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Antigenic Characterization and Analysis of the Human Immune Response to Outer Membrane Protein E of *Branhamella catarrhalis*

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Outer membrane protein E (OMP E) is a 50-kDa major OMP of *Branhamella catarrhalis*. Polyclonal antisera and four monoclonal antibodies (MAbs) to OMP E were generated to study its antigenic structure. All antibodies recognized epitopes in all 19 *B. catarrhalis* strains tested by immunoblot assays. By flow cytometry, it was determined that MAbs 1B3 and 9G10d recognized epitopes which are expressed on the surface of the intact bacterium, while MAbs 1C11 and 7C10 recognized epitopes which were buried within the outer membrane. A competitive enzyme-linked immunosorbent assay showed that MAbs 1B3 and 9G10d recognize the same or closely related epitopes. Proteinase K treatment of whole bacterial cells revealed that MAbs 1B3 and 9G10d recognize a surface-exposed epitope located in the 17-kDa region towards the amino terminus of OMP E. The human serum and mucosal antibody responses to OMP E in adults with chronic bronchitis were studied. A majority of these patients had immunoglobulin A to OMP E in sputum supernatants. None of ten adults who experienced lower respiratory tract infections due to *B. catarrhalis* demonstrated a clear-cut rise in antibody titer to OMP E in serum or sputum supernatant. This study has demonstrated that OMP E has at least one surface-exposed epitope which is highly conserved among strains of *B. catarrhalis* and which is located in the amino-terminal 184 amino acids of the molecule.

Branhamella catarrhalis, also called *Moraxella catarrhalis*, is an important human respiratory tract pathogen (2, 4, 21). It is the third most common cause of otitis media in children after *Haemophilus influenzae* and *Streptococcus pneumoniae* (4, 20, 21). It is estimated that 83% of children have at least one episode of otitis media by the age of 3 years, and many children have recurrent episodes (13). Approximately 20% of these infections are caused by *B. catarrhalis* (20, 21, 31).

B. catarrhalis is also an important lower respiratory tract pathogen in adults with chronic obstructive pulmonary disease (COPD) (5, 11, 24). Recently, *B. catarrhalis* has also been identified as a nosocomial pathogen, causing outbreaks of lower respiratory tract infections in hospital and community settings (4, 21, 26).

Several major outer membrane proteins (OMPs) of *B. catarrhalis*, including OMP E, have been identified (1, 20, 23). Heating alters the electrophoretic migration of OMP E in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Its apparent molecular masses are ~35 kDa at 25°C and ~50 kDa at 100°C (20, 23). The gene encoding OMP E has been cloned, and its nucleotide sequence has been determined (3). OMP E is synthesized as a precursor possessing a signal peptide of 25 amino acids cleaved by signal peptidase 1 during export to the outer membrane (3, 25, 32). The molecular mass of the mature OMP E is 47.03 kDa, which correlates well with SDS-PAGE results (3). The transcription initiation start site has been identified by primer extension (3).

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Previous work has also established that the OMP E gene is conserved in all strains tested (3) and that OMP E contains determinants on the surface of the intact bacterial cell (22). However, little is known about the antigenic structure of this protein.

The goal of the present study is to develop monoclonal antibodies (MAbs) to OMP E and to study the antigenic structure of OMP E, with particular interest in surface-exposed epitopes. In addition, the antibody responses to OMP E in the serum and sputum samples of adults with COPD are characterized.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. catarrhalis* strains were grown on chocolate agar plates at 37°C and 5% CO_2 in Mueller-Hinton broth or in brain heart infusion broth. *B. catarrhalis* ATCC 25240 was obtained from the American Type Culture Collection (Rockville, Md.). Clinical isolates of *B. catarrhalis* used in the study were recovered from sputum (9), middle ear fluid (6), transtracheal aspirate (1), sinus (1), and blood (1).

Production of polyclonal antibodies. Purified outer membranes were prepared as described previously by the Zwittergent extraction method (22). Purified outer membranes of *B. catarrhalis* ATCC 25240 were electrophoresed on a preparative SDS-PAGE gel. The gel was stained with amido black, and the OMP E band was excised from the gel and electrophoretically eluted with Bio-Rad (Hercules, Calif.) electroeluter model 422 according to the manufacturer's instruction. The eluted protein was dialyzed against phosphate-buffered saline (PBS) before immunizations. Protein was quantitated by comparison with standards of known concentrations by SDS-PAGE. OMP E was gel purified for the purpose of generating antibodies. The purity of OMP E was assessed by Coomassie blue and silver staining of SDS-PAGE gels. A total of 250 to 300 μg of OMP E was recovered when 5 mg of OMP extracts were used in preparative gels.

New Zealand White rabbits were immunized with gel-purified OMP E of *B. catarrhalis* ATCC 25240. OMP E emulsified with incomplete Freund's adjuvant in aliquots of 50 μg was injected subcutaneously on days 0, 14, and 27, and blood samples were obtained on day 46. The antiserum was tested by immunoblot assay with whole-cell lysates, purified outer membranes, and purified OMP E.

Development of MAbs. Two separate fusions were performed to develop MAbs to OMP E. For the first fusion, mice were immunized intraperitoneally with 50 μg of gel-purified OMP E of strain ATCC 25240 on day 0 with complete

Freund's adjuvant and on days 19, 33, 47, and 53 with incomplete Freund's adjuvant. An intravenous boost was given on day 67, and the fusion was performed on day 70 by standard methods (12). Culture supernatants of hybridomas were tested by enzyme-linked immunosorbent assay (ELISA) with purified OMP E. Clones which were reactive by ELISA were tested by immunoblot assay with whole bacterial cell lysates, purified outer membrane, and purified OMP E. Reactive hybridomas were cloned by limiting dilution. Ascites fluid was prepared by injecting the cells into the peritoneums of pristane-primed mice. MAbs 1C11 and 7C10 were produced from this fusion.

