Moraxella catarrhalis Outer Membrane Protein CD Elicits Antibodies That Inhibit CD Binding to Human Mucin and Enhance Pulmonary Clearance of *M. catarrhalis* in a Mouse Model[†]

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Received 5 January 2007/Returned for modification 25 February 2007/Accepted 24 March 2007

The outer membrane protein CD of *Moraxella catarrhalis* is considered to be a potential vaccine antigen against *Moraxella* infection. We purified the native CD from isolate O35E, administered it to mice, and detected considerable titers of anti-CD antibodies. Anti-CD sera were cross-reactive towards six different *M. catarrhalis* isolates and promoted bacterial clearance of O35E in a pulmonary challenge model. To circumvent the difficulty of generating large quantities of CD from *M. catarrhalis* for vaccine use, the CD gene from O35E was cloned into *Escherichia coli*, and the recombinant CD, expressed without a signal sequence or fusion tags, represented ~70% of the total *E. coli* proteins. The recombinant CD formed inclusion bodies that were solubilized with 6 M urea and then purified by ion-exchange chromatography, a procedure that produced soluble CD of high purity and yield. Mice immunized with the purified recombinant CD had significant titers of anti-CD antibodies that were cross-reactive towards 24 different *M. catarrhalis* isolates. Upon challenge, these mice showed enhanced bacterial clearance of both O35E and a heterologous *M. catarrhalis* isolate, TTA24. In an in vitro assay, antisera to either the native or the recombinant CD inhibited the binding activity of CD to human tracheobronchial mucin in a serum concentration-dependent manner, and the extent of inhibition appeared to correlate with the corresponding anti-CD antibody titer and whole-cell enzyme-linked immunosorbent assay titer. Our results demonstrate that the recombinant CD is a promising vaccine candidate for preventing *Moraxella* infection.

Moraxella catarrhalis is an important human mucosal pathogen of the respiratory tract (20, 29, 44). It is the third most common cause of bacterial otitis media in infants and young children (3, 40), following *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae*. Among adults, *M. catarrhalis* is often associated with bronchitis, laryngitis, and other respiratory diseases (1, 5). Patients with chronic obstructive pulmonary disease (COPD) are particularly vulnerable to exacerbations caused by *M. catarrhalis* (1, 6, 35). Interest in the development of a *Moraxella* vaccine is further stimulated by the increasing prevalence of antibiotic resistance among *M. catarrhalis* strains (2, 8, 19).

The CD outer membrane protein of *M. catarrhalis* has been identified as a potential vaccine against *Moraxella* infection (9, 26) and is a safe and effective carrier for *M. catarrhalis* detoxified lipooligosaccharide (LOS)-based conjugates (18). Serum immunoglobulin G (IgG) antibodies specific to CD are present in infants with otitis media (25) and in children with otitis media with effusion (11). Analysis of salivary immunoglobulin A (IgA) in children with acute respiratory tract infection indicates that CD may be one of the outer membrane antigens eliciting a mucosal immune response (27). IgA antibodies against CD as well as several other surface components of *M. catarrhalis* are also detected in the saliva of healthy adults (28). Furthermore, adults with COPD develop mucosal IgA against CD

in the sputum in addition to CD-specific IgG in the serum (30, 33). These observations strongly suggest that CD is a target of both systemic and mucosal immune responses following *M. catarrhalis* infection.

CD is a heat-modifiable protein of ~45 kDa that displays an apparent molecular mass of ~60 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) when heated under reducing conditions (41). Since the CD gene sequence shows that CD shares homology with the OprF outer membrane porin protein of *Pseudomonas* species, CD may also function as a porin (32). The CD gene is highly conserved based on gene sequence and PCR restriction fragment length polymorphism analysis of more than 30 *M. catarrhalis* isolates recovered from diverse clinical and geographic sources (17, 32). Using a panel of mouse monoclonal antibodies (MAbs) against CD, two surface-exposed epitopes have been identified, one near the amino terminus and the other within the central region of the protein (41). Human antibodies from adults with COPD also target these surface-exposed epitopes (31, 41). Native *M. catarrhalis* CD elicited bactericidal antibodies in mice and guinea pigs (45), and mice immunized with a histidine-tagged recombinant CD showed enhanced pulmonary clearance of *M. catarrhalis* (34).

CD is the only outer membrane protein of *M. catarrhalis* capable of binding to purified human salivary mucin, nasopharyngeal mucin, middle ear mucin, and tracheobronchial mucin, suggesting that CD-mucin interaction may facilitate adherence of *M. catarrhalis* in the respiratory tract (4, 39). Furthermore, CD is thought to interact with host target cells. Recently, *M. catarrhalis* CD gene mutants were generated by transposon

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[‡] Published ahead of print on 2 April 2007.

mutagenesis; these mutants exhibited significantly reduced binding to A549 human lung cells (15).

