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## The C-Terminal Fragment of the Internal 110-Kilodalton Passenger Domain of the Hap Protein of Nontypeable *Haemophilus influenzae* Is a Potential Vaccine Candidate

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Nontypeable *Haemophilus influenzae* is a major causative agent of bacterial otitis media in children. *H. influenzae* Hap autotransporter protein is an adhesin composed of an outer membrane Hap $\beta$  region and a moiety of an extracellular internal 110-kDa passenger domain called Hap $\alpha$ . The Hap $\alpha$  moiety promotes adherence to human epithelial cells and extracellular matrix proteins, and it also mediates bacterial aggregation and microcolony formation. A recent work (D. L. Fink, A. Z. Buscher, B. A. Green, P. Fernsten, and J. W. St. Geme, Cell. Microbiol. 5:175-186, 2003) demonstrated that Hap $\alpha$  adhesive activity resides within the C-terminal 311 amino acids (the cell binding domain) of the protein. In this study, we immunized mice subcutaneously with recombinant proteins corresponding to the C-terminal region of Hap $\alpha$  from *H. influenzae* strains N187, P860295, and TN106 and examined the resulting immune response. Antisera against the recombinant proteins from all three strains not only recognized native Hap $\alpha$  purified from strain P860295 but also inhibited *H. influenzae* Hap-mediated adherence to Chang epithelial cells. Furthermore, when mice immunized intranasally with recombinant protein plus mutant cholera toxin CT-E29H were challenged with strain TN106, they were protected against nasopharyngeal colonization. These observations demonstrate that the C-terminal region of Hap $\alpha$  is capable of eliciting cross-reacting antibodies that reduce nasopharyngeal colonization, suggesting utility as a vaccine antigen for the prevention of nontypeable *H. influenzae* diseases.

Nontypeable *Haemophilus influenzae* (NTHi), a nonencapsulated gram-negative bacterium, is the cause of a number of human respiratory tract diseases, such as otitis media, sinusitis, bronchitis, and pneumonia (15, 16). Otitis media is among the most common infections in young children. By 3 years of age, approximately 80% of children have had at least one episode of acute otitis media (25). Recurring bouts of otitis media may lead to significant hearing loss, which in turn may result in developmental delay. A vaccine that prevents nontypeable *H. influenzae* disease would provide major benefits to the health of children and the general population.

The pathogenesis of *H. influenzae* disease begins with colonization of the nasopharynx. Subsequently, organisms spread to other sites in the respiratory tract, including the middle ear, sinuses, and lower airways (21). Based on in vitro and animal studies, a number of factors appear to influence the process of colonization. One such factor is the Hap adhesin, which promotes bacterial interaction with human respiratory epithelial cells and extracellular matrix proteins as well as mediates bacterial aggregation and microcolony formation (10, 23).

Hap belongs to the autotransporter family of proteins common among gram-negative pathogens (9). It is synthesized as a

155-kDa precursor protein, which consists of an N-terminal 25-amino-acid signal peptide, an internal 110-kDa passenger domain called Hap $\alpha$ , and a C-terminal 45-kDa outer membrane domain called Hap $\beta$  (9). Hap $\alpha$  has serine protease activity and is released from the precursor protein via autoproteolysis. Of note, autoproteolysis is inhibited by secretory leukocyte protease inhibitor, which is a natural component of respiratory secretions. The Hap $\alpha$  domain is responsible for all the adhesive properties of Hap (10, 23). Furthermore, purified Hap $\alpha$  is immunogenic in mice, eliciting significant anti-Hap $\alpha$  antibody titers. In a mouse intranasal challenge model, animals immunized with purified Hap $\alpha$  from NTHi strain P860295 or N187 in the presence of mutant cholera toxin CT-E29H as an adjuvant are protected against nasopharyngeal colonization (5). These findings suggest that Hap $\alpha$  has potential as a vaccine antigen against NTHi. However, the development of a Hap $\alpha$ -based vaccine has been hindered by difficulties in purifying adequate quantities of Hap $\alpha$  from the bacterium and the tendency of this protein to self associate.

Fink et al. recently reported that the domain in Hap responsible for promoting adherence to epithelial cells resides in the C-terminal 311 amino acids of Hap $\alpha$  (6). Additional work revealed that this region mediates bacterial aggregation via Hap $\alpha$ -Hap $\alpha$  interaction between molecules on neighboring organisms and is a part of the C-terminal 511 amino acids required for adherence to selected extracellular matrix proteins, including fibronectin, laminin, and collagen IV (7). To address whether the C-terminal 311 amino acids of Hap $\alpha$  (the cell binding domain [CBD]) are capable of eliciting a protective

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immune response, we prepared recombinant CBD (rCBD) either from glutathione *S*-transferase (GST) fusion proteins or from urea-solubilized inclusion bodies. Using the mouse intranasal challenge model, we found that immunization with rCBD derived from three different NTHi strains leads to significant reduction in nasopharyngeal colonization. These results should facilitate efforts to develop a Hap<sub>S</sub>-based vaccine to prevent nontypeable *H. influenzae* disease.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** NTHi strains N187 (obtained from Eric Hansen, University of Texas), P861454, P860295 (obtained from Charles Brinton, University of Pittsburgh), and SR7332 (11) were isolated from middle ear fluid of children with acute otitis media. NTHi strain TN106 (obtained from Eric Hansen) was isolated from a patient with pneumonia (19, 23). TN106.P2 is a streptomycin-resistant derivative of TