Construction and Immunogenicity of Recombinant Adenovirus Vaccines Expressing the HMW1, HMW2, or Hia Adhesion Protein of Nontypeable Haemophilus influenzae

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The objective of the present study was to construct and assess the immunogenicity of recombinant adenovirus vectors expressing the HMW1, HMW2, or Hia protein of nontypeable Haemophilus influenzae (NTHi). These proteins are critical adhesins and potential protective antigens expressed by NTHi. Segments of the hmwlA and hmw2A structural genes that encode the distal one-half of mature HMW1 or HMW2 were cloned into the T7 expression vector pGEMEX-2. These constructs encoded stable HMW1 or HMW2 recombinant fusion protein that expresses B-cell epitopes common to most NTHi strains. A segment of the hia gene that encodes the surface-exposed portion of mature Hia was also cloned into pGEMEX-2. The resulting T7 gene 10 translational fusions were excised from the parent plasmids and cloned into the shuttle plasmid pDC316. Cotransfection of HEK 293 cells with the pDC316 derivatives and pHGlOxΔE1,3Cre resulted in the production of viral plaques from which recombinant adenoviruses expressing fusion proteins were recovered. Chinchillas immunized intraperitoneally with a single 10^8-PFU dose of either the HMW2 or Hia adenoviral construct developed high anti-HMW2 or anti-Hia serum antibody titers within 4 weeks of immunization. Chinchillas immunized intranasally with a single 10^7- to 10^9-PFU dose of the Hia adenoviral construct also developed high anti-Hia serum antibody titers within 8 weeks of immunization. Recombinant adenoviruses represent a promising system to induce mucosal and systemic immunity and protection against mucosal diseases such as otitis media. Recombinant adenoviruses expressing recombinant HMW1, HMW2, or Hia protein will be important new tools in NTHi vaccine development efforts.

Otitis media remains a significant health problem for children in this country and elsewhere in the world (15, 16). Most children in the United States have had at least one episode of otitis by the third birthday, and one-third have had three or more episodes (51). In addition to the short-term morbidity and costs of this illness, the potential for delay or disruption of normal speech and language development in children with persistent middle ear effusions is a subject of considerable concern (50). Experts in the field have strongly recommended that efforts be made to develop safe and effective vaccines for prevention of otitis media in young children (26).

Bacteria, usually in pure culture, can be isolated from middle ear exudates in approximately two-thirds of cases of acute otitis media (20, 53). Streptococcus pneumoniae has been the most common bacterial pathogen recovered in all age groups, with isolation rates commonly ranging from 35% to 40% (20, 53). Nontypeable Haemophilus influenzae (NTHi) is the second most common bacterium recovered and accounts for 20% to 30% of cases of acute otitis media and a larger percentage of cases of chronic and recurrent disease (37). Interestingly, since introduction of the pneumococcal conjugate vaccine as part of the regular childhood vaccination schedule, nontypeable Haemophilus influenzae has become an even more common cause of acute and recurrent middle ear disease, often surpassing Streptococcus pneumoniae in frequency of recovery from middle ear fluid specimens (12, 18).

Many different antigens have been suggested as possible nontypeable Haemophilus influenzae vaccine candidates (1, 5, 23, 43, 44, 63). In our early work, we demonstrated that development of bactericidal antibody in the sera of children who had recovered from acute NTHi otitis media was associated with the appearance of serum antibodies directed against highly immunogenic high-molecular-weight (HMW) proteins (7). This work led subsequently to the identification and characterization of the HMW1 and HMW2 (HMW1/HMW2) family of proteins (8). The HMW1/HMW2 proteins have subsequently been shown to be major adhesins of nontypeable Haemophilus influenzae (57) as well as targets of opsonophagocytic (65, 66) and protective (6) antibodies. The HMW1/HMW2-like proteins are expressed by approximately 75% of NTHi strains (8, 58). The 25% of NTHi strains that do not express HMW1/HMW2-like proteins also express immunogenic high-molecular-weight proteins that are recognized by human convalescent-phase serum antibodies (11). Almost all the HMW1/HMW2-negative strains have subsequently been shown to express a second distinct class of adhesins known as Hia proteins (11). The Hia proteins are members of a large family of bacterial proteins known as autotransporters that are found in many Gram-negative bacteria (28, 69). The Hia proteins have also recently been shown to serve as targets for opsonophagocytic antibodies (64). Nearly all NTHi strains that lack HMW1/HMW2 proteins contain a hia gene and express a...
TABLE 1. Bacterial strains, plasmids, and cell lines