In this study, we purified the native CD (nCD) from *M. catarrhalis* outer membrane and a recombinant CD (rCD) without a signal sequence or fusion tags from *Escherichia coli*. Both purified nCD and rCD were examined in a murine pulmonary challenge model for bacterial clearance of homologous and heterologous isolates. Antibodies against nCD and rCD were also assessed for their ability to inhibit the interaction between CD and purified human tracheobronchial mucin.

MATERIALS AND METHODS

Moraxella catarrhalis isolates, MABs, and human tracheobronchial mucin.

Isolates O35E and TTA24 were kindly provided by E. Hansen (University of Texas Southwestern Medical School, Dallas, TX); 4608, 15P9B1, and 5193 were provided by T. Murphy (The State University of New York at Buffalo and Veterans Affairs Medical Center, Buffalo, NY); and all other strains were provided by D. Hardy (University of Rochester, Rochester, NY) (23). MABs 7D6 and 3.9H specific for CD (31, 41) were prepared as ammonium sulfate concentrates of culture supernatants from hybridoma clones provided by T. Murphy. Purified human tracheobronchial mucin was provided by M. Reddy (The State University of New York at Buffalo, Buffalo, NY). The bacteria were stored at -70°C in Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, MI) containing 40% glycerol and routinely passaged on MH agar at 37°C with 5% CO_2 before use. MABs and purified tracheobronchial mucin were stored at -20°C .

Expression of CD in *E. coli* and construction of an *M. catarrhalis* CD knockout mutant. The CD gene from *M. catarrhalis* O35E was cloned using forward (5'-CATATGGGTGTGACAGTCAGCCCACTAC) and reverse (5'-GGATCC CTGGCGATATGCCGAACTG) primers for PCR amplification of chromosomal DNA. After ligation of the ~ 1.3 -kb PCR product into an intermediate vector, pCR2.1 (Novagen, Madison, WI), the CD coding sequence was subcloned into pRSET T7 expression plasmid (Novagen) as an NdeI-BamHI fragment without a signal sequence or fusion tags. The ampicillin resistance (Amp^r) marker was subsequently replaced with the kanamycin resistance (Kan^r) marker from pUC4K. The resulting plasmid, designated pLP150, was sequenced to verify the CD gene identity and used to transform *E. coli* BL21(DE3). The transformed *E. coli* BL21(DE3)pLP150 was grown in Luria-Bertani (LB) media supplemented with 30 $\mu\text{g}/\text{ml}$ of kanamycin (LB-Kan; Sigma Chemical Co., St. Louis, MO).

A CD knockout mutant of *M. catarrhalis* was constructed by digesting the CD gene of isolate O35E with EspI and ligating it into the site of a Kan^r marker that disrupted the CD coding region at a position corresponding to Ala117. The pPX191 vector backbone used to contain CD::Kan^r is a pUC9 derivative with the gene for chloramphenicol resistance (Cam^r) in the SspI site of the Amp^r marker of pUC9, which confers a Cam^r Amp^r phenotype. The resulting plasmid, pPX2921 (Amp^r Cam^r CD::Kan^r), was electroporated into *M. catarrhalis* O35E. Selection on MH agar containing 30 $\mu\text{g}/\text{ml}$ of kanamycin gave rise to Kan^r transformants, approximately half of which had a small-colony Cam^r phenotype. The remaining Kan^r transformants were Cam^r and possessed a normal colony morphology. The transformants were screened for disruption of CD expression by Western blot analysis using CD-specific MAB 7D6 as well as polyclonal anti-CD sera. All the Kan^r Cam^r transformants, but not the Kan^r Cam^r transformants, failed to react with either antibody reagent. One of these Kan^r Cam^r isolates was saved and designated PBCC2508.

Purification of nCD. nCD was purified from outer membrane vesicles of O35E. MH broth was inoculated with bacteria to an optical density at 600 nm (OD_{600}) of 0.01 and grown in a 37°C shaking incubator to an OD_{600} of ~ 3.5 . Cells resuspended in phosphate-buffered saline (PBS) containing 0.5 M sodium chloride were subjected to vigorous mixing before centrifugation at $10,000 \times g$ for 20 min. Outer membrane vesicles in the supernatant were solubilized with 1% *n*-octyl- β -D-glucopyranoside (Sigma), dialyzed against 10 mM sodium phosphate buffer at pH 6.2 with 1 mM EDTA and 1% *n*-octyl- β -D-glucopyranoside (buffer A), and then loaded onto an SP Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) column in tandem with a hydroxylapatite (ceramic hydroxylapatite; Bio-Rad, Hercules, CA) column previously equilibrated with buffer A. The flowthrough fraction from the SP-hydroxylapatite columns was dialyzed against 10 mM Tris buffer at pH 9 with 1 mM EDTA and 1% *n*-octyl- β -D-glucopyranoside (buffer B) and loaded onto a TMAE (VWR, Bridgeport, NJ) column equilibrated with buffer B. After the column was washed with 80 mM sodium chloride in buffer B, nCD was eluted with 100 mM sodium chloride in buffer B.

