, outer membrane proteins have been shown to be targets of bactericidal antibodies (8, 21, 22) or to be capable of stimulating the production of protective antibodies in animal models of infection (1, 2, 15, 16, 25). Proteins P2 and P6 have been shown to be targets of human bactericidal antibody (21, 22), and antibody directed against purified P2 was demonstrated to enhance pulmonary clearance of NTHI in a mouse model of infection (15). However, from the standpoint of possible vaccine development, the P2 protein is poorly immunogenic in infants recovering from acute otitis media caused by NTHI (3), and P2 also demonstrates significant strain heterogeneity (20, 23) as well as potential instability (13, 14). The P6 protein is highly conserved and remains an attractive vaccine candidate (11, 22). Recombinant P6 protein, truncated at the amino terminus and no longer fatty acylated, has also been prepared, and it too elicits bactericidal antibody and is protective against infection in a rat bacteremia model (10). Unfortunately, P6 offered little if any protection when evaluated in the chinchilla otitis model (12). In addition, P6 appears to be poorly immunogenic in children with recurrent otitis media, a group that would be a prime target for a nontypeable-*Haemophilus* vaccine (29). Thus, there is reason to continue efforts to define additional protective antigens.

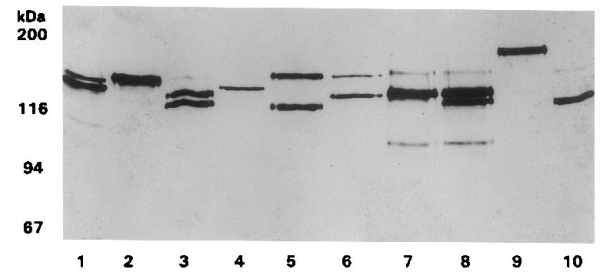

FIG. 6. Western immunoblot assay with MAb AD6 and a panel of unrelated NTHI strains that express HMW1-HMW2-like proteins. Cell sonicates were prepared from freshly grown samples of each strain prior to analysis in the Western blot. For reference, the strain in lane 1 is prototype strain 12 and the upper and lower immunoreactive bands are HMW1 and HMW2, respectively.

Attachment of NTHI to the epithelial surface of the upper respiratory tract is a critical first step in colonization of the human host. In theory, interruption of this colonization process should prevent disease. With a variety of other bacterial pathogens, adhesion molecules which, when administered as components of vaccines, are capable of preventing or modifying disease have been identified (6). In our earlier studies, we identified the HMW1-HMW2 family of high-molecular-weight proteins as critical adhesion molecules for NTHI (26). Furthermore, we recently demonstrated that immunization with these proteins was capable of modifying disease in the chinchilla model of nontypeable-*Haemophilus* otitis media (2). Thus, these high-molecular-weight adhesion proteins also are important proteins to consider as possible vaccine candidates.

The degree to which these proteins are conserved among strains and the extent to which they share common surface-accessible epitopes is critical to their potential usefulness as vaccine components. The HMW1-HMW2 family of adhesion proteins demonstrates significant size heterogeneity when proteins of different strains are compared (Fig. 6). This size variability is likely indicative of some degree of antigenic variation, a characteristic which would be important for survival of these organisms in the human host (7). Although such antigenic variability could preclude the use of these proteins as components of a vaccine providing broad protection against nontypeable-*Haemophilus* infection, our MAb data suggest that the situation might not be so bleak. We were able to identify at least one surface-exposed B-cell epitope, defined by MAb AD6, that was shared by most or all NTHI strains which express HMW1-HMW2-like proteins. Furthermore, we previously reported that 70 to 75% of nontypeable *Haemophilus* strains express proteins of the HMW1-HMW2 family (4). The identification of shared surface-exposed epitopes on the high-molecular-weight adhesion proteins suggests that it may be possible to develop recombinant or synthetic peptide-based vaccines based upon these proteins which would be protective against disease caused by the majority of NTHI strains.

ACKNOWLEDGMENTS

We thank Linda Winter and David Cutter for expert technical assistance.

This work was supported by Public Health Service grants AI-21707 from the National Institute of Allergy and Infectious Diseases to S.J.B. and DC-02873 from the National Institute on Deafness and Other Communication Disorders to J.W.S. and by a grant-in-aid from the American Heart Association to J.W.S.

REFERENCES

1. **Barenkamp, S. J.** 1986. Protection by serum antibodies in experimental nontypable *Haemophilus influenzae* otitis media. Infect. Immun. **52:**572–578.
2. **Barenkamp, S. J.** 1996. Immunization with high-molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae* modifies experimental otitis media in chinchillas. Infect. Immun. **64:**1246–1251.

