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Evaluation of Purified UspA from *Moraxella catarrhalis* as a Vaccine in a Murine Model after Active Immunization

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Moraxella catarrhalis **causes otitis media, laryngitis, and respiratory infections in humans. A high-molecular-weight outer membrane protein from this bacterium named ubiquitous surface protein A (UspA) is present on all isolates. A monoclonal antibody (MAb) to UspA that recognizes a conserved epitope of this protein has been shown to promote pulmonary clearance of bacteria in passively immunized mice. In the present study, *M. catarrhalis* heterologous isolates were screened by dot blot with a panel of four additional MAbs specific for surface-exposed epitopes of UspA from *M. catarrhalis* isolate O35E. Three of the MAbs were specific for O35E, and the fourth reacted with 17 (74%) of the 23 isolates tested. Thus, UspA contains highly conserved, semiconserved, and variable surface-exposed epitopes. The UspA was purified from the O35E isolate by ion-exchange and size-exclusion chromatography, formulated with the adjuvant QS-21, and used to immunize BALB/c mice. Upon pulmonary challenge with either O35E or the heterologous isolate TTA24, significantly fewer bacteria were recovered from the lungs of immunized mice 6 h postchallenge than from control mice. The immune sera from mice or guinea pigs contained high titers of antibodies to the homologous isolate and heterologous isolates in a whole-bacterial-cell enzyme-linked immunosorbent assay. Sera against UspA, whether prepared in mice or guinea pigs, had complement-dependent bactericidal activity toward homologous and 11 heterologous *M. catarrhalis* isolates. These results indicate that the conserved epitopes of the UspA are highly immunogenic and elicit broadly reactive and biologically functional antibodies. UspA may offer protection against *M. catarrhalis* infections and is being further evaluated as a vaccine candidate.**

Moraxella catarrhalis is a gram-negative, nonencapsulated bacterium recently recognized as an important human pathogen. It is frequently isolated from patients with otitis media (12, 25), laryngitis (27, 28), and other respiratory diseases (26). The populations most affected are infants, young children, and the elderly, and the incidence of disease appears to be increasing (8). In some hospitals, *M. catarrhalis* now accounts for half of all the respiratory infections (3). In addition, the percentage of clinical isolates producing β-lactamase has increased significantly over the last two decades; in some localities, more than 90% of the isolates are resistant to β-lactam antibiotics (3, 8, 13). Currently, there is no vaccine to prevent *M. catarrhalis* infections.

The feasibility of a prophylactic vaccine against *M. catarrhalis* is suggested by several lines of evidence. First, normal adults with background immunity resulting from natural infection have a lower carriage rate than children and the elderly do (9, 11, 30) and suffer fewer infections. Second, children develop serum antibodies to *M. catarrhalis* after age 4 (14), and this appears to correlate with a decrease in the frequency of otitis media caused by *M. catarrhalis*. Third, antibodies to *M. catarrhalis* antigens have been detected in both the acute-phase and convalescent-phase sera of adult patients (7, 15). These data suggest that an appropriate humoral response to the surface antigens of this bacterium is likely to provide protection.

M. catarrhalis outer membrane proteins and other surface structures are considered potential vaccine candidates. Several of the major outer membrane proteins are relatively well conserved among *M. catarrhalis* isolates (16–18, 21, 22) and elicit antibodies with biological activity (5, 16, 17). Among these is a high-molecular-weight protein that Helminen et al. (17) have named the ubiquitous surface protein A (UspA). UspA appears to be identical to the protein identified by Klingman and Murphy (18), which they designate the high-molecular-weight outer membrane protein. The molecular weight of UspA varies among isolates. In most isolates, it usually migrates as multiple bands upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a dominant band of greater than 300 kDa and a minor band at 120 to 140 kDa. In some isolates, it appears to have only the high-molecular-weight form. Two monoclonal antibodies (MAbs) that react with all the bands of nearly every isolate have been described (17, 18). Passive immunization with one of these MAbs, 17C7, enhanced pulmonary clearance of both homologous and heterologous isolates in a murine pulmonary challenge model (17). The observation that human convalescent-phase sera contain antibodies to UspA indicates that this protein is expressed by *M. catarrhalis* in vivo (7, 15, 17). *M. catarrhalis* gains entry to the host by attachment to and colonization on the epithelial cell surface of the respiratory tract (1, 2). Polyclonal antibodies to UspA block the adherence of a homologous *M. catarrhalis* isolate to HEp-2 cells in vitro (6), indicating that UspA may be a virulence factor. These results suggested that this protein warranted closer examination as a vaccine candidate.