The purity of nCD ($>99\%$) was estimated by densitometric scanning of a Coomassie blue-stained SDS-polyacrylamide gel.

Purification of rCD. HySoy broth (DMV International Nutritionals, Delhi, NY) supplemented with 30 $\mu\text{g}/\text{ml}$ of kanamycin and 1% glycerol was inoculated with *E. coli* BL21(DE3)pLP150 to an OD_{600} of 0.05, grown in a 10-liter Biostat B fermentor (Sartorius, New Brunswick, NJ) to an OD_{600} of ~ 1.0 , and induced with isopropyl-1-thio- β -D-galactopyranoside. Rifampin (Sigma) was added 10 min later to a final concentration of 0.15 mg/ml. Three hours after induction, cells were harvested by low-speed centrifugation.

The cells were resuspended in PBS containing 1% Triton X-100, 5 mM Pefabloc (Boehringer Mannheim, Indianapolis, IN), 10 mM EDTA, 0.5 mg/ml of lysozyme, and 10 $\mu\text{g}/\text{ml}$ of RNase and DNase. The cell suspension was incubated at 37°C for 1 h and then passed through a Microfluidizer (Microfluidics International Corp., Newton, MA). The cell lysate was centrifuged at $12,000 \times g$ for 20 min. The pellet, consisting mainly of rCD inclusion bodies, was extracted at 4°C with 6 M urea in 0.1 M Tris buffer at pH 8.0 with 5 mM Pefabloc and then centrifuged at $45,000 \times g$ for 20 min. The solubilized rCD was diluted twofold with 1% Triton X-100 containing 4 mM EDTA and loaded onto a TMAE column equilibrated with 50 mM Tris buffer at pH 8 containing 3 M urea, 2.5 mM Pefabloc, 0.5% Triton X-100, and 2 mM EDTA (buffer C). After the TMAE column was washed with 5 mM sodium chloride in buffer C, rCD was eluted with 0.1 M sodium chloride in buffer C. The partially purified rCD was dialyzed against 5 mM sodium phosphate buffer at pH 6 containing 1 M urea, 0.5% Triton X-100, and 1 mM EDTA (buffer D) and loaded onto an SP Sepharose (Amersham Biosciences, Piscataway, NJ) column equilibrated with buffer D. After the SP Sepharose column was washed with buffer D, rCD was eluted with 0.5 M sodium chloride in 0.25 M sodium phosphate buffer at pH 7 containing 1 M urea, 0.5% Triton X-100, and 1 mM EDTA. The purified rCD was dialyzed against PBS containing 0.1% Triton X-100 and stored at -20°C . The purity of rCD was estimated by densitometric scanning of a Coomassie blue-stained SDS-polyacrylamide gel.

As a negative control, *E. coli* BL21(DE3) containing the plasmid vector pRSET with Kan^r was grown and induced, and a cell lysate was subsequently used to generate a mouse polyclonal antiserum.

Amino acid composition, N-terminal sequence, molecular mass, and protein concentration determination. Amino acid analysis was performed on a Biochrom 20 Plus amino acid analyzer (Biochrom Ltd., Cambridge, United Kingdom). N-terminal sequencing was performed on a Procise HT amino-terminal protein sequencer (Applied Biosystems, Framingham, MA). Molecular mass was determined with a Voyager DE-sSTR matrix-assisted laser desorption/ionization-time of flight mass spectrometer (Perseptive Biosystems, Framingham, MA). Protein concentrations were determined by the Peterson-Lowry method (38).

SDS-PAGE, Western blot analysis, and LOS content determination. SDS-PAGE analysis was performed in 12% polyacrylamide gels (21). For Western blot assays, proteins transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) (43) were probed with anti-CD MABs or mouse anti-nCD sera. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG and IgM antibodies (BioSource, Camarillo, CA). In the experiments in which cell lysates were used for Western blot analysis, *M. catarrhalis* grown in MH broth at 37°C to an OD_{600} of 1.0 was lysed in SDS-PAGE sample buffer; cell lysates from 2×10^7 cells were subjected to SDS-PAGE followed by protein transfer. LOS contents were determined by the *Limulus* amoebocyte lysate assay according to the manufacturer's instructions (Cape Cod/VWR, Bridgeport, NJ).

Generation of mouse antisera. Female BALB/c mice, 6 to 8 weeks old (Taconic Farms, Germantown, NY), were vaccinated subcutaneously at weeks 0 and 4 with antigens with one of the following adjuvants: 50 μg of 3-O-deacylated monophosphoryl lipid A (MPL; Corixa Corp., Hamilton, MT) plus 100 μg of aluminum phosphate (alum), 100 μg of alum alone, or 20 μg of QS21 (Antigenics, Inc., Framingham, MA). Sera collected at weeks 0, 4, and 6 were pooled from 10 mice for analysis.