| Strain, plasmid, or cell line | Relevant genotype or description | Reference or source |
|-----------------------------|----------------------------------|---------------------|
| E. coli strain DH5α          | Δ(lacZYA-argF)U169 phyA           | Invitrogen Life Technologies |
| Plasmids                    |                                  |                     |
| pHMW1-15                    | pT7-7 derivative containing the hmw1 gene cluster and flanking DNA | 9                    |
| pHMW2-21                    | pT7-7 derivative containing the hmw2 gene cluster and flanking DNA | 9, 57                |
| pGEMEX-2                    | T7 expression plasmid for construction of in-frame translational fusions with T7 gene 10, Amp⁴ | Promega |
| pDC316                      | E1 shuttle plasmid, derived from the left end of the Ad5 genome, which contains the human cytomegalovirus immediate early promoter, a polycloning site, and the SV40 polyadenylation signals | Microbix Biosystems Inc. |
| pBHGIoxΔE1,3Cre             | Plasmid complementary to pDC316 derived from the nearly full-length Ad5 genome but with deletions in the Ad E1 and E3 regions | Microbix Biosystems Inc. |
| pGEMEX-HMW1 Eco             | pGEMEX-2 derivative with an in-frame fusion of T7 gene 10 and the portion of the hmw1A gene encoding amino acids 1020 to 1536 of the HMW1 protein | 10                   |
| pGEMEX-HMW2 Hind            | pGEMEX-2 derivative with an in-frame fusion of T7 gene 10 and the portion of the hmw24 gene encoding amino acids 973 to 1477 of the HMW2 protein | 10                   |
| pGEMEX-Hia BstEII del       | pGEMEX-2 derivative with an in-frame fusion of T7 gene 10 and the portion of the hia gene encoding amino acids 200 to 788 of the strain 11 Hia protein | 64                   |
| pDC316-HMW1 Eco             | pDC316 derivative with the BglII-HindIII fragment from pGEMEX-HMW1 Eco, containing the T7 gene 10 hmw1A gene fusion described above, cloned into the polycloning site of Smal-HindIII-digested pDC316 | This study |
| pDC316-HMW2 Hind            | pDC316 derivative with the BglII-HindIII fragment from pGEMEX-HMW2 Hind, containing the T7 gene 10 hmw2A gene fusion described above, cloned into the polycloning site of Smal-HindIII-digested pDC316 | This study |
| pDC316-Hia BstEII del       | pDC316 derivative with the BglII-HindIII fragment from pGEMEX-Hia BstEII del, containing the T7 gene 10 hia gene fusion described above, cloned into the polycloning site of Smal-HindIII-cut pDC316 | This study |
| Cell lines                  |                                  |                     |
| HEK 293                     | Human embryonic kidney cell line transfected with the left side of the Ad5 genome | Microbix Biosystems Inc. |
| A549                        | Lung carcinoma respiratory epithelium cell line | ATCC |

Hia protein, and conversely, strains that express HMW1/ HMW2 proteins lack a hia gene (11, 58).

Several groups have begun exploring mucosal and, in particular, nasopharyngeal immunization strategies to stimulate a protective immune response in the upper respiratory tract and middle ear (19, 24), and results to date have been encouraging (4, 29, 32, 47). Intranasal immunization has a number of potential advantages over traditional parenteral immunization approaches for prevention of otitis media. The presence of abundant microvilli in the nasal cavity greatly increases the available surface area of this anatomical site, thereby generating a large absorptive surface (34). Immunization via the nasal cavity also allows direct delivery of immunogens of interest to the nasal cavity, which is readily accessible and allows for noninvasive delivery of antigens or vaccines, thus eliminating the need for trained staff or the use of sterile needles and syringes. The option of treating or immunizing against human disease with such a noninvasive technique would almost certainly result in increased patient compliance if intranasal vaccines advance to the clinic.

A number of mucosal vaccination strategies continue to be actively explored (41, 49). Adenoviruses have been identified as promising live recombinant vaccine vectors based upon their ability to induce high levels of heterologous gene expression (13, 25, 55, 60) and to stimulate mucosal immunity in the upper respiratory tract, their natural site of replication. E1-deletion-containing replication-defective adenoviral recombinants based on human serotype 5 (Adhu5) have been tested as vaccine candidates for prevention of a number of infectious diseases (21, 60). Studies of adenoviral vaccines based upon the H5N1 hemagglutinin protein (30), surface proteins of Plasmodium falciparum (54), and the E6 and E7 oncoproteins of human papillomavirus type 16 (HPV-16) (27) have demonstrated that E1-deletion-containing vaccines induce excellent B-cell and CD8⁺-T-cell responses in experimental animals, even if given at moderate doses.