In this report, purified UspA from *M. catarrhalis* O35E strain was evaluated as a vaccine antigen by active immunization. The immunogenicity of UspA, its ability to elicit a bactericidal antibody response and the ability of anti-UspA antibodies to promote bacterial clearance in a murine pulmonary challenge model are presented.

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MATERIALS AND METHODS

Bacteria. The *M. catarrhalis* isolates O35E and TTA24 were provided by Eric Hansen of the University of Texas Southwestern Medical Center. Additional isolates were provided by Dwight Hardy of the University of Rochester and Gary Doern of the University of Massachusetts Medical Center. The bacteria were routinely passaged on Mueller-Hinton agar incubated at 35°C with 5% carbon dioxide. The bacteria used for the purification of UspA were grown in Mueller-Hinton broth, while those used for the murine challenge experiments were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.). The isolates were stored at −70°C in Mueller-Hinton broth containing 40% glycerol.

Purification of UspA. UspA was purified from outer membrane vesicles prepared from isolate O35E. To make the vesicles, bacteria were grown in Fernbach flasks containing 1.3 liters of Mueller-Hinton broth at 37°C for 22 h with shaking at 200 rpm. The bacteria were harvested by centrifugation (10,000 × *g* for 20 min) and then resuspended in 200 ml of phosphate buffer (10 mM, pH 7.5) containing 600 mM NaCl. This suspension was vigorously vortexed for 2 min prior to the removal of the bacterial bodies by centrifugation at 10,000 × *g* for 60 min. The supernatant containing the salt wash outer membrane vesicles was retained. When compared with heat-EDTA-induced vesicles prepared by the method of Murphy and Loeb (23), a similar protein pattern was found by 10% SDS-PAGE. The salt wash vesicles were dialyzed extensively against 10 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl. Proteins were then solubilized by adding EDTA and Triton X-100 (J. T. Baker Inc., Phillipsburg, N.J.) to final concentrations of 10 mM and 1%, respectively, followed by stirring for 60 min at room temperature. The soluble fraction was loaded on a 10-ml bed volume of Q Sepharose high-performance gel column (Pharmacia, Piscataway, N.J.) and eluted with a NaCl step gradient in a buffer containing 10 mM Tris-HCl, 1% Triton X-100, and 10 mM EDTA. The bulk of the UspA eluted at 250 to 500 mM NaCl. The fractions enriched in UspA were pooled and concentrated with a Centriprep-30 (Amicon, Beverly, Mass.). The small-molecular-weight contaminants were removed by passage over a column (2.6 by 100 cm) of Sephadex G-200 (Pharmacia) in 10 mM Tris-HCl buffer containing 1% Triton X-100, 150 mM NaCl, and 10 mM EDTA at a flow rate of 0.2 ml/min. The purified UspA was dialyzed against phosphate-buffered saline (PBS) at 4°C prior to determination of the protein concentration by a microbicinchoninic acid assay (Pierce, Rockford, Ill.).

ELISA. The anti-*M. catarrhalis* antibody titers elicited by immunization with UspA were determined by a whole-cell enzyme-linked immunosorbent assay (ELISA). For this assay, colonies of *M. catarrhalis* grown overnight on BHI agar were resuspended in PBS and diluted to an A_{550} of 0.2, and 50 μl of this suspension was added to each well of 96-well microtiter plates (Nunc, Roskilde, Denmark). The plates were dried at 37°C and stored at 4°C until needed. After blocking with 200 μl of PBS containing 5% (wt/vol) dry milk for 1 h, the plates were washed five times with PBS and incubated with mouse test sera diluted in PBS containing 5% (wt/vol) dry milk for 1.5 h. The plates were then washed and incubated with biotin-labeled rabbit antibodies specific for mouse immunoglobulin G (IgG) or IgG subclasses (1:10,000 in PBS; Brookwood Biomedical, Birmingham, Ala.) for 1 h. Following three additional washes with PBS, the plates were incubated with streptavidin-horseradish peroxidase conjugate (1:10,000 in PBS; Zymed Laboratories, South San Francisco, Calif.) for 1 h at room temperature. Finally, the plates were washed with PBS and developed with a substrate consisting of 2,2′-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) at 0.3 mg/ml in pH 4.0 citrate buffer containing 0.03% hydrogen peroxide (KPL, Gaithersburg, Md.). End point titers of the immune sera were determined as the highest dilution with an A_{415} which exceeded the mean background absorbance by 2 standard deviations. The mean background absorbance was determined from eight wells which received all reagents except sera.