ELISAs. The antibody response of mice to CD was measured using a standard enzyme-linked immunosorbent assay (ELISA) procedure. Costar 96-well plates (Corning Inc., Corning, NY) were coated with rCD diluted to 5 $\mu\text{g}/\text{ml}$ with carbonate buffer. Whole-cell titers were measured by coating 96-well plates with *M. catarrhalis* cells suspended in PBS (7). Anti-CD antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG and IgM antibodies (BioSource, Camarillo, CA). Antibody titers and whole-cell titers were the reciprocals of serum dilutions with an absorbance at 405 nm of 0.1 extrapolated from a linear plot of the logarithm of absorbance versus the logarithm of serum dilution.

CD-mucin binding inhibition assay. Costar 96-well plates were coated overnight at 4°C with purified human tracheobronchial mucin diluted to 20 $\mu\text{g}/\text{ml}$ in PBS. The diluent for rCD and antisera was PBS containing 5% fetal bovine

TABLE 1. CD-specific antibody response elicited by either nCD or rCD in mice^a

Mouse serum	Anti-CD antibody titer elicited by CD (μ g) ^b		
	nCD (1)	nCD (5)	rCD (5)
Preimmune	<50	<50	<50
Wk 4	19,222	93,110	89,515
Wk 6	58,451	284,657	247,008

^a Mice were immunized with either nCD or rCD with MPL plus alum as adjuvant.

^b Anti-CD antibody titers were the reciprocal of the serum dilution with an absorbance at 405 nm of 0.1 extrapolated from a linear plot of the logarithm of absorbance versus the logarithm of serum dilution. A negative-control antiserum from mice immunized with 5 μ g of an induced *E. coli* BL21(DE3)/pRSET/Kan^r cell lysate exhibited a titer of <50.

serum and 0.3% Tween 20. A CD-mucin binding curve was included in each plate that expanded from 0 to ~20 μ g/ml of rCD. For inhibition of CD-mucin binding, sera were serially diluted, starting at 1:50; mixed with an equal volume of rCD at 5 μ g/ml; and incubated at 25°C for 40 min. The mixtures were then transferred to a mucin-coated plate and incubated at 37°C for 1 h. The plate was washed to remove unbound material. CD bound to mucin was detected with mouse anti-CD serum, and bound anti-CD antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG and IgM antibodies. The results were plotted as absorbance at 405 nm versus serum dilution $\times$ 100; thus, a serum dilution of 1:100 had a value of 1 on the x axis (see Fig. 4B). The inhibition titer is the serum dilution at which a 50% reduction of CD-mucin binding was observed.

Murine pulmonary clearance model. Groups of 10 female BALB/c mice, 6 to 8 weeks old, were immunized subcutaneously at weeks 0 and 4 with either nCD or rCD plus adjuvants and bled at weeks 0, 4, and 6. Two days after the final bleed, intratracheal challenge with 3.5×10^5 live bacteria, either O35E or TTA24, was conducted as described previously (7). Viable bacteria recovered from the lungs of individual mice 6 h postchallenge were recorded as CFU. In each challenge experiment, a sham group of mice immunized with genetically detoxified diphtheria toxin (CRM197) was included as a negative control. The percent bacterial clearance was determined by comparing the number of bacteria recovered from the test group with that from the sham group. Statistical analysis of the data was performed using the Wilcoxon signed-rank test (JMP software; SAS Institute, Cary, NC). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Antibody response elicited by nCD in mice. nCD purified from outer membrane vesicles of *M. catarrhalis* O35E was highly immunogenic in mice. After one immunization with 1 μ g of nCD with MPL plus alum as adjuvant, the week 4 antiserum had an anti-CD antibody titer of >19,000. Upon a second immunization at week 4, the anti-CD antibody titer of week 6 antiserum was boosted by approximately threefold (Table 1). In addition to the antigen-specific antibody titers toward CD, these antisera also reacted with whole cells of *M. catarrhalis*. Whole-cell titers ranging from 5,000 to over 13,000 were detected with the week 6 antiserum toward five different isolates besides O35E (Table 2), indicating that the antibodies elicited by nCD were cross-reactive and recognized CD surface epitopes of the homologous isolate as well as several heterologous isolates.

Pulmonary clearance of *M. catarrhalis* in mice immunized with nCD. To investigate if immunization with nCD would promote pulmonary clearance of *M. catarrhalis*, mice were administered 5 μ g of nCD with MPL plus alum as adjuvant at weeks 0 and 4. Upon challenge with the homologous isolate O35E at week 6, the amount of bacteria recovered from the

TABLE 2. Antibodies elicited by either nCD or rCD recognize homologous and heterologous isolates of *M. catarrhalis*^a

<i>M. catarrhalis</i> isolate	Whole-cell titer of wk 6 antiserum elicited by CD (μ g) ^b	
	nCD (1)	rCD (5)
O35E	9,644	30,012
TTA24	5,099	67,955
430:345	7,258	42,596
216:96	9,614	69,881
111:210	13,322	64,955
1230:359	11,473	91,943

^a Mice were immunized with either nCD or rCD with MPL plus alum as adjuvant.