For the second fusion, mice were immunized with a preparation purified as follows. *B. catarrhalis* ATCC 25240 was grown overnight on Mueller-Hinton plates. Sarcosyl-insoluble preparations were made as described previously (14). An aliquot of the Sarcosyl-insoluble pellet was suspended in 1.5% sodium deoxycholate–0.05 M Tris–0.2 M NaCl–0.05 M EDTA (pH 9.0) and incubated for 10 min at room temperature. The suspension was centrifuged for 10 min at 10,000 × *g* at 4°C. The resulting pellet consisted of a preparation which is enriched for OMP E, HMW-OMP, and selected other OMPs. The pellet was suspended in PBS without adjuvant and used to immunize BALB/c mice intraperitoneally on days 0, 14, and 28 with 100 μg at each immunization. On day 31, splenocytes were harvested and fused with Sp2/0-Ag14 plasmacytoma cells by previously described methods (29). Hybridomas were screened by testing tissue culture supernatants in immunodot assays with a whole-cell lysate of strain ATCC 25240. Reactive clones were tested by immunoblot assays and were cloned by limiting dilution. MAbs 1B3 and 9G10d were produced from this fusion.

SDS-PAGE and immunoblot assay. Whole-cell lysates, purified outer membrane preparation, and purified OMP E were subjected to SDS-PAGE on 10% gels (Novex, San Diego, Calif.) (15). The gels were subjected to Coomassie brilliant blue R250 or silver staining (Bio-Rad). Immunoblot assays were performed essentially as described previously (22). The extracts were transferred to nitrocellulose membrane (30). The membrane was blocked with 5% BLOTTO buffer (5% nonfat dry milk in PBS) for 2 h at 37°C. For the detection of OMP E, immunoblots were incubated with polyclonal antiserum at a dilution of 1:2,000 or 1:4,000, and MAbs in ascites were used at a 1:32,000 or 1:81,000 dilution. Tissue culture supernatants of hybridomas were used undiluted. The antibodies were diluted in fresh 5% BLOTTO buffer, and the blots were incubated with antibodies overnight at 4°C. The membrane was washed three times with PBS and incubated with protein A linked to horseradish peroxidase (Zymed Laboratories, Inc., San Francisco, Calif.) or goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Boehringer Mannheim, Indianapolis, Ind.) for 2 h at 37°C. The membrane was washed again four times and then reacted with 4-chloro-1-naphthol–horseradish peroxidase color developer (Bio-Rad) and H_2O_2 for color development.

Flow cytometry. Flow cytometry was performed to determine whether antibodies recognized epitopes which were present on the surface of the intact bacterial cell. Bacteria were grown to mid-logarithmic phase in broth. An equal volume of PBS containing 25 μg of DNase (PBS-DNase) per ml to reduce clumping was added, and the cells were harvested by centrifugation. Cells were then added to the MAb diluted in PBS-DNase. After incubation at 37°C for 1 h, the cells were centrifuged, suspended in 200 μl of anti-mouse IgG or IgM conjugated to fluorescein (Kirkegaard & Perry Laboratories, Inc.), diluted in PBS-DNase, and incubated for 30 min at 37°C. A volume of 1.8 ml of PBS-DNase was added, and the cells were subjected to flow cytometry with a fluorescence-activated cell sorter (FACScan; Becton Dickinson). A total of 20,000 cells was counted in a gated region corresponding to unclumped cells. Data were acquired by using an instrument status with a logarithmic mode for forward scatter, side scatter, and fluorescence.

Immunofluorescence microscopy. Mid-logarithmic-phase *B. catarrhalis* cells were harvested, suspended in PBS, placed onto glass slides, air dried, and briefly passed through a flame. Polyclonal antiserum or preimmunization serum (negative control) was placed on the slides at dilutions of 1:4,000 and 1:2,000 and incubated for 30 min at 37°C. Slides were washed three times with PBS. Goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (Boehringer Mannheim) was added, and the mixture was incubated at 37°C for 30 min. Cells were washed again with PBS and water and examined with a fluorescence microscope.

Proteinase K digestion of whole cells of *B. catarrhalis*. *B. catarrhalis* ATCC 25240 was grown to mid-logarithmic phase. Cells were harvested by centrifugation, washed, and suspended in 10 mM Tris-HCl (pH 8.0). Proteinase K (Boehringer Mannheim) was added to one aliquot of cells to a concentration of 75 μg/ml and incubated for 1 h at 37°C. A second aliquot of cells was incubated in buffer alone at 37°C. Phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM after incubation of the cells with proteinase K. Cells were washed twice, and half of the cells were suspended in sample buffer, heated at 100°C for 10 min, and subjected to SDS–12% PAGE. The other half of the cells were used to extract outer membranes and also subjected to SDS–12% PAGE. Immunoblotting was performed with polyclonal antiserum and MAbs. Incubation of the cells for 10, 30, and 45 min and 2 h with 75 or 38 μg of proteinase K per ml gave the same results. Proteinase K hydrolyzes native proteins and cleaves peptide bonds adjacent to the carboxyl group of aliphatic, aromatic amino acid residues (7).

Determination of amino-terminal sequence. Purified OMPs after proteinase K digestion were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (16). The band of interest was cut out, and the amino-terminal sequence was determined by Edman degradation.

Assays for bactericidal activity. To determine whether MAbs 1B3 and 9G10d have bactericidal activity against *B. catarrhalis*, assays were performed by previously described methods (10). The complement source was prepared by adsorbing normal human serum with protein G. A positive control was polyclonal antiserum directed at OMP CD. Negative controls included buffer in place of antibody and buffer in place of the complement source.

Cloning of the OMP E gene into pRSET. Oligonucleotide primers corresponding to the 5′ and 3′ ends of the OMP E gene sequence were designed with *Eco*RI and *Bam*HI restriction sites. The OMP E gene was amplified by PCR with genomic DNA from *B. catarrhalis* ATCC 25240 as the template and Vent DNA polymerase. The resulting 1.3-kb fragment was ligated into pRSET (Invitrogen, San Diego, Calif.) which was restricted with *Eco*RI and *Bam*HI, followed by electroporation into *Escherichia coli* BLR(DE3)pLysS (Novagen, Madison, Wis.). Colonies were picked and grown in broth, and plasmids were purified. A clone with the appropriate insert confirmed by restriction digestion patterns was chosen. The nucleotide sequence of the entire insert was determined to confirm that the correct clone was obtained. This clone, which expresses the full-length OMP E protein with six histidines on the amino terminus, is called pESA.