Guinea pig immune sera were assayed by a similar procedure except that the secondary antibody was goat anti-guinea pig IgG-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.; 1:3,000). Plates were developed with disodium *r*-nitrophenyl phosphate at 1 mg/ml in diethanolamine buffer.

SDS-PAGE and immunoblotting. Samples for SDS-PAGE were prepared by mixing with sample buffer at a 1:2 ratio and boiling for 5 min unless otherwise indicated. Proteins were separated on a 4 to 15% gradient SDS-polyacrylamide gel (Bio-Rad Laboratories, Melville, N.Y.) and stained with Coomassie brilliant blue R-250 (19).

For the Western blot (immunoblot) assay, proteins were electrophoretically transferred from the acrylamide gels onto nitrocellulose membranes (26). The membranes were blocked for 1 h with PBS containing 5% (wt/vol) dry milk and then incubated with mouse immune sera to UspA (1:1,000 in PBS containing 5% dry milk) or tissue culture supernatants containing the *M. catarrhalis* MAb for 2 h at room temperature. The membranes were washed three times with distilled water and then incubated with goat anti-mouse immunoglobulin (heavy plus light chain)-alkaline phosphatase conjugate at a 1:2,000 dilution (Sigma) in PBS–5% dry milk for 1 h at room temperature. Finally, the membrane was washed and developed with a substrate solution containing nitroblue tetrazolium and 5-bromo-chloro-3-indolyl phosphate in 0.1 M Tris-HCl buffer (pH 9.8). Dot blotting was done by a similar method except that 1-μl samples were applied directly to the nitrocellulose membrane.

MAbs to UspA. The UspA-specific MAb 17C7 was provided by Eric Hansen (University of Texas Southwestern Medical Center, Dallas, Tex.) Four additional MAbs were made at our Hybridoma Core Facility. They were obtained by immunizing mice intraperitoneally with 10 μg of outer membrane preparations from *M. catarrhalis* isolate O35E on experimental days 0, 14, 28, and 60. Splenocytes from two immunized mice were fused with hypoxanthine-aminopterin-thymidine-sensitive Ag8 myeloma cells at a 5:1 ratio by using 50% polyethylene glycol 1500 containing 10% dimethyl sulfoxide. Hybridomas were selected by culture in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 10% NCTC 109 (GIBCO BRL, Grand Island, N.Y.), 0.1 mM nonessential amino acids (GIBCO BRL), gentamicin (10 μg/ml), and HAT supplement (GIBCO BRL). *M. catarrhalis*-specific hybridomas were selected by ELISA with O35E whole cells as the coating antigen and by Western blotting with purified UspA from the same isolate. The four UspA-specific hybridomas were cloned by limiting dilution. The immunoglobulin isotype of the MAbs were determined with an Isostrip kit (Boehringer Mannheim, Indianapolis, Ind.).

Preparation of immune sera against UspA in mice and guinea pigs. Female BALB/c mice (Taconic Farms, Germantown, N.Y.), 6 to 8 weeks old, were subcutaneously immunized at experimental weeks 0 and 4 with 25 μg of purified UspA protein or salt wash vesicles formulated with 25 μg of the adjuvant QS-21 (Cambridge Biotech, Worcester, Mass.). In some studies, a third immunization was given at week 8. Control mice were injected with QS-21 alone or tetanus toxoid with QS-21. Serum samples were collected at weeks 0, 4, 8, and 9.

Three female guinea pigs (Taconic Farms; 400 g [body weight]) were subcutaneously immunized at weeks 0, 4, and 8 with 25 μg of purified UspA protein formulated with 20 μg of QS-21. Serum samples were collected at weeks 0 and 10.

Mice and guinea pigs were housed in a specific-pathogen-free facility during the study. They were allowed food and water ad libitum and not entered into study until 7 days after receipt from the vendor.