^b Whole-cell titers were the reciprocal of the serum dilution with an absorbance at 405 nm of 0.1 extrapolated from a linear plot of the logarithm of absorbance versus the logarithm of serum dilution. A whole-cell titer of <50 was obtained for the preimmune serum and a negative-control antiserum from mice immunized with 5 μ g of an induced *E. coli* BL21(DE3)/pRSET/Kan^r cell lysate.

lungs of the immunized mice was reduced by 65.2% (*P* = 0.0007) compared to the sham group in which mice received CRM197 instead (Fig. 1). Compared to the antisera from mice immunized with 1 μ g of nCD, the week 4 and 6 antisera of mice immunized with 5 μ g of nCD showed almost a fivefold increase in the anti-CD antibody titer (Table 1), a clear demonstration of dose response toward the antigen. In contrast, mice from the CRM197 sham group had a titer of <50 at weeks 4 and 6. These results indicated that the antibodies elicited by nCD were functional in promoting pulmonary clearance of *M. catarrhalis* O35E in mice.

Purification and characterization of rCD. In order to generate large quantities of CD, we constructed an *E. coli* strain that overproduced a full-length mature rCD under the control of the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible T7 expression system. The amount of rCD represented ~70% of the total protein of the induced *E. coli* cells (Fig. 2, lane 2), and the bulk of rCD formed inclusion bodies with a purity of ~88% (Fig. 2, lane 3). The rCD inclusion bodies were solubilized with 6 M urea and purified on a TMAE column that resulted in rCD preparations with a purity of >99% (Fig. 2,

FIG. 1. Enhanced pulmonary clearance of *M. catarrhalis* isolate O35E in mice immunized with nCD. Mice immunized twice with 5 μ g of nCD with MPL plus alum as an adjuvant were challenged intratracheally with 3.5×10^5 CFU of O35E. Six hours after challenge, CFU recovered from the lungs of individual mice were determined. Each bar represents the standard deviation of the mean. *, *P* = 0.0007 compared to the sham group CRM197 + MPL/alum.

FIG. 2. SDS-PAGE analysis of fractions obtained at different stages of rCD purification. A 12% Coomassie blue-stained SDS gel was loaded with 5 μ g of protein in each lane. Lane 1, Bio-Rad prestained low-molecular-mass markers in kDa; lane 2, cell lysate of an induced culture of *E. coli* containing pLP150; lane 3, rCD inclusion bodies solubilized with 6 M urea; lane 4, rCD eluted off the TMAE column; lane 5, rCD eluted off the SP column and then dialyzed into PBS containing 0.1% Triton X-100.

lane 4). Following TMAE chromatography, rCD was further purified on an SP Sepharose column to remove residual LOS. The final purified rCD protein (Fig. 2, lane 5) remained soluble upon multiple freeze/thaw cycles from -20°C storage.

The overall yield from the purification scheme was ~ 10 mg of rCD per g of cell mass (wet weight). The purified rCD protein had very low levels of LOS, ~ 0.05 EU per μ g of protein, and was reactive with the two anti-CD MAbs 7D6 and 3.9H as well as mouse anti-nCD sera (data not shown). The identity of rCD was verified by amino acid analysis, N-terminal sequencing, and mass spectrometry. The amino acid content of rCD for the majority of residues was equal to the theoretical value ± 0 to ~ 0.67 . The first 20 residues, GVTVSPLLLGYH YTDEAHND, were confirmed by N-terminal sequencing. Mass spectrometry analysis yielded a molecular mass of 45,809 Da, whereas the CD protein sequence predicts a theoretical molecular mass of 45,916 Da.

Antibody response elicited by rCD in mice. Mice were twice administered 5 μ g of rCD with MPL plus alum as adjuvant, using the same vaccination schedule described for nCD. The rCD protein was as immunogenic as the nCD protein, eliciting equivalent anti-CD antibody titers at week 4 after one immunization and at week 6 upon a second immunization (Table 1). In addition to the CD-specific antibody titers, the week 6 antiserum also reacted with whole cells of six different *M. catarrhalis* isolates. A comparison of the whole-cell titers elicited by 5 μ g of rCD to the whole-cell titers elicited by 1 μ g of nCD reflected a dose response to the antigen (Table 2). A negative-control antiserum from mice immunized with 5 μ g of an induced *E. coli* BL21(DE3)/pRSET/Kan^r cell lysate exhibited a titer of <50 in both the antigen-specific and whole-cell ELISAs.