Recombinant adenovirus (rAd) vectors have yet to be investigated as potential vaccine candidates for prevention of otitis media. However, they are particularly attractive candidates for the reasons noted above. The objective of the present study was to construct E1-deletion-containing replication-defective recombinant adenovirus vectors expressing the HMW1, HMW2, or Hia adhesion protein of nontypeable Haemophilus influenzae and to assess their immunogenicity in the chinchilla experimental model.

MATERIALS AND METHODS

Bacteria, plasmids, and cell lines. The bacterial strain and plasmids used in this study are listed in Table 1. Escherichia coli strain DH5α has been described previously (67). The plasmid pGEMEX-2 is a T7 expression plasmid (Promega, Madison, WI) that was used for construction of translational fusions with the hmw1A, hmw2A, and hia genes (10, 64). Plasmid pDC316 is an E1 shuttle
plasmid, derived from the left end of the adenovirus type 5 (Ad5) genome, which contains the human cytomegalovirus immediate early promoter upstream of a polycoding site and simian virus 40 (SV40) polyadenylation signal (Microbix Biosystems Inc., Toronto, Ontario, Canada). pBH1GloxE1.3Cre is a plasmid complementary to pDC316 that is derived from a nearly full-length Ad5 genome but contains deletions in the Ad E1 and E3 regions (Microbix Biosystems Inc.). Low-passage-number human embryonic kidney 293 cells were obtained from Microbix Biosystems Inc. and were used for recovery and growth of recombinant adenoviruses. A59 respiratory epithelial cells were obtained from the American Type Culture Collection (Manassas, VA) and were used for transient expression experiments with recombinant adenoviruses.

Construction of pGEMEX recombinant plasmids containing T7 gene 10 translationally fused with the hmw1A, hmw2A, or hia gene. Plasmid pGEMEX-HMW1 Eco contains a 5' truncated hmw1A gene fused in frame to T7 gene 10 present in pGEMEX-2. pGEMEX-HMW1 Eco was constructed by excising a 2.1-kbp EcoRI fragment from pHMW1-13 (9) and ligating it into EcoRI-digested pGEMEX-2. This fragment encodes amino acids 1020 to 1536 of the strain 12 HMW1 protein and includes the 3' terminus of the hmw1A gene as well as additional downstream DNA. Plasmid pGEMEX-HMW2 Hind contains a 5'-truncated hmw2A gene fused in frame to T7 gene 10 present in pGEMEX-2. Plasmid pGEMEX-HMW2 Hind was constructed by excising a 2.4-kbp HindIII fragment from pHMW2-21 (9, 57) and ligating it into HindIII-digested pGEMEX-2. This fragment encodes amino acids 973 to 1477 of the strain 12 HMW2 protein and includes the 3' terminal 35 amino acids of hmw2A gene as well as additional downstream DNA. Plasmid pGEMEX-Hia BstEII del encodes amino acids 200 to 788 of the strain 11 Hia protein. The construction of this plasmid was described previously (64).

Construction of pDC316 recombinant plasmids containing T7 gene 10 translational fusions with the hmw1A, hmw2A, or hia gene. Plasmid pDC316-HMW1 Eco was constructed by excising the 3.2-kbp SmaI-Mand-HindIII fragment, containing the hmw1A gene-T7 gene 10 fusion described above, from pGEMEX-HMW1 Eco and ligating it into SmaI-Mand-HindIII-digested pDC316. Plasmid pDC316-HMW2 Hind was constructed by excising the 3.3-kbp SmaI-Mand-HindIII fragment, containing the hmw2A gene-T7 gene 10 fusion described above, from pGEMEX-HMW2 Hind and ligating it into SmaI-Mand-HindIII-digested pDC316. Plasmid pDC316-Hia BstEII del was constructed by excising the 2.9-kbp BglII fragment, containing the hia gene-T7 gene 10 fusion from pGEMEX-Hia BstEII del (64), and ligating it into BglII-digested pDC316.

The fidelity of the plasmid constructs was determined by the selection of constructs with the expected restriction endonuclease gel profiles and by the demonstration of abundant recombinant protein expression when DH5α was transformed with the respective plasmids. The construction of this plasmid was described previously (64).