Murine model of *M. catarrhalis* pulmonary clearance. Mice immunized with UspA prepared from isolate O35E were challenged with either the homologous or a heterologous (TTA24) isolate of *M. catarrhalis*. The challenge procedure was a modification of that described by Unhanand et al. (29). A few colonies of O35E or TTA24 from an overnight BHI agar plate culture were inoculated into 10 ml of BHI broth. These cultures were incubated at 37°C until mid-logarithmic growth, diluted to the desired concentration with saline, and stored on ice until use. The mice were anesthetized by intramuscular injection of a mixture of 2 mg of ketamine HCl (Fort Dodge Laboratory, Ford Dodge, Iowa) and 0.2 mg of acepromazine maleate (Butler Co., Columbus, Ohio) in a 50-μl volume. The bacterial suspension (3.5×10^5 CFU in 30 μl of saline) was dispensed intratracheally into the lungs of the mice with a blunt-end lavage needle attached to a 26-gauge Hamilton syringe via 80-mm (inside diameter) Tygon tubing. The number of bacteria administered was confirmed by plating dilutions of the inoculum on Mueller-Hinton agar plates. Lungs were collected aseptically 6 h after challenge and homogenized in 3 ml of sterile saline with a tissue homogenizer (Ultra-Turax T25; Janke & Kunkel IKA-Labortechnik, Staufen, Germany). The homogenate was serially diluted in saline and plated on Mueller-Hinton agar plates which were incubated at 37°C with 5% CO_2 for 24 h. Bacterial recovery was expressed as the percentage of the geometric mean number of CFU recovered from the lungs of immunized animals compared with the number recovered from the nonimmunized control animals. Statistical analysis was performed by the Wilcoxon rank sum test (JMP Software; SAS Institute, Cary, N.C.). A probability (*P*) of less than 0.05 was considered significant.

Complement-dependent bactericidal assay. The complement source for these experiments was a pool of immunoglobulin-depleted human sera from five healthy individuals. The immunoglobulin was removed by passing 5 ml of pooled human serum over a protein G column twice at 4°C. The immunoglobulin-depleted sera were stored in small aliquots at −20°C. The bactericidal assay was performed by mixing 50 μl of a bacterial suspension (approximately 1,200 CFU in PBS containing 1 mM $CaCl_2$ and 0.2 mM $MgCl_2$) with 25 μl of heat-inactivated test serum and incubating for 30 min at 4°C. The complement-containing serum was then added to a final concentration of 20%, and the mixture was incubated for an additional 30 min at 35°C. At the end of the second incubation, the assay was stopped by diluting with 200 μl of PBS. The number of remaining viable bacteria was determined by plating 50 μl of the assay mixture on Mueller-Hinton agar plates. The plates were incubated for 24 h at 35°C with 5% carbon dioxide. The control for this assay consisted of bacteria incubated with immune sera and complement which had been heat inactivated at 55°C for 30 min. The percentage of bacteria killed was calculated by the following formula: % killing = 100 × [(CFU from control − CFU from sample)/CFU from control]. The level of killing was considered significant when greater than 50% of the bacteria were killed.

The bactericidal activities of the MAbs were determined in a similar fashion. The MAbs used in this assay were concentrated tissue culture supernatants prepared by adding ammonium sulfate to the tissue culture supernatant to 50% saturation. The precipitate was collected by centrifugation (3,000 × *g*, 30 min), resuspended in 1/10th the initial volume in PBS, and dialyzed extensively against PBS.

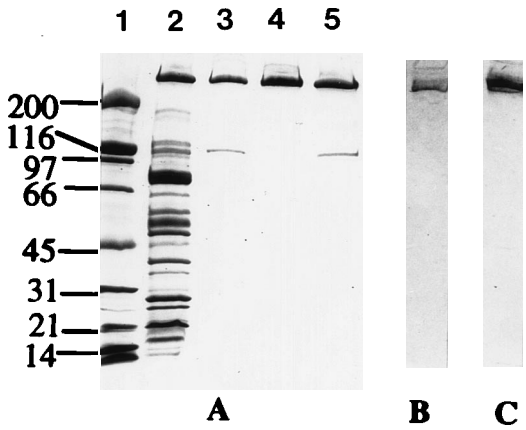

FIG. 1. Characterization of purified UspA protein by SDS-PAGE and Western blotting. (A) Salt wash vesicles (lane 2) and UspA (lanes 3 to 5) separated on a 4 to 15% gradient SDS-polyacrylamide gel and stained with Coomassie blue. The sample in lane 3 is partially purified UspA off the Q column, and the samples in lanes 4 and 5 are purified UspA off the size-exclusion column. The molecular weight standards are shown in lane 1 (values on the left are molecular weights in thousands). The UspA in lane 4 was mixed with SDS-PAGE sample buffer and loaded without heating, while all other samples were mixed with sample buffer and boiled for 5 min before loading. The reactivities of MAb 17C7 and mouse immune serum with the purified UspA on Western blots are shown in panels B and C, respectively. The 120-kDa UspA band is not seen in panels B and C because the UspA sample was not heated prior to electrophoresis.