While characterizing an additional 18 *M. catarrhalis* isolates using anti-CD MAbs in Western blot assays, we found that 13 isolates reacted with both MAb 7D6 and MAb 3.9H and five isolates reacted only with MAb 7D6. To determine if anti-rCD sera might react with selected isolates, we examined these 18 isolates in a whole-cell ELISA along with a CD knockout mutant, strain PBCC2508, as a negative control. Anti-rCD

serum displayed whole-cell titers ranging from 16,000 to 161,000 toward 3.9H-positive as well as 3.9H-negative isolates, except for PBCC2508 as expected (Table 3). That PBCC2508 had a whole-cell titer of 1,277, higher than the preimmunization titer of <50 , is due to cross-reactive epitopes on other *M. catarrhalis* surface proteins. We found two such proteins, of ~ 59 kDa and ~ 28 kDa, respectively, in PBCC2508 (data not shown). These results demonstrated that the antibodies elicited by rCD were cross-reactive and recognized surface epitopes of at least 23 heterologous isolates in addition to the homologous isolate O35E.

Pulmonary clearance of *M. catarrhalis* in mice immunized with rCD. To determine whether immunization with rCD would promote pulmonary clearance of *M. catarrhalis*, mice were twice administered 20 μ g of rCD in the presence of different adjuvants and then challenged at week 6 with live bacteria. Upon challenge with the homologous isolate O35E, the CFU recovered from the lungs of mice that received rCD with either alum or QS21 were reduced by 43% ($P = 0.012$) and 51.8% ($P = 0.0095$), respectively, compared to the CRM197 sham groups (Fig. 3A). In a separate experiment, mice were challenged with the heterologous isolate TTA24, and the CFU recovered from the lungs of mice that received rCD with either alum or MPL plus alum were reduced by 62.3% ($P = 0.0008$) and 50.6% ($P = 0.0023$), respectively, compared to the CRM197 sham group (Fig. 3B). High anti-CD antibody titers and anti-O35E whole-cell titers (Table 4, values indicated by footnote letter *b*) were detected in the anti-rCD sera raised with either alum alone, QS21 alone, or MPL plus alum, whereas both the preimmune and anti-CRM197 sera exhibited titers of <50 . These results indicated that anti-rCD antibodies were functional, capable of promoting pulmonary

TABLE 3. Antibodies against rCD recognize multiple *M. catarrhalis* isolates regardless of whether the isolates are reactive toward MAb 3.9H^a

<i>M. catarrhalis</i> isolate	Reactivity toward MAb ^b :		Whole-cell titer of wk 6 anti-rCD serum ^c
	7D6	3.9H	
125:114	+	+	148,824
115:142	+	+	39,453
205:211	+	+	35,006
110:070	+	+	33,134
306:155	+	+	22,930
204:206	+	+	97,119
B46	+	+	31,062
422:318	+	+	25,710
312:171	+	+	160,972
J261	+	+	100,826
113:136	+	+	25,663
205:333	+	+	78,120
046E	+	+	52,217
218:038	+	—	57,518
417:082	+	—	16,160
4608	+	—	103,523
5193	+	—	110,915
15P9B1	+	—	106,339
PBCC2508	—	—	1,227

^a Mice were immunized with 5 μ g of rCD with MPL plus alum as adjuvant.

^b Reactivity of cell lysates in Western blot assay.

^c Preimmune serum had a whole-cell titer of <50 .

FIG. 3. Enhanced pulmonary clearance of *M. catarrhalis* isolates O35E and TTA24 in mice immunized with rCD. Mice immunized twice with 20 μ g of rCD mixed with different adjuvants were challenged intratracheally with either O35E or TTA24 as described in the Fig. 1 legend. (A) Pulmonary clearance of O35E. *, $P = 0.012$ compared to the sham group CRM197 + alum; **, $P = 0.0095$ compared to the sham group CRM197 + QS21. (B) Pulmonary clearance of TTA24. *, $P = 0.0008$, and **, $P = 0.0023$, compared to the sham group CRM197 + alum. Each bar represents the standard deviation of the mean.

clearance of both the homologous isolate O35E and the heterologous isolate TTA24 of *M. catarrhalis*.

Inhibition of CD-mucin binding by antisera against CD.

The binding activity of CD for human tracheobronchial mucin was measured by an ELISA-based method. In this assay, CD bound to mucin in a protein concentration-dependent manner

and the binding curve was fairly linear up to 10 μ g/ml of rCD (Fig. 4A). Bovine serum albumin and the purified recombinant CopB protein (rCopB) of *M. catarrhalis* (23), included as negative controls, did not display affinity to mucin. To assess functional characteristics of the antisera against CD, their effects on the CD-mucin binding activity were examined. Binding of rCD to mucin was strongly inhibited by preincubating rCD with anti-rCD serum at the lowest dilution of 1:100, and this inhibition was gradually relieved when the antiserum concentration was reduced by serial dilution (Fig. 4B). In contrast, this serum concentration-dependent inhibition of CD-mucin binding by the anti-rCD serum was not observed with the preimmune serum. The slight interference at high concentrations of the preimmune serum appeared to be nonspecific, since all preimmune sera did not have anti-CD activity (Tables 1 to 4).