Expression and purification of recombinant OMP E. A single colony of clone pESA was inoculated into 2 ml of Terrific Broth (TB) broth containing 200 μg of carbenicillin per ml, and the culture was incubated at 37°C until the cells were in logarithmic phase. Cells were centrifuged and resuspended in 2 ml of fresh TB broth; a volume of 0.1 ml was used to inoculate 8 ml of TB broth containing 500 μg of carbenicillin per ml. This culture was incubated at 37°C until cells were in logarithmic phase. Cells were centrifuged for 5 min at 6,000 × *g*. The cells were resuspended in 50 ml of TB broth containing 500 μg of carbenicillin per ml and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation at 30°C for 2 h, cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. The cells were resuspended in 5 ml of 50 mM NaH_2PO_4–10 mM Tris–6 M guanidine–100 mM NaCl–1 mM Pefabloc (Boehringer Mannheim) (pH 8.0) (lysis buffer) and mixed on a nutator for 20 min at room temperature to lyse cells. The suspension was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant, which represented the bacterial lysate, was saved.

An aliquot of Talon resin (Clontech, Palo Alto, Calif.) was centrifuged at 750 × *g* for 5 min at 4°C. The resulting pellet was suspended in 10 volumes of lysis buffer. After being mixed, the resin was centrifuged, and the supernatant was discarded. The resin was resuspended in the bacterial lysate (1 ml of resin per 75 ml of culture) and mixed on a nutator for 20 min at room temperature. The suspension was centrifuged at 750 × *g* for 5 min at 4°C, and the resin was saved. The resin was washed four times in 10 volumes of lysis buffer by sequential centrifugation and resuspension. To elute recombinant OMP E, the washed resin was suspended in 2 volumes of 50 mM NaH_2PO_4–100 mM NaCl–20 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (PIPES)–8 M urea (pH 5.2) and incubated at room temperature on a nutator for 20 min. The elution was repeated two additional times. The eluted protein was tested by SDS-PAGE, and the protein concentration was determined by the bicinchoninic acid assay (Pierce).

In later experiments, the method was altered so that OMP E was purified into a buffer containing detergent rather than 8 M urea. The Talon resin was suspended and mixed in bacterial lysate as described above. The resin was washed twice with 10 volumes of lysis buffer and collected by centrifugation. The resin was resuspended in TON buffer (0.02 M Tris [pH 8], 1% β-octylglucoside, 0.5 M NaCl), mixed for 10 min at room temperature, and centrifuged to collect the resin. The protein was eluted by suspending the resin in 2 volumes of TON buffer containing 0.05 M EDTA. The resin was removed by centrifugation, and the supernatant, which contained purified OMP E, was collected. The eluted protein was tested by SDS-PAGE, and the protein concentration was determined by the bincinchoninic acid assay (Pierce).

Competitive ELISA with biotinylated OMP E. To determine whether MAbs 1B3 and 9G10d recognized the same or different epitopes, a competitive ELISA with OMP E labeled with biotin was performed. To biotinylate OMP E, the ECL Protein Biotinylation Module (Amersham) was used. Purified OMP E was dialyzed against 0.5% Triton X-100–0.4 M sodium bicarbonate (pH 8.6) overnight at 4°C. After the mixture was warmed to room temperature, 8 μl of biotinylation reagent (Amersham) was added, and the solution was mixed by agitation for 1 h at room temperature. A G25 Sephadex column (Amersham) was washed with 5 ml of 1% bovine serum albumin in PBS (pH 7.5) followed by 20 ml of PBS (pH 7.5). The OMP E-biotin solution was brought up to 2.5 ml with 0.04 M sodium bicarbonate (pH 8.6) and was applied to the G25 Sephadex column. The column was eluted with PBS (pH 7.5), and 1-ml fractions were collected. Fractions were tested by immunodot assay with polyclonal anti-E serum, and fractions which contained OMP E were pooled and saved.

To perform the competitive ELISA, the wells of Immulon 4 flat-bottom microtiter plates (Dynatech Laboratories, Chantilly, Va.) were coated with MAb 1B3 at 10 μg/ml in 0.1 M sodium carbonate–0.1 M sodium bicarbonate (pH 9.6) (CBC buffer) overnight at 4°C. The wells were washed three times between each step with PBS plus 0.05% Tween 20 (PBS-Tween) and blocked with 3% nonfat dry milk in PBS-Tween for 1 h at room temperature. Biotinylated OMP E (1:200 dilution) was added to the wells and incubated for 3 h at room temperature. To assay competition between antibodies, OMP E was preincubated with various concentrations of MAb 9G10d for 30 min at 37°C before the OMP-E-MAb

solution was added to the wells. After the wells were washed, biotinylated OMP E, which was bound to the wells, was detected by the addition peroxidase-labeled streptavidin (Kirkegaard & Perry) (1:3,000 in PBS-Tween) and incubation for 1 h at room temperature. After the wells were washed, color was developed by adding 0.1 mg of 3,3′,5,5′-tetramethylbenzidine TMB)-dimethyl sulfoxide–0.02% hydrogen peroxide per ml in 0.1 M sodium acetate adjusted to pH 4.5 with citric acid. After approximately 5 min, the reaction was stopped by adding 4 N H_2SO_4. The same assay was also performed by coating the wells with MAb 9G10d and using MAb 1B3 as the inhibitor.

The percent inhibition was calculated as follows. For each well coated with MAb, a corresponding well in which the coating antibody was left out and replaced with coating buffer only was included. These wells received biotinylated OMP E, streptavidin, and color developer exactly as did the wells coated with antibody. To control for nonspecific background, the values obtained for these wells which were sham coated were subtracted from the values for the corresponding wells which were coated with MAbs. To calculate the percent inhibition, the optical density for wells containing the competing MAb was subtracted from the optical density for wells in the absence of competing MAb. This result was divided by the value obtained with no competing MAb and multiplied by 100.