RESULTS

Purification of UspA. UspA was purified from outer membrane vesicles prepared by exposing the bacteria to a high concentration of salt. UspA accounted for approximately 5% of the total protein in these vesicles. The UspA was soluble in 10 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X-100, 300 mM NaCl, and 10 mM EDTA and was selectively bound to Q Sepharose resin, while most of the other proteins and lipooligosaccharides were present in the flowthrough. The UspA was eluted from the Q Sepharose by raising the salt concentration to 500 mM. Lower-molecular-weight contaminants were then removed by size-exclusion chromatography on Sephadex G-200. Approximately 2 mg of purified UspA was obtained from 1 liter of bacterial culture.

The purity of the UspA was initially verified by SDS-PAGE and Coomassie blue staining. The migratory behavior of purified UspA from the isolate O35E on SDS-polyacrylamide gels (Fig. 1A) depended on the method of sample preparation. With or without reducing reagent, heated samples exhibited multiple bands, one dominant band with a molecular mass greater than 300 kDa and less intense bands of >300 and 120 kDa. The 120-kDa band was not observed in unheated samples. The MAb 17C7 reacted with all bands on Western blots (Fig. 1B). This confirms the existence of multiple forms of UspA as reported by both Helminen et al. (17) and Klingman and Murphy (18). Klingman and Murphy speculated that the higher-molecular-weight forms arise as polymerization products of a 120-kDa subunit (18). We also confirmed that the UspA proteins from different isolates have different migration rates and multiple migratory forms (data not shown).

Reactivity of MAbs with UspA. Five UspA-specific MAbs were used to examine the antigenic variability of UspA among diverse isolates. Because of the way they were selected, all of the MAbs reacted with UspA on the surface of isolate O35E when assayed by a whole-cell ELISA, with purified O35E UspA in an ELISA, and with denatured O35E UspA in Western blots.

TABLE 1. Reactivity of UspA-specific MAbs to heterologous *M. catarrhalis* isolates

MAb	Isotype	Heterologous reactivity[a]	Bactericidal titer[b]
17C7	IgG2a	81/82 (99)	1:200
13-1	IgG1	0/23 (0)	<1:5
6-3	IgM	0/23 (0)	<1:5
29-31	IgG1	0/23 (0)	<1:5
45-2	IgG1	17/23 (74)	<1:5

[a] Values are number of reactive isolates/number of isolates tested, with percentages in parentheses. Reactivity to the UspA from heterologous isolates was established by dot blotting against whole bacterial lysates. All MAbs react with homologous isolate O35E.

[b] Tenfold-concentrated tissue culture supernatants were assayed for bactericidal activity against isolate O35E. The bactericidal titer was the highest dilution resulting in killing of greater than 50% of the bacteria.

The reactivity of these MAbs indicated that not all UspA epitopes were conserved (Table 1). The MAb 17C7 reacted with 81 of 82 isolates by dot blotting. This is consistent with the result reported by Helminen et al. (17). The MAb 45-2 reacted with 17 (74%) of 23 isolates by dot blot. Both MAbs 45-2 and 17C7 reacted with the same multiple bands of UspA in Western blots. In contrast, MAbs 13-1, 6-3, and 29-31 only reacted with UspA from the immunizing isolate (O35E) by dot blot and only with the large-molecular-mass form (>300 kDa) in Western blots (data not shown). Thus, there are at least three types of surface-exposed epitopes on UspA, namely, conserved, semiconserved, and strain specific.

Immunogenicity of UspA. Mice administered a single dose of UspA formulated with QS-21 exhibited an ELISA IgG titer of 114,000 toward O35E whole cells; the antibody titer was boosted to 1,327,000 after the second immunization. The third immunization did not result in an increase of the antibody titer, and the titer was actually slightly lower (i.e., 727,000). A significant portion of antibodies induced by purified UspA formulated with QS-21 were IgG2a and IgG2b, the complement-fixing subclasses important for bactericidal activity (Table 2). Salt wash vesicles, which contain many antigens besides UspA, were used as a control, and these elicited an equivalent level of antibodies after two immunizations and even higher proportions of IgG2a and IgG2b antibodies than immunization with UspA did (Table 2). Immune sera to UspA reacted with purified UspA on a Western blot (Fig. 1C) and only with the UspA bands in the salt wash vesicles (data not shown). This provided another measure of the purity of this material.