Generation of recombinant plasmids expressing the HMW1, HMW2, or Hia protein. For recombinant adenoviruses, the pDC316 recombinant plasmids which contained fragments of the hmw1A, hmw2A, or hia structural gene were cotransfected into HEK 293 cells with plasmid pBH1GloxE1.3Cre (46). Recovery of recombinant adenovirus is greatly enhanced in this system by the contribution of Cre-mediated site-specific recombination (46). Transfections were performed with HEK 293 cells in 60-mm dishes maintained at 37°C in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 0.4 g per ml hydrocortisone, and 2 µg per ml isopropenol (45). Cells grown in 60-mm dishes were passaged 1 day prior to transfection and were at 80% confluent when used in the experiments. To transfected the cells, the medium was removed from the cells immediately prior to overlaying the cell layers with 0.5 ml of tissue culture medium containing the recombinant adenovirus under study. The adenoviral concentrations in these transfection preparations were approximately 10⁶ PFU per ml. The adenoviruses were allowed to adsorb to the cell monolayer for 30 min at 37°C, and then 5 ml of the tissue culture medium was added to the cells. The cells were then maintained in a standard tissue culture incubator at 37°C with 5% CO2 for 10 days before the cells were harvested for additional study.

Western immunoblot assays to monitor expression of recombinant proteins in HEK 293 and A59 cell lines. Adherent cells were released from the tissue culture dishes with citrate saline, in the case of the HEK 293 cells, and trypsin-EDTA solution, in the case of the A59 cells. The released cells were then concentrated by centrifugation at 750 × g at 4°C, the supernatant was discarded, and the cells were suspended in 0.5 ml of phosphate-buffered saline (PBS). Filtration of each cell suspension was solubilized in electrophoresis sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% acrylamide gels, and transferred to nitrocellulose with a Genie electrophoretic blotter (Idea Scientific Company, Corvallis, OR) for 45 min at 24 V. After transfer, the nitrocellulose sheet was blocked with a 3% gelatin solution in Tris-buffered saline and then probed with anti-HMW1/ HMW2 monoclonal antibody (MAb) 4G4 (10, 65) or anti-Hia MAb 1F4 (64), followed by alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody. Bound antibodies were detected by incubation with nitroblue tetrazolium–5-bromo–4-chloro–3-indolylphosphate solution.

Immunization of chinchillas with recombinant adenoviruses expressing the HMW2 or Hia protein. For the immunogenicity experiments, adult outbred chinchillas were immunized with one of the purified recombinant adenoviruses described above. For the parenteral immunization studies, the animals received 10⁹ PFU of either the recombinant adenovirus (rAd) HMW2 or rAd Hia suspended in 1 ml of PBS and administered by intraperitoneal injection. For the intranasal immunization studies, the animals received 10⁷ to 10⁸ PFU of either the rAd HMW2 or rAd Hia constructed in 0.5 ml of PBS. For administration of the intranasal doses, chinchillas were first sedated with keta-
enzyme recognition sites are as indicated. of the respective mature protein. The locations of several restriction

FIG. 1. Schematic representation of the hmw1, hmw2, and hia
genotypes and respective fragments used for recombinant adenoviral
carrier construction. Mature 5’ refers to the locations within the respectiv
gene of the codons that encode the amino acids at the N termini
term of the respective mature protein. The locations of several restriction

RESULTS

Construction of recombinant plasmids expressing segments of the hmw1A, hmw2A, or hia gene of prototype NTHi strains 11 and 12. Efforts to generate recombinant adenoviruses expressing full-length HMW1/HMW2 and Hia proteins were unsuccessfu

Expression of recombinant HMW1, HMW2, or Hia protein in HEK 293 cells. After generation of the pGEMEX-2-hmw1, hmw2, and hia recombinant plasmids just described, the T7 gene 10-adhesin gene fusion from each construct was excised from the respective parent plasmid and cloned into pDC316 (Fig. 2B). Recall that the pDC316 derivatives are required for cotransfection of HEK 293 cells with pBHGlloxΔE1,3Cre and generation of recombinant adenoviruses, pDC316 derivatives with the expected endonuclease restriction enzyme digestion patterns were selected for further use. High-level HMW1, HMW2, or Hia recombinant protein expression was demonstrated by Western immuno