From the inhibition curve, we derived an inhibition titer for a given anti-CD serum as described in Materials and Methods. The various anti-CD sera generated from the murine pulmonary clearance studies were tested for their abilities to inhibit CD-mucin binding and compared based on the inhibition titers (Table 4). Anti-nCD and anti-rCD sera, obtained from mice immunized with 5 μ g of either nCD or rCD with MPL and alum as adjuvants, exhibited equivalent inhibition titers, and the inhibition titer increased almost threefold with antiserum from mice immunized with 20 μ g of rCD. Besides the dosage of rCD, the choice of adjuvant also affected the inhibition titer. The highest inhibition titer was obtained when rCD at 20 μ g was used with QS21 as an adjuvant, compared to MPL plus alum or alum alone. Neither preimmune serum nor antisera against CRM197, rCopB, native UspA from *M. catarrhalis*, or an induced *E. coli* BL21(DE3)/pRSET/Kan^r cell lysate blocked the CD-mucin binding activity. The CD-mucin binding inhibition titers rose in parallel with increased anti-CD antibody titers and anti-O35E whole-cell titers, although there was not a direct linear relationship among these titers (Table 4).

DISCUSSION

A number of investigations have reported that the CD outer membrane protein is a target of both systemic and mucosal immune responses following *M. catarrhalis* infection. To dem-

TABLE 4. Correlation between CD-mucin binding inhibition titers and antibody titers

Wk 6 antiserum	CD dose (μ g)	Adjuvant	Titer		
			CD-mucin binding inhibition ^a	Anti-CD	Anti-O35E whole cell
Anti-nCD	5	MPL/alum	650	284,657	34,588
Anti-rCD	5	MPL/alum	664	247,008	30,012
Anti-rCD	20	MPL/alum	1,556	3,049,725 ^b	240,860 ^b
Anti-rCD	20	Alum	2,157	4,448,261 ^b	406,666 ^b
		QS21	4,211	7,540,524 ^b	1,411,385 ^b
Anti-rCopB	20	MPL/alum	<50	<50	100,000
Anti-UspA	5	MPL/alum	<50	<50	<50
Anti-CRM197	5	MPL/alum	<50	<50	<50
Anti- <i>E. coli</i> lysate ^c	5	MPL/alum	<50	<50	<50
Preimmune			<50	<50	<50

^a The inhibition titer is the dilution of serum at which a 50% reduction of CD-mucin binding was observed.

^b Mice were immunized with 20 μ g of rCD for the murine pulmonary clearance studies.

^c Antiserum to an induced *E. coli* BL21(DE3)/pRSET/Kan^r cell lysate.

FIG. 4. (A) Binding to human tracheobronchial mucin as a function of protein concentration of rCD (◆), rCopB (○), or bovine serum albumin (△). (B) Inhibition of rCD-mucin binding by anti-rCD serum. The extent of rCD-mucin binding was measured with rCD at 2.5 μ g/ml previously incubated with either preimmune serum (◇) or anti-rCD serum (◆). The lowest serum dilution was 1:100 and the highest 1:12,800. For each data point, the bar represents the standard deviation of the mean.

onstrate that CD is able to elicit a protective immune response, we first examined nCD purified from the outer membrane of *M. catarrhalis*. The nCD was immunogenic in mice, eliciting cross-reactive antibodies that recognized surface epitopes of homologous and heterologous isolates (Tables 1 and 2). This observation is consistent with the reports that CD is highly conserved (17, 32) and contains surface-exposed epitopes (31, 41). In the murine pulmonary challenge model, immunization with nCD promoted significant bacterial clearance of the homologous strain, indicating that anti-nCD antibodies are functional (Fig. 1). This is the first demonstration that the CD protein isolated from the outer membrane of *M. catarrhalis* elicits a protective immune response in mice.

Since production of large quantities of nCD from *M. catarrhalis* for vaccine use is not feasible, we constructed an *E. coli* strain that expresses a full-length rCD without a signal sequence or any fusion tags. Due to overproduction by *E. coli*, the rCD forms insoluble inclusion bodies. A method was developed to solubilize, purify, and renature the rCD protein, and this process can easily be adapted to large-scale production. The integrity of purified rCD (Fig. 2) is preserved based on its reactivity toward anti-CD MAbs, amino acid composition, N-terminal sequence, and molecular mass. The immunogenicity of the rCD was indistinguishable from that of the nCD (Tables 1 and 2). Both anti-nCD and anti-rCD sera were cross-reactive, able to recognize surface epitopes of homologous and heterologous isolates, including those isolates that are not recognized by MAb 3.9H (Table 3). Most importantly, mice immunized with the rCD showed enhanced pulmonary clearance of the homologous isolate O35E as well as the heterologous isolate TTA24 (Fig. 3). The degree of bacterial clearance that we observed with the nCD and the rCD from *M. catarrhalis* O35E is comparable to that reported by other investigators using either a histidine-tagged rCD or different *Moraxella* immunogens in the same murine pulmonary clearance model of *M. catarrhalis* (7, 12, 13, 24, 34).