The immune sera to purified UspA from mice and guinea pigs were assayed against 11 additional *M. catarrhalis* isolates

TABLE 2. Immunogenicity of UspA and IgG subclass distribution[a]

Immunogen	Total IgG titer	IgG subclass titer to O35E isolate			
		IgG1	IgG2a	IgG2b	IgG3
UspA	727,000[b]	1,133,000	59,600	50,600	1,000
Vesicles	1,829,000[c]	421,000	228,000	304,000	94,000

[a] Groups of 10 mice were immunized subcutaneously with purified UspA or salt wash vesicles by using 25 μg of protein with 25 μg of adjuvant QS-21 on weeks 0 and 4. The UspA group received another dose on week 8. End point titers are for pooled sera from 10 mice assayed in a whole-cell ELISA with the O35E strain as the detection antigen.

[b] Immune serum was from the week 9 bleed of the mice immunized with UspA.

[c] Immune serum was from the week 7 bleed of the mice immunized with salt wash vesicles.

TABLE 3. Effect of immunization with UspA protein from the O35E isolate on pulmonary clearance of *M. catarrhalis*

Expt[a]	UspA vaccine	IgG titer[b]	Challenge strain	% Clearance[c]
1	Lot 1	727,294	O35E	49[d]
2	Lot 1	727,294	TTA24	63[d]
3	Lot 2	184,228	O35E	47[d]
3	Lot 3	616,123	O35E	60[d]

[a] Experiments are described in Materials and Methods and Results.
[b] Sera, collected 3 to 7 days before challenge, were assayed against O35E in a whole-cell ELISA. Results are expressed as IgG end point titers on pooled samples. The IgG titers for experiments 1 and 2 are the same because both groups of mice were immunized with the same preparation of UspA and the sera were pooled prior to the assay.
[c] Mice were challenged intratracheally with 3.5×10^5 CFU of bacteria, and viable bacteria were recovered from the lungs 6 h after challenge. Numbers are the percentages of bacteria cleared from the immunized mice compared with that of the control.
[d] The bacterial clearance relative to controls for each experiment was statistically significant by the Wilcoxon signed rank test ($P < 0.05$).

of diverse geographic and anatomical origin. The immune sera were highly cross-reactive against the heterologous isolates by whole-cell ELISA, although the end point titers were lower than those toward the homologous isolate O35E (see Table 4). The pooled mouse sera to UspA was shown to be specific for UspA by an immunoblot assay of whole-cell lysates from heterologous isolates. The sera detected only those bands that reacted with MAbs specific for UspA (data not shown). Thus, while the UspA of *M. catarrhalis* is antigenically diverse, there are epitopes exposed on the bacterial surface that are conserved and accessible to antibodies elicited by the purified UspA from O35E.

Enhanced pulmonary clearance of bacteria after immunization with UspA. The results of three challenge studies are summarized in Table 3. In these studies, groups of BALB/c mice were immunized three times with 25 μg of UspA (experiment 1 and 2) or twice with 5 μg of UspA (experiment 3) plus 25 μg of QS-21 as an adjuvant at 4-week intervals. Mice were then challenged 2 weeks after the final immunization with the homologous O35E isolate (experiments 1 and 3) or the heterologous isolate TTA24 (experiment 2) by intratracheal instillation. Nonimmunized mice (experiments 1 and 2) and mice immunized with saline mixed with QS-21 or with tetanus toxoid, an irrelevant antigen, mixed with QS-21 (experiment 3) challenged at the same time served as controls. The challenge dose was 3.5×10^5 CFU of bacteria per mouse. Bacterial clearance was determined from the number of bacteria (CFU) recovered from homogenized lung tissue 6 h after challenge. When challenged with the homologous O35E isolate, between 49% (experiment 1) and 60% (experiment 3) fewer bacteria were recovered from the immunized mice than from the control groups challenged in parallel. When the heterologous TTA24 isolate was used as the challenge organism, an average of 63% fewer bacteria were recovered (experiment 2). The degree of bacterial clearance by mice immunized with UspA was statistically significant in all three experiments ($P < 0.05$) by the Wilcoxon signed rank test when compared with mice immunized with tetanus toxoid or adjuvant controls. Thus, immunization with UspA from a single isolate promoted rapid clearance of both the homologous and a heterologous isolate of *M. catarrhalis* from the lungs of mice.