Expression of rHMW1, rHMW2, or rHia protein in A549 cells. The 293 cells are a permissive cell line that allows active replication of the E1-deleted recombinant adenoviruses. We also wished to assess the degree and persistence of expression of the recombinant proteins in a nonpermissive cell line, a situation analogous to the in vivo environment where the replication-defective adenoviruses would be unable to multiply. Therefore, we next used the hmw1, hmw2, and hia constructs
FIG. 2. Schematic representation of the steps used in generation of the hmw1 recombinant adenoviral construct. The hmw2 and hia adenoviral constructs were generated using similar strategies. Ap', ampicillin resistance gene; T7 term, T7 terminator region; MCMV, murine cytomegalovirus promoter; SV40 poly A, SV40 polyadenylation signal; loxP, loxP gene; ITR, inverted terminal repeat; HCMV IE, human cytomegalovirus immediate early promoter; Cre, cre gene; ORF, open reading frame.
to transfect nonpermissive A549 respiratory epithelial cells and monitored the resulting infected cells for recombinant protein expression. We were able to demonstrate high-level expression of each of the recombinant fusion proteins in the infected A549 cell line for at least 10 days following infection. Shown in Fig. 4 is a comparison of recombinant HMW2 (rHMW2) and rHia protein expression in A549 cells (lanes 1 and 2) and HEK 293 cells (lanes 3 and 4). Comparable results were seen with rHMW1 protein expression in the two cell lines.

The largest rHMW2 and rHia immunoreactive bands in the A549 cells had apparent molecular masses identical to those observed when the proteins were expressed in the HEK 293 cells (compare lanes 1 and 3 or lanes 2 and 4 in Fig. 4). However, several lower-molecular-weight bands were also observed when the proteins were expressed in A549 cells. We previously reported that both the HMW1/HMW2 and rHia proteins are quite susceptible to proteolysis in vitro (65, 66). This property likely explains the lower-molecular-weight immunoreactive bands that are seen in Fig. 4.

Immunization of chinchillas with rAd HMW2 or rAd Hia.

The long-term objective of this work is to test the recombinant HMW1, HMW2, and Hia adenoviral constructs as vaccines for prevention of disease caused by nontypeable *Haemophilus influenzae*. As an initial step in this process, we assessed the immunogenicity of two of our constructs when administered to chinchillas by an intraperitoneal or intranasal route. The reason for selecting this animal is that the chinchilla model is...
considered the preferred animal model for studies of otitis media disease pathogenesis and vaccine efficacy.

Two of our recombinant adenoviral constructs were used to immunize 10 chinchillas by either the intraperitoneal or the intranasal route. Four chinchillas were immunized with a single intraperitoneal dose of $10^8$ PFU of rAd HMW2, and four others were immunized with the same dose of rAd Hia. With this route of administration and dose of the recombinant vaccine, we observed robust serum antibody responses in the immunized animals to the respective *Haemophilus influenzae* proteins. As summarized in Table 2, by 1 month following immunization all chinchillas had developed serum antibody titers of $\geq 200$ ELISA units (EU), with most animals demonstrating considerably higher titers. These same boosts in titer were also observed when the sera were examined in immunoblot assays. Shown in Fig. 5 is a Western immunoblot demonstrating the serum antibody responses in acute and convalescent-phase sera of representative chinchillas immunized intraperitoneally with rAd HMW2 (lanes 1 and 2) or rAd Hia (lanes 3 to 6). The acute-phase sera (lanes 1, 3, and 5) lack detectable antibody whereas the convalescent-phase sera (lanes 2, 4, and 6) demonstrate strong antibody responses to the respective proteins.

The same two adenoviral constructs were also used to immunize chinchillas by the intranasal route, a route that would be expected to stimulate the local mucosal immune system in the upper respiratory tract. Six animals were immunized with $10^7$ to $10^9$ PFU of either rAd HMW2 or rAd Hia virus. As summarized in Table 2, by 2 months following immunization, all animals that received the intranasal rAd Hia had developed serum ELISA titers of $\geq 100$ EU. In contrast, the serum antibody responses in the chinchillas that received rAd HMW2 intranasally were more variable. Two months following immunization, two of the six animals had developed serum ELISA titers of $\geq 100$ EU, but the other animals did not demonstrate a measurable boost in serum antibody. The reason that rAd HMW2 was less immunogenic than rHia when delivered intranasally has yet to be fully explained but could be due to lesser stability of the recombinant HMW2 protein than of the Hia protein.