Human serum and sputum supernatant samples. Human serum samples were obtained from adults with chronic bronchitis who are enrolled in a prospective study at the Chronic Bronchitis Study Clinic of the Department of Veterans Affairs Western New York Healthcare System. Paired samples of sera from 10 adults who experienced purulent exacerbations characterized by increases in coughing and sputum production and in which *B. catarrhalis* was recovered from sputum were obtained. Preexacerbation serum was collected 1 month prior to the exacerbation and postexacerbation serum was collected 1 month following the exacerbation.

Paired serum samples from 10 patients in the Chronic Bronchitis Study Clinic whose respiratory tracts have not been colonized by *B. catarrhalis* were obtained. Monthly sputum cultures documented the absence of *B. catarrhalis* in the sputa of these patients. A serum sample obtain upon the patient's enrollment in the clinic and a second sample obtained a mean of 20.1 months later (range 15 to 29 months) were tested for antibodies to OMP E. Ten serum samples obtained from apparently healthy adults who did not have COPD and who did not have an infection were assayed as well. Blood was obtained by venipuncture and was allowed to clot. Serum was obtained by centrifugation and was stored at −80°C.

Paired sputum supernatants from the 20 patients from the study clinic were obtained during the same clinic visits at which the serum samples described above were obtained. The first morning sputum was expectorated and brought by the patient to the clinic. An equal volume of 6.5 mM dithiothreitol in PBS was added to the sputum. The sputum was mixed by vortexing and incubated at 37°C for 20 min. The mixture was centrifuged at 27,000 × *g* for 30 min at 4°C. The supernatants were saved by storage at −80°C. Levels of immunoglobulin to OMP E were assayed in sputum supernatants.

ELISA. The levels of immunoglobulin to OMP E were assayed in human serum and sputum supernatants. The wells of a 96-well microtiter plate (Immulon 4; Dynatech) were coated overnight at room temperature with 10 μg of recombinant, purified OMP E per ml in CBC buffer. The wells were washed three times between each step with PBS-Tween. Serum or sputum supernatant was diluted in 1% nonfat dry milk in PBS-Tween, added to the wells, and incubated at 37°C for 2 h. At the same time, a microtiter plate for immunoglobulin standards was prepared. The wells were coated with rabbit anti-human IgG (1:2,000), IgM (1:1,000), or IgA (1:1,000) (Kirkegaard & Perry, Gaithersburg, Md.) in CBC buffer and incubated overnight at room temperature. The wells were washed three times with PBS-Tween. Decreasing concentrations of IgG, IgM, or IgA (Cappel Organon Teknika, Durham, N.C.) were added to the wells and incubated for 2 h at 37°C. After the wells were washed with PBS-Tween, horseradish peroxidase-conjugated rabbit anti-human F(ab′)2 IgG (1:1,000), IgM (1:1,000), or IgA (1:1,000) (Dako, Carpenteria, Calif.) diluted in 3% goat serum was added to the wells with serum, sputum supernatant, and immunoglobulin standards and incubated for 1 h at room temperature. Substrate and color developer were added to wells as described above and incubated for 15 min before the reactions were stopped by the addition of 4 N H_2SO_4. The optical density at 450 nm was read.

Samples were run in duplicate. For each sample which was assayed, corresponding wells were sham coated with CBC buffer and run with each serum tested. The optical density for these wells was subtracted from the value obtained with the serum or sputum supernatant to control for nonspecific background. To control for plate-to-plate and day-to-day variability, duplicate wells were run with OMP E and a serum sample known to yield an optical density of approximately 1.0 for each peroxidase conjugate. The results for plates which varied more than 15% were eliminated and repeated. The results for samples were standardized to the results for these wells.

The amount of antibody in each sample was calculated from the standard curve run with each experiment, and the result was expressed as micrograms per milliliters. Paired samples from the same patient were always run together.

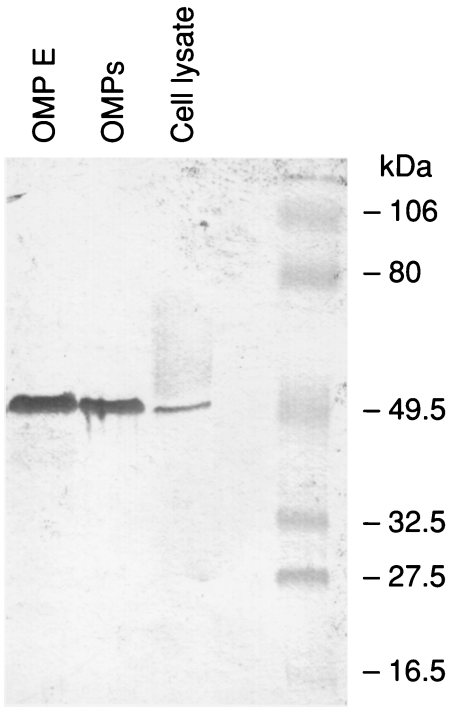

FIG. 1. Immunoblot assay with anti-OMP E antiserum. The lanes contain OMP E (purified OMP E), OMPs (purified outer membrane), and cell lysate (whole-cell lysate). All preparations were made with *B. catarrhalis* ATCC 25240. Immunoblotting was performed with anti-OMP E (1:4,000 dilution) and developed with goat anti-rabbit IgG conjugated to peroxidase. Molecular mass standards are noted on the right.

RESULTS

Characterization of anti-OMP E antiserum. Immunization of rabbits with gel-purified OMP E resulted in a strong antibody response, indicating that OMP E is immunogenic in vivo. The rabbit antiserum recognized OMP E in whole-cell lysates, purified outer membranes, and purified OMP E by immunoblotting (Fig. 1).

To assess the degree of conservation of OMP E among strains of *B. catarrhalis*, whole-cell preparations of 19 different strains were subjected to immunoblot assay with the polyclonal anti-OMP E. Anti-OMP E recognized OMP E in all strains tested. This result indicates that at least some determinants recognized by anti-OMP E antisera are conserved among all *B. catarrhalis* strains tested.