Complement-dependent bactericidal activity of anti-UspA antibodies. Guinea pig antisera elicited by immunization with purified UspA were tested for their ability to kill *M. catarrhalis* isolate O35E (homologous strain) in a complement-dependent bactericidal assay. The O35E isolate was killed when incubated with heat-inactivated guinea pig immune sera against UspA if immunoglobulin-depleted human serum was added as a complement source. The bacterial killing was abolished when the complement serum was heat inactivated or omitted from the assay. Preimmune sera from guinea pigs did not exhibit detectable bactericidal activity. Thus, the bactericidal activity was both complement and antibody dependent. Mouse immune sera to UspA also had complement-dependent bactericidal activity toward O35E (data not shown). Interestingly, a prozone effect was observed in which anti-UspA sera from mice or guinea pigs caused significantly less bacterial killing of the homologous isolate at dilutions of less than 1:100. The guinea pig immune serum had a bactericidal titer of 2,000. Maximum bacterial killing (80 to 90%) was seen with serum diluted to 1:200 to 1:1,000.

TABLE 4. Complement-dependent bactericidal activity of guinea pig immune sera to UspA from the O35E isolate assayed against heterologous isolates[a]

Isolate	ELISA titer[b]	Reactivity with MAb 45-2[c]	% Killing[d] at dilution of:		
			1:50	1:500	1:5,000
O35E	1,285,757	+	32	82	37
TTA24	310,333	+	73	80	8
ATCC 25238	118,266	±	87	61	62
128-179	ND[e]	+	79	70	62
216-96	98,488	+	82	62	54
125-144	229,589	±	97	79	21
205-211	ND	+	89	81	22
301-221	155,178	−	100	100	32
111-210	260,477	+	89	86	29
324-171	278,208	−	99	98	9
4320-345	190,264	−	98	88	10
1230-359	460,501	+	87	57	−5

[a] Sera were pooled from three guinea pigs immunized with the UspA from the O35E isolate. Preimmune pooled serum was not bactericidal at dilutions of 1:50 to 1:500.
[b] End point titers of the sera against the isolate in a whole-cell ELISA. Preimmune sera had a titer of <1,000.
[c] Reactivity with MAb 45-2 was determined by both dot blot and Western blot with whole bacterial lysates. Those which reacted poorly were scored ±.
[d] The level of killing was considered significant when greater than 50% of the bacteria were killed.
[e] ND, not determined.

All five UspA-specific MAbs were assayed for their ability to induce complement-dependent killing of isolate O35E. Only MAb 17C7 had bactericidal activity. It killed greater than 50% of the bacteria at dilutions up to 1:200 of the concentrated tissue culture supernatant (Table 1).

Guinea pig immune sera to the purified UspA from strain O35E were also examined for bactericidal activity against heterologous *M. catarrhalis* isolates. The isolates were selected on the basis of their reactivity with MAb 45-2. Seven of the isolates were reactors, two were nonreactors, and two reacted poorly. Because of the number of samples assayed, the sera were tested at only three dilutions, 1:50, 1:500, and 1:5,000 (Table 4). The bactericidal titers against all 11 heterologous isolates were greater than 500, and the titers against 3 of them were greater than 5,000. The prozone effect was not observed against the heterologous isolates.

DISCUSSION

Purified UspA from *M. catarrhalis* isolate O35E was evaluated as a vaccine antigen by use of a murine pulmonary challenge model, an in vitro bactericidal assay, and a whole-cell

ELISA. The UspA protein was found to be highly immunogenic in mice and guinea pigs and to elicit antibodies recognizing the intact bacterium. Characterization of the UspA with a panel of specific MAbs revealed that this protein expressed several epitopes exposed on the bacterial surface and that those epitopes had a variable degree of conservation. Antibodies elicited by the purified UspA from isolate O35E, however, were broadly reactive with heterologous isolates in an ELISA using whole *M. catarrhalis* as the coating antigen (Table 4). Antibodies against UspA made in mice and guinea pigs had complement-dependent bactericidal activity against both the homologous isolate and 11 heterologous isolates. In addition, mice immunized with UspA exhibited enhanced clearance of both the homologous isolate and a heterologous isolate in a pulmonary challenge model (Table 3).