Earlier we reported that mice immunized with the UspA protein of *M. catarrhalis* developed bactericidal antibodies (7). However, unlike anti-UspA antibodies, neither mouse anti-nCD nor mouse anti-rCD sera were bactericidal (data not shown). We routinely included anti-UspA serum in the bactericidal assay as a positive control and tested it against more

than eight different *M. catarrhalis* isolates. Our finding is consistent with the observation that antibodies elicited by the histidine-tagged rCD were not bactericidal (34). The only report to date on bactericidal activity of anti-CD antibodies involves a CD preparation from *M. catarrhalis* 4223 (45). The discrepancy may arise from the use of different methods for measuring bactericidal activity in different laboratories, since there is no standardized bactericidal assay for *M. catarrhalis*. It is also possible that this CD preparation, unlike rCD preparations, might have contained low levels of other *M. catarrhalis* antigens capable of eliciting bactericidal antibodies. One factor that can readily affect the results is the strain used to measure bactericidal activity. Yang et al. (45) examined the bactericidal activities of anti-CD mouse and guinea pig sera against only two strains, RH408, which is a nonclumping mutant of strain 4223, and Q8, another nonclumping strain isolated from an expectorate. Their observation raises the question of whether the mouse or guinea pig antisera raised by the CD derived from *M. catarrhalis* 4223 are bactericidal to *M. catarrhalis* strains other than these two nonclumping strains. Should bactericidal activity play a crucial role in protective immunity, it is essential that anti-CD antibodies be bactericidal toward not just a few selected but many different clinical isolates. Our findings demonstrate that there is no correlation between bactericidal activity and total antibody titers or pulmonary clearance as a result of immunization with either nCD or rCD.

To facilitate bacterial colonization and establish infection, *M. catarrhalis* utilizes adhesins to interact with tissues of the respiratory tract. UspA1 and UspA2 (14, 22), Hag (16, 37), MID (10, 36), and McaP (42) are among the adhesins that have been identified. *M. catarrhalis* CD may be a porin, based on the homology with the OprF protein of *Pseudomonas* species (32). Later evidence shows that CD is able to bind to human mucin isolated from saliva, the nasopharynx, the middle ear, and tracheobronchial tissue (4, 39) as well as human lung cells (15). We developed an assay that measures binding of purified rCD to human tracheobronchial mucin and demonstrated that this CD-mucin binding activity is specifically inhibited by either anti-nCD or anti-rCD serum in a serum concentration-dependent manner (Fig. 4). Furthermore, the CD-mucin binding inhibition titer of a given anti-CD serum correlates with the corresponding antibody titer and whole-cell titer (Table 4).

These results support the idea that CD functions as an adhesin, promoting bacterial adherence to the respiratory tract, and are consistent with the hypothesis that immunizing mice with either nCD or rCD enhanced pulmonary clearance of *M. catarrhalis* (Fig. 1 and 3) by blocking adherence. In contrast, recombinant fusion proteins such as the histidine-tagged rCD and glutathione *S*-transferase rCD did not bind to mucin (39). The authors suggested that the mucin binding domain might be located in the amino-terminal region of the CD protein, because they were able to restore the binding activity of CD by removing glutathione *S*-transferase from the fusion protein. Although the histidine-tagged rCD failed to bind mucin, immunization of mice with this tagged protein promoted pulmonary bacterial clearance (34), suggesting that bacterial adherence may be mediated by other pathways besides CD-mucin binding. Further investigation is required to gain insight into the mechanisms involved in pulmonary clearance of *M. catarrhalis*.

Our investigation is the first to evaluate the vaccine potential of the native CD outer membrane protein of *M. catarrhalis* by active immunization of mice. To produce large quantities of CD for vaccine use, we constructed an *E. coli* strain that overexpresses an rCD without a signal sequence or any fusion tags and developed a scalable purification scheme. We showed that both nCD and rCD can elicit functional antibodies that inhibit CD binding to human tracheobronchial mucin. Furthermore, immunization of mice with either nCD or rCD enhances clearance of *M. catarrhalis* in a pulmonary challenge model. These results demonstrate that the rCD described in this report is a promising vaccine candidate to prevent *Moraxella* infection.

ACKNOWLEDGMENTS

We thank Tom Baroody, Michael Wetherell, Terri Mininni, and Karl VanDerMeid for technical assistance.

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Editor: A. Camilli