Characterization of MAbs to OMP E. To determine the antigenic specificities of MAbs 1C11, 7C10, 1B3, and 9G10d, ELISAs and immunoblot assays were performed. All four MAbs were reactive with gel purified OMP E by ELISA. Immunoblot assays were performed to further assess the specificities of the MAbs. All four MAbs bound to a single band with a size of ~50 kDa in the whole-cell lysate by immunoblot assay. When the purified outer membrane of strain ATCC 25240 was subjected to immunoblot assay, all four MAbs bound exclusively to the OMP E band. SDS-PAGE of the purified outer membrane shows a single band in the molecular mass region of 50 kDa. All four MAbs were identified as the IgG1 isotype. All four MAbs also recognized purified OMP E by immunoblotting. These experiments establish that MAbs 1C11, 7C10, 1B3, and 9G10d recognize epitopes on OMP E.

To assess the degree of antigenic conservation of OMP E among strains, whole-cell preparations from 19 strains of *B.*

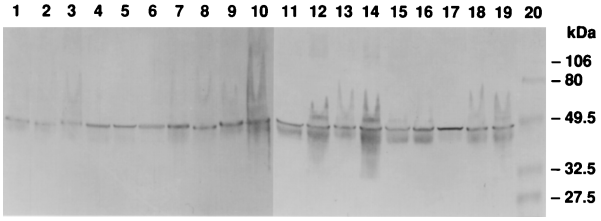

FIG. 2. Immunoblot assay with MAb 1C11. The lanes contain whole-cell lysates of 19 strains of *B. catarrhalis* Lanes: 1, 25240; 2, 5191; 3, 555; 4, 585; 5, 7221; 6, 135; 7, 14; 8, 701; 9, 931; 10, 42; 11, 1; 12, 56; 13, 621; 14, 690; 15, 45; 16, 9483; 17, 3584; 18, 58; 19, Tal2. The immunoblot was developed with goat anti-mouse IgG conjugated to peroxidase. Molecular mass standards (lane 20) are noted on the right.

catarrhalis from diverse clinical and geographic sources were subjected to SDS-PAGE and immunoblot assays. All four MAbs recognized epitopes on all strains of *B. catarrhalis* tested (Fig. 2). Furthermore, the OMP E band in all 19 strains showed identical patterns of migration by SDS-PAGE and immunoblotting.

To determine the species specificities of MAbs 1C11, 7C10, 1B3, and 9G10d, whole-cell preparations from the following gram-negative species were tested by immunoblot assays: *Proteus mirabilis*, *H. influenzae*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Haemophilus ducreyi*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. None of the antibodies reacted with any of these gram-negative bacteria.

Since MAbs 1B3 and 9G10d recognized surface exposed epitopes (see below), bactericidal assays were performed with these two antibodies. Neither antibody had bactericidal activity against *B. catarrhalis* with the addition of an exogenous complement source. This result is consistent with the observation that most mouse IgG1 antibodies do not fix complement.

Analysis of surface-exposed epitopes on OMP E. (i) Flow cytometry. The four MAbs were subjected to flow cytometry to determine whether the antibodies recognized epitopes which are expressed on the surface of the intact bacterium. Undiluted tissue culture supernatants were used. For each assay, negative controls, including (i) SP2 tissue culture supernatant in place of antibody, (ii) buffer in place of antibody, and (iii) an irrelevant IgG1 MAb, were run in parallel. In addition, each antibody was tested with *E. coli* HB101 to establish the specificity of binding of antibodies to the surface of *B. catarrhalis*. All of these controls consistently yielded a negative result. Figure 3 shows that antibodies 9G10d and 1B3 bind to epitopes which are on the surface of the intact bacterial cell. Antibodies 1C11 and 7C10 bind to epitopes which are not available on the bacterial surface. The presence of a single peak corresponding to cells bound by 9G10d and 1B3 in Fig. 3 indicates that the epitopes recognized by these antibodies are expressed on the surfaces of all cells detected by flow cytometry.

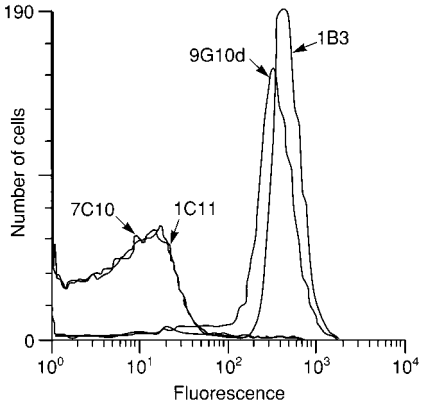

FIG. 3. Results of flow cytometric analysis of *B. catarrhalis* ATCC 25240. The results with each of the four antibodies, MAbs 7C10, 1C11, 9G10d, and 1B3, are shown.

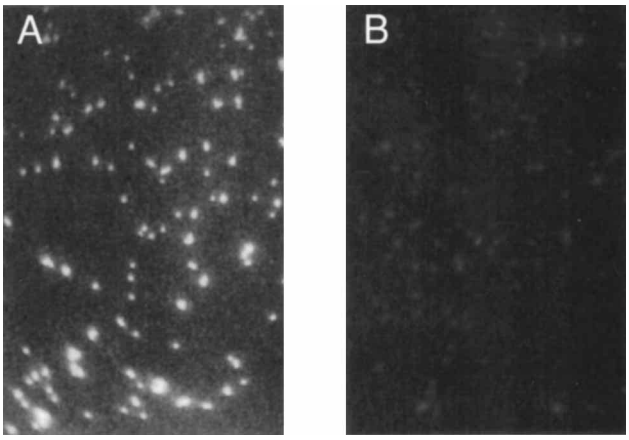

FIG. 4. Immunofluorescence assay. *B. catarrhalis* ATCC 25240 cells were incubated with anti-OMP E (1:4,000) (A) or with pre-immune rabbit antiserum at the same dilution (B). The cells were then incubated with anti-rabbit IgG conjugated to fluorescein and examined under a fluorescence microscope.