The results confirm earlier reports that UspA is an outer membrane protein with multiple molecular forms that vary from isolate to isolate and that the predominant form has a molecular mass greater than 300 kDa (17, 18). The same pattern of multiple bands was seen in Western blots of the purified protein and fresh whole-cell lysates, suggesting that the multiple bands were not the result of protein degradation during the purification process. It has been proposed that the high-molecular-weight form is a multimer of the 120-kDa band on the basis of the observation that UspA treated with formic acid migrates as a single 120-kDa band (18). This speculation is also supported by the observation that a single MAb reacts with both the 120-kDa and the larger-molecular-mass bands in Western blots. However, multiple bands were not observed for a recombinant UspA. It migrated as a single high-molecular-weight form (17). Whether the different forms of UspA are oligomers of different subunits or a single gene product at different processing stages can be addressed only after the gene is sequenced.

Because of its size, it was not surprising that UspA had multiple exposed epitopes on the bacterial surface. Clearly, the epitope recognized by MAb 17C7 is highly conserved among *M. catarrhalis* isolates and is a target of bactericidal antibodies and antibodies that enhance pulmonary clearance of both homologous and heterologous isolates (17). The epitope recognized by MAb 45-2 was conserved to a lesser extent and was present on about 75% of the isolates tested. In contrast, MAbs 13-1, 6-3, and 29-31 reacted with isolate O35E but not with 23 heterologous isolates. The antigenic heterogeneity of the UspA protein from different isolates may explain the observation that guinea pig immune sera raised against UspA from the O35E isolate exhibited lower reactivity with heterologous isolates in the whole-cell ELISA (Table 4). It also raised a question as to whether UspA purified from one isolate would offer protection against heterologous isolates.

The lack of a good animal model for *M. catarrhalis* disease presents difficulty for evaluating vaccine candidates (4, 10). At present, the only small animal model available measures enhanced pulmonary clearance of bacteria in mice (29). In this model, bacteria are delivered directly into the lungs of anesthetized mice and the number of surviving bacteria is determined 6 h later. Previous reports have shown that naive mice are able to clear *M. catarrhalis* within 48 h of endotracheal challenge, and this is accompanied by an influx of polymorphonuclear neutrophils (24, 31). However, mice actively immunized with outer membrane vesicles of *M. catarrhalis* or passively immunized with MAbs to certain outer membrane proteins exhibit accelerated pulmonary clearance of bacteria (16, 17, 20). While a correlation between enhanced bacterial clearance in this model and protection against disease has not been established, the model does provide a means to select antigens capable of eliciting antibodies with in vivo activities.

In addition to the data provided by MAbs, immunization studies demonstrated that UspA contained conserved immunogenic epitopes. Polyclonal antisera were found to be highly reactive with bacteria of diverse clinical and geographic origins (Table 4). Mice actively immunized with purified UspA from isolate O35E exhibited accelerated pulmonary clearance of both O35E and TTA24 in the murine pulmonary clearance model (Table 3). These two isolates are the only challenge strains known to colonize in this model (17) and have distinctly different patterns of reactivity with the MAbs to UspA and the CopB protein (17). However, it should be noted that both O35E and TTA24 react with MAb 45-2.

There is evidence that *M. catarrhalis* infections in humans induce serum bactericidal antibodies (3). Vaccine candidates capable of eliciting circulating bactericidal antibodies should be effective in preventing bacteremia and perhaps pneumonia and otitis media. This study demonstrated that guinea pig and mouse antisera to UspA were bactericidal against diverse isolates. However, the killing pattern for the homologous isolate was different from that for heterologous isolates. High concentrations of serum did not kill the homologous isolate but did kill heterologous isolates. This may be a prozone effect in which antibodies at high concentration interfere with each other for binding to the bacteria, leading to unstable antibody-complement complexes or competition for complement components. However, this is difficult to accept since high bactericidal titers were seen for three heterologous isolates without a prozone effect at lower serum dilutions. It is also possible that antibodies lacking bactericidal activity bind to nonconserved epitopes on the homologous isolate that are not present on the heterologous isolates, resulting in interference of bactericidal antibodies. Further studies are needed to understand the basis of this phenomenon.

In summary, this is the first study to evaluate purified UspA protein from *M. catarrhalis* as a vaccine candidate by active immunization. UspA elicited antibodies that promoted clearance of both homologous and heterologous isolates in the murine pulmonary challenge model. In addition, antibodies to UspA induced complement-dependent bacterial killing of all the *M. catarrhalis* isolates tested. These data indicate that UspA is a promising vaccine candidate for the prevention of the diseases caused by *M. catarrhalis.*

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