3. **Barenkamp, S. J., and F. F. Bodor.** 1990. Development of serum bactericidal activity following nontypable *Haemophilus influenzae* acute otitis media. Pediatr. Infect. Dis. J. **9:**333–339.
4. **Barenkamp, S. J., and E. Leininger.** 1992. Cloning, expression, and DNA sequence analysis of genes encoding nontypeable *Haemophilus influenzae* high-molecular-weight surface-exposed proteins related to filamentous hemagglutinin of *Bordetella pertussis*. Infect. Immun. **60:**1302–1313.
5. **Barenkamp, S. J., and J. W. St. Geme III.** 1994. Genes encoding high-molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae* are part of gene clusters. Infect. Immun. **62:**3320–3328.
6. **Beachey, E. H.** 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. **143:** 325–345.
7. **Brunham, R. C., F. A. Plummer, and R. S. Stephens.** 1993. Bacterial antigenic variation, host immune response, and pathogen-host coevolution. Infect. Immun. **61:**2273–2276.
8. **Gnehm, H. E., S. I. Pelton, S. Galati, and P. A. Rice.** 1985. Characterization of antigens from nontypable *Haemophilus influenzae* recognized by human bactericidal antibodies: role of *Haemophilus* outer membrane proteins. J. Clin. Invest. **75:**1645–1658.
9. **Goding, J. W.** 1986. Monoclonal antibodies: principles and practice, p. 59–141. Academic Press Limited, London.
10. **Green, B. A., B. J. Metcalf, T. Quinn-Dey, D. H. Kirkley, S. A. Quataert, and R. A. Deich.** 1990. A recombinant non-fatty acylated form of the Hi-PAL (P6) protein of *Haemophilus influenzae* elicits biologically active antibody against both nontypeable and type b *H. influenzae*. Infect. Immun. **58:**3272–3278.
11. **Green, B. A., T. Quinn-Dey, and G. W. Zlotnick.** 1987. Biologic activities of antibody to peptidoglycan-associated lipoprotein of *Haemophilus influenzae* against multiple clinical isolates of *H. influenzae* type b. Infect. Immun. **55:**2878–2883.
12. **Green, B. A., M. D. Vasquez, G. W. Zlotnick, G. Quigley-Reape, J. D. Swarts, I. Green, J. L. Cowell, C. D. Bluestone, and W. J. Doyle.** 1993. Evaluation of mixtures of purified *Haemophilus influenzae* outer membrane proteins in protection against challenge with nontypeable *H. influenzae* in the chinchilla otitis media model. Infect. Immun. **61:**1950–1957.
13. **Groeneveld, K., L. van Alphen, P. P. Eijk, H. M. Jansen, and H. C. Zanen.** 1988. Changes in outer membrane proteins of nontypable *Haemophilus influenzae* in patients with chronic obstructive pulmonary disease. J. Infect. Dis. **158:**360–365.
14. **Groeneveld, K., L. van Alphen, C. Voorter, P. P. Eijk, H. M. Jansen, and H. C. Zanen.** 1989. Antigenic drift of *Haemophilus influenzae* in patients with chronic obstructive pulmonary disease. Infect. Immun. **57:**3038–3044.
15. **Hansen, E. J., D. A. Hart, J. L. McGehee, and G. B. Toews.** 1988. Immune enhancement of pulmonary clearance of nontypable *Haemophilus influenzae*. Infect. Immun. **56:**182–190.
16. **Karasic, R. B., C. E. Trumpp, H. Gnehm, P. A. Rice, and S. I. Pelton.** 1985. Modification of otitis media in chinchillas rechallenged with nontypable *Haemophilus influenzae* and serologic response to outer membrane antigens. J. Infect. Dis. **151:**273–279.
17. **Kilian, M.** 1985. *Haemophilus*, p. 387–393. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
18. **Kuklinska, D., and M. Kilian.** 1984. Relative proportions of *Haemophilus* species in the throat of healthy children and adults. Eur. J. Clin. Microbiol. **3:**249–252.
19. **Murphy, T. F., and M. A. Apicella.** 1987. Nontypable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection. Rev. Infect. Dis. **9:**1–15.
20. **Murphy, T. F., and L. C. Bartos.** 1988. Purification and analysis with monoclonal antibodies of P2, the major outer membrane protein of nontypeable *Haemophilus influenzae*. Infect. Immun. **56:**1084–1089.
21. **Murphy, T. F., and L. C. Bartos.** 1988. Human bactericidal antibody response to outer membrane protein P2 of nontypeable *Haemophilus influenzae*. Infect. Immun. **56:**2673–2679.
22. **Murphy, T. F., L. C. Bartos, P. A. Rice, M. B. Nelson, K. C. Dudas, and M. A. Apicella.** 1986. Identification of a 16,600-dalton outer membrane protein of nontypable *Haemophilus influenzae* as a target for human serum bactericidal antibody. J. Clin. Invest. **78:**1020–1027.
23. **Murphy, T. F., A. A. Campagnari, M. B. Nelson, and M. A. Apicella.** 1989. Somatic antigens of *Haemophilus influenzae* as vaccine components. Pediatr. Infect. Dis. J. **8:**S66–68.
24. **Rabinovich, N. R., P. McInnes, D. L. Klein, and B. F. Hall.** 1994. Vaccine technologies: view to the future. Science **265:**1401–1404.
25. **Sirakova, T., P. E. Kolattukudy, D. Murwin, J. Billy, E. Leake, D. Lim, T. DeMaria, and L. Bakaletz.** 1994. Role of fimbriae expressed by nontypeable *Haemophilus influenzae* in pathogenesis of and protection against otitis media and relatedness of the fimbrin subunit to outer membrane protein A. Infect. Immun. **62:**2002–2020.
26. **St. Geme, J. W., III, S. Falkow, and S. J. Barenkamp.** 1993. High-molecular-weight proteins of nontypable *Haemophilus influenzae* mediate attachment to human epithelial cells. Proc. Natl. Acad. Sci. USA **90:**2875–2879.
27. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
28. **Wallace, R. J., Jr., D. M. Musher, E. J. Septimus, J. E. McGowan, Jr., F. J. Quinones, K. Wiss, P. H. Vance, and P. A. Trier.** 1981. *Haemophilus influenzae* infection in adults: characterization of strains by serotypes, biotypes, and beta-lactamase production. J. Infect. Dis. **144:**101–106.
29. **Yamanaka, N., and H. Faden.** 1993. Antibody response to outer membrane proteins of nontypeable Haemophilus influenzae in otitis-prone children. J. Pediatr. **122:**212–218.

Editor: J. R. McGhee