(ii) Immunofluorescence assay. Immunofluorescence studies were performed to further determine if OMP E has determinants that are exposed on the surface of the bacterium. Anti-OMP E produced prominent fluorescence of cells (Fig. 4A). No staining was observed when normal rabbit sera (pre-immune) was used (Fig. 4B).

(iii) Proteinase K treatment of *B. catarrhalis* cells. To further investigate the regions of OMP E exposed on the surface, whole cells were incubated with proteinase K. Purified outer membranes were extracted from cells incubated with proteinase K and were analyzed by immunoblot assays (Fig. 5). Immunoblotting with anti-OMP E detected a band at approximately 32 kDa (Fig. 5A) when the cells were digested with proteinase K. MAbs 1C11 and 7C10, which recognize non-surface-exposed epitopes, also recognize this band (Fig. 5B and C) and the intact 50-kDa band in the same lane. MAbs 1B3 and 9G10d, which recognize surface-exposed epitopes, did not recognize the 32-kDa band (Fig. 5D and E) or any other band in cells incubated with proteinase K. We conclude that incubation of whole bacterial cells with proteinase K results in cleavage of a surface-exposed region of OMP E. The 32-kDa band represents the portion of OMP E which remains in the outer membrane. All antibodies recognized OMP E at 50 kDa when cells were incubated in buffer with no proteinase K (Fig. 5). When whole-cell lysates incubated with proteinase K were prepared, none of the antibodies detected the presence of a band, most likely because of the relatively lower concentrations of OMP E in these preparations compared to that of the purified outer membrane.

The amino-terminal sequence of the 32-kDa band was ATDGQKTN, indicating that proteinase K digests at glutamine 184. Therefore, MAbs 1B3 and 9G10d recognize a surface-exposed epitope(s) which is located in the 17-kDa region of the amino terminus of the protein molecule.

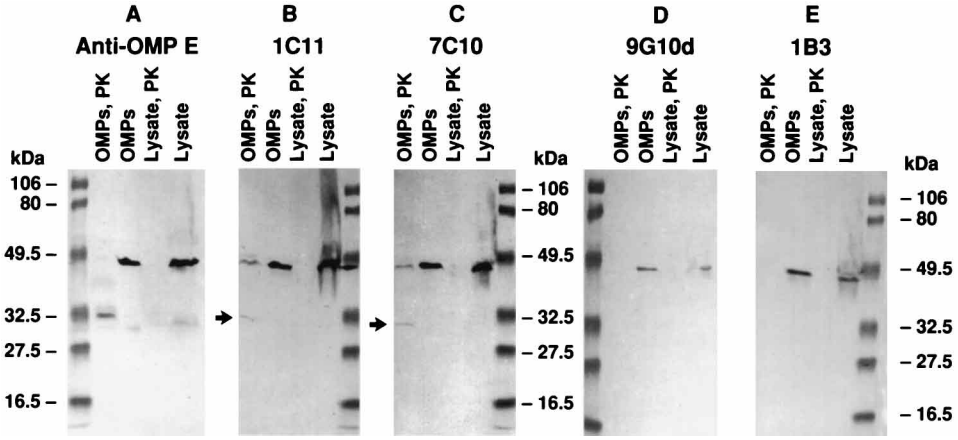

FIG. 5. Proteinase K digestion of whole cells of *B. catarrhalis*. Cells were incubated with proteinase K (75 μg/ml) at 37°C for 1 h. As a control, cells were incubated in buffer alone. Outer membranes were extracted from cells incubated in proteinase K and buffer alone. Cells and purified OMPs were suspended in sample buffer, heated at 100°C for 10 min, and subjected to SDS–12% PAGE and immunoblotting. Lanes: OMPs, PK, purified outer membrane extracted after incubation with proteinase K; OMPs, purified outer membrane; lysate, PK, whole-cell lysates incubated with proteinase K; lysate, whole-cell lysates in buffer. The cells were probed with antibodies as noted above the lanes. Molecular mass markers are indicated on the right.

(iv) Competitive ELISA. To determine whether MAbs 1B3 and 9G10d recognized the same or different epitopes, a competitive ELISA with OMP E labeled with biotin was performed. MAb 1B3 was coated onto the wells of a microtiter plate, and the ability of MAb 9G10d to inhibit the binding of biotinylated OMP E was assessed. The reverse experiment was also performed, in which MAb 9G10d was coated onto wells and MAb 1B3 was tested for its ability to inhibit the binding of biotinylated OMP E. Figure 6 shows that MAb 9G10d inhibits the binding of MAb 1B3 (Fig. 6A) and that MAb 1B3 inhibits the binding of MAb 9G10d (Fig. 6B). The degree of inhibition by the heterologous MAb was similar to that observed when the homologous MAb was used as the competing antibody. These results indicate that MAbs 1B3 and 9G10d recognize the same or closely related epitopes on OMP E.

Human antibody response to OMP E. To characterize the human antibody responses to OMP E, IgG, IgM, and IgA to OMP E were quantitated in the serum and sputum supernatants from adults with COPD. Ten patients who experienced purulent exacerbations with an increased amount and purulence of sputum associated with the recovery of *B. catarrhalis* from sputum were identified. Serum and sputum supernatants from samples collected 1 month before and 1 month after the exacerbation were studied. Serum and sputum supernatants from an additional 10 adults with COPD who had not experienced exacerbations associated with *B. catarrhalis* were also studied. Table 1 shows the results of these assays along with results for 10 serum samples from healthy adults. In general, many of these samples contained undetectable (<0.1 μg/ml) or low levels of antibodies to OMP E. Several observations are apparent. (i) Of 150 assays of 50 serum samples, 6 had immunoglobulin levels of >1 μg/ml and 41 had levels of <1 and >0.1 μg/ml. (ii) Of 120 assays of 40 sputum samples, 1 had an immunoglobulin level of >1 μg/ml and 32 had levels of <1 and >0.1 μg/ml. (iii) None of the 10 patients with COPD who experienced exacerbations due to *B. catarrhalis* demonstrated a clear-cut rise in antibody titer to OMP E of any isotype in serum or sputum following infection. (iv) Nine of 10 patients with COPD who experienced exacerbations due to *B. catarrhalis* had detectable levels of IgA to OMP E in sputum supernatants and 7 of 10 patients with COPD who did not experience exacerbations had detectable IgA in sputa. (v) IgG was not present in any of 40 sputum supernatants. Only 4 of 40 sputum supernatants had detectable levels of IgM. (vi) Four of 10 patients in each of the COPD groups had serum IgG levels of >0.1 μg/ml, whereas none of the 10 normal human serum samples had detectable levels of IgG.