**DISCUSSION**

Recombinant adenovirus vectors are promising vaccine candidates for the prevention of an array of infectious diseases in animals and humans (21). These vectors are capable of eliciting robust innate and adaptive immune responses in immunized subjects (60). Prototype adenoviral vaccines against such major human pathogens as *Plasmodium falciparum* (39), *Mycobacterium tuberculosis* (31, 40), and human immunodeficiency virus (17) have moved into human clinical trials (21). While the successful development and approval of recombinant adenovirus vaccines for human use must still overcome a number of scientific (22) and regulatory hurdles, the progress to date with several candidate vaccines strongly supports the continued investigation of this promising technology. Experts in the field of otitis media vaccine research have strongly advocated the development of mucosal immunization strategies for the prevention of otitis media (26). Recombinant adenoviral vectors are particularly attractive as vaccine candidates for immunization of the upper respiratory tract (61). The middle ear cavity is lined by a very thin mucosa that is continuous with the mucosa that lines the nasopharynx and the Eustachian tube. Components of both the innate and adaptive immune systems are thought to mediate protection of the middle ear space against infection (36, 38, 59). The nasal mucosa-associated lymphoid tissue (NALT) has been proposed as a primary inductive site for upper respiratory tract mucosal immune responses (36, 56), and recent studies have begun to investigate the trafficking of lymphocytes to the middle ear mucosa (35, 48, 59). In response to antigenic stimulation, dendritic cells in the chinchilla nasal mucosa have been shown to traffic to the NALT, where they presumably initiate an antigen-specific immune response (48). Recombinant adenoviral vectors have been shown to be potent activators of dendritic cell maturation in several experimental systems (42, 52). Presentation of a vaccine antigen to nasal dendritic cells by a recombinant adenoviral vector could be a particularly effective way to induce a protective middle ear immune response.

The nontypeable *Haemophilus influenzae* HMW1/HMW2 and Hia proteins upon which our prototype recombinant adenoviral vaccines are based have a number of features that make them attractive as vaccine candidates (6, 64, 65). Ideally, the recombinant adenoviral vector expressing one’s antigen of interest would contain the entire gene or gene cluster responsible for encoding the relevant protein. As noted earlier, we were unable to construct vectors with a full-length *hmw1/hmw2*...
or hia gene. The mature HMW1 and HMW2 proteins are each encoded by clusters of three genes with total spans of over 10 kbp (8, 9), an insert size too large to be accommodated by the adenoviral vector system that we used (46). In the case of the hia construct, we were unsuccessful in generating constructs containing the full-length gene, presumably because of the toxicity of the recombinant Hia protein in the HEK 293 cell culture system used for recovery of the recombinant viruses. For both the hmw1/hmw2 and hia adenoviral constructs that we generated, we expressed defined segments of the respective genes as translational fusions with T7 gene 10 derived from the pGEMEX vector. In constructs in which the hmw1/hmw2 and hia genes were not fused to T7 gene 10, expression of recombinant HMW1/HMW2 and Hia proteins was quite poor, presumably due to relatively rapid degradation of the proteins (data not shown) (65). The segments of the hmw1/hmw2 or hia gene that were used to generate the fusion constructs encoded domains of the respective proteins that are known to be highly immunogenic, in the case of HMW1 and HMW2 (10), or capable of eliciting opsonophagocytic antibodies, in the case of Hia (64).

The studies described in this work represent an initial but important step in the evaluation of recombinant adenoviral vectors expressing the HMW1/HMW2 or Hia protein as potential vaccine candidates. We demonstrated the immunogenicity of these constructs when delivered by the parenteral and the intranasal route in the chinchilla model. As noted earlier, the chinchilla model is the system favored by most research groups in the field for the study of otitis media pathogenesis (2) and for assessment of the protective potential of otitis media vaccine candidates (5, 48). Future studies of our adenoviral constructs will be needed to define the optimal immunization regimen for generation of a protective immune response in the chinchilla model. Important issues yet to be addressed include the ability of the immunization regimens that we develop to induce a functional antibody response (64, 65), the ability of the constructs to induce a mucosal immune response in the upper airway (48), and the potential benefit of prime-boost immunization regimens to enhance the protective immune response in the vaccinees (21).

Recombinant adenoviruses represent a promising system to achieve mucosal and systemic immunity and protection from mucosal diseases such as otitis media. Recombinant adenovirus vectors expressing recombinant HMW or Hia protein will be important new tools in nontypeable Haemophilus influenzae vaccine development efforts.

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