A competition ELISA was performed to determine whether

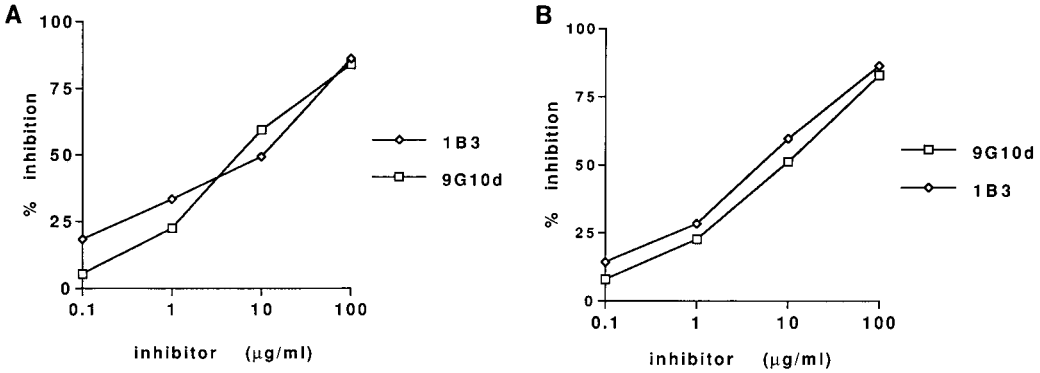

FIG. 6. Results of competitive ELISA in which MAb 1B3 (A) or 9G10d (B) was coated onto the wells of a microtiter plate. MAbs 1B3 and 9G10 were tested for their ability to inhibit the binding of biotinylated OMP E to the immobilized antibody. The *x* axis shows the concentration of the inhibiting MAb, and the *y* axis shows the percent inhibition relative to binding of OMP E in the absence of an inhibiting MAb.

TABLE 1. Immunoglobulin levels to OMP E in serum and sputum samples from adults[a]

Patient category and no.	Concn (μg/ml) in serum						Concn (μg/ml) in sputum					
	IgG		IgA		IgM		IgG		IgA		IgM	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
COPD with colonization												
1E17/19	—[b]	—	0.40	0.35	0.14	0.14	—	—	0.39	0.54	—	—
3E10/12	—	—	—	—	—	—	—	—	0.51	0.42	—	—
10E10/12	0.21	0.22	—	0.14	—	—	—	—	0.33	0.39	—	—
12E14/16	—	—	0.125	0.125	0.44	0.36	—	—	—	—	—	—
22E6/11	2.0	1.0	—	—	0.14	0.55	—	—	0.74	0.48	—	0.145
29E1/4	—	—	—	—	0.19	0.14	—	—	0.43	0.67	—	0.175
34E4/6	0.42	0.20	1.75	0.78	0.16	0.13	—	—	0.64	0.70	—	—
39E9/11	—	0.11	—	—	0.28	0.35	—	—	0.50	0.39	—	—
51E8/10	—	—	—	—	—	—	—	—	0.15	0.40	—	—
52E1/4	—	—	—	—	—	—	—	—	0.74	0.65	—	—
COPD without colonization												
6E1/26	0.12	0.33	—	—	0.53	0.95	—	—	—	—	—	—
7E1/30	0.29	0.34	—	—	—	—	—	—	—	—	—	—
11E1/25	0.35	0.25	—	—	—	—	—	—	—	—	—	—
24E2/22	—	—	0.16	—	—	—	—	—	2.0	0.47	0.14	—
27E1/19	0.16	0.15	—	—	0.32	0.26	—	—	0.18	0.16	—	—
28E1/15	—	—	0.18	—	—	—	—	—	0.27	—	0.13	—
35E1/20	—	—	0.3	0.16	—	—	—	—	0.52	—	—	—
36E1/17	—	—	—	—	—	—	—	—	0.27	0.16	—	—
40E1/16	—	—	—	—	—	—	—	—	0.49	—	—	—
44E1/18	—	—	—	—	—	—	—	—	0.33	—	—	—
Healthy												
NHS1	—		1.80		—							
NHS2	—		—		—							
NHS3	—		—		—							
NHS4	—		1.05		—							
NHS5	—		—		0.23							
NHS6	—		—		1.85							
NHS7	—		—		—							
NHS8	—		0.24		—							
NHS9	—		—		—							
NHS10	—		—		—							

[a] Ten patients with COPD experienced exacerbations and *B. catarrhalis* was recovered from sputum. Pre and post refer to serum and sputum samples obtained 1 month prior to the exacerbation and 1 month after the exacerbation, respectively, for this group. Ten patients with COPD were free of colonization by *B. catarrhalis* as confirmed by monthly sputum cultures. Pre refers to serum and sputum samples obtained upon enrollment of the patients in the study clinic, and post refers to samples obtained an average of 20 months later.

[b] —, level of <0.1 μg/ml.

the human serum and sputum supernatant antibodies were directed at the same OMP E epitope which is recognized by MAbs 1B3 and 9G10d. The seven human samples which had antibody levels of >1 μg/ml were tested. The wells of a microtiter plate were coated with OMP E. After being blocked and washed, human serum and sputum supernatant samples (1:10 and 1:50 dilutions) were added and incubated for 2 h. After the supernatant samples were removed, MAb 1B3 was added and allowed to bind. MAbs were detected with anti-mouse IgG–peroxidase. None of the seven samples caused a reduction in binding of MAb 1B3 to OMP E. Separate wells were tested with anti-human peroxidase conjugates of the appropriate specificity to confirm that the human antibodies bound to OMP E. In addition, human antibodies were tested with anti-mouse immunoglobulin–peroxidase conjugate to establish that the anti-mouse conjugate did not detect human antibody. These experiments indicate that antibodies in the human serum and sputum supernatants bind to epitopes on OMP E different from the epitope recognized by MAbs 1B3 and 9G10d.

DISCUSSION

In this study we developed polyclonal antibodies and MAbs to OMP E. MAbs 1C11 and 7C10 recognize non-surface-exposed epitopes, and MAbs 1B3 and 9G10d recognize epitopes that are present on the surface of the intact bacterium. All four MAbs are specific for *B. catarrhalis*. The epitopes are present in strains from diverse clinical origins. Finally, proteinase K digestion of whole cells allowed us to map conserved surface epitopes to a 17-kDa region towards the N terminus of the protein molecule.

These observations extend earlier studies which showed that OMP E is conserved among strains of *B. catarrhalis*. Previous studies showed that OMP E was present in all of 50 strains of *B. catarrhalis* by SDS-PAGE. The molecular masses of OMP E were similar in all 50 strains (1). Analysis of PCR restriction fragment length polymorphisms of 19 *B. catarrhalis* strains demonstrated that the OMP E gene was present and was the same size (1.4 kb) in all strains (3). The sequences recognized at 11 sites digested by restriction endonuclease were identical

among all strains tested (3). However, analysis of restriction fragments of the OMP E gene does not enable reliable detection of minor sequence differences which could result in altered epitopes. Furthermore, bacteria often express OMPs that have heterogeneous surface epitopes but are otherwise conserved. Analysis of strains with MAbs which are specific for surface epitopes in the present study established that at least one surface epitope is conserved among strains.

It is important to study surface epitopes because antibodies to surface-exposed epitopes are more likely to be protective against infections. For example, antiserum to the P2 protein of *H. influenzae* type b was protective against infection by the homologous strain in the infant rat model (19). The same antiserum was not protective against infection by a strain when the P2 protein differed by a single amino acid in a surface-exposed region of the molecule (18, 19).

Neisseria species and *B. catarrhalis* are phenotypically indistinguishable on chocolate agar plates and by Gram staining. The frequency of *B. catarrhalis* in sputum cultures is probably underestimated because its appearance is similar to that of commensal *Neisseria* species; therefore, better tools are needed in the clinical microbiology laboratory to differentiate the two species. All four MAbs in this study recognize epitopes which are conserved among all *B. catarrhalis* strains tested. Furthermore, all four MAbs do not react with the other gram-negative bacteria tested. These observations suggest that the MAbs can be used to identify *B. catarrhalis* in clinical samples, assuming that the MAbs will not react with other species when tested more extensively. Alternatively, DNA probes corresponding to the regions recognized by these species-specific MAbs could be developed. This is important because it will become easier to differentiate *B. catarrhalis* from commensal *Neisseria* species in sputa from patients with lower respiratory tract infections.

Proteinase K treatment of whole cells has been used for structural and functional studies of OMPs of other bacteria (17, 27, 28, 33). For example, proteinase K treatment was used to locate a region of OmpC of *E. coli* that was cell surface exposed and also showed that this region was a phage receptor site. Proteinase K treatment of the intact cells completely abolished the ability of OmpC to act as a receptor to phage Tulb (17).

Incubation of whole cells of *B. catarrhalis* with proteinase K was done to map regions of OMP E which have surface-exposed epitopes. The portions of OMP E which are buried inside the membrane are protected from the enzyme following incubation of proteinase K with whole bacterial cells. MAbs 1B3 and 9G10d do not recognize the 32-kDa OMP E band resulting from proteinase K digestion in immunoblots, indicating that the 17-kDa region which was cleaved contains the surface epitopes recognized by 1B3 and 9G10d. On the basis of the amino-terminal sequence of the 32-kDa protein, MAbs 1B3 and 9G10 recognize epitopes which are located in the N-terminal 184 amino acids of OMP E.

Analysis of the serum and mucosal antibody responses to OMP E in adults with COPD revealed that these patients had relatively low levels of antibodies. IgA levels of >0.1 and <1 μg/ml were present in the sputum supernatants of a majority of these patients. The absence of a consistent antibody response to OMP E following well-documented lower respiratory tract infections with *B. catarrhalis* indicates that OMP E is not an immunodominant antigen when the human host is presented with the whole bacterium. Analysis of T-cell epitopes on outer membrane proteins of *Neisseria meningitidis* has revealed a hierarchy of T-cell immunogenicity among the proteins (34). A strong antibody response to class 5 OMPs has been observed following meningococcal infection. Wiertz et al. (34) showed that the T-cell immunogenicity of OMPs parallels the antibody response. One might speculate that in the case of the antibody response to *B. catarrhalis*, OMP E does not have immunodominant T-cell epitopes. Therefore, a prominent antibody response to OMP E does not occur following infection in which the human immune system is presented with many antigens in the form of the whole bacterium. One might speculate further that the organism uses this mechanism to "hide" OMP E from the human host because the induction of an antibody response to a conserved, surface-exposed antigen might lead to the elimination of the bacterium.

The immune response to a single protein following presentation of the immune system with the whole organism does not predict the immune response to immunization with a single protein. For example, animals challenged with whole bacterial cells of nontypeable *H. influenzae* show an antibody response primarily to OMP P2 and minimal or undetectable antibodies to OMPs P6 and P4 (35). On the other hand, immunization of animals with purified OMP P6 and P4 results in high-titer antibody responses (6, 8, 9). Immunization of animals with OMP E of *B. catarrhalis* in this study produced prominent antibody responses to OMP E. It will be important to study the antibody response to immunization of humans with purified OMP E to assess its immunogenicity and potential as a vaccine antigen.

OMP E has several characteristics which indicate that it may be an effective vaccine antigen. Previous work and the present study have shown that OMP E is conserved among *B. catarrhalis* strains. The current study has established that OMP E is immunogenic because immunization of animals produced antibodies. The present study also demonstrates that OMP E expresses epitopes that are surface exposed and highly conserved. Future work should be performed to determine if OMP E can generate a protective immune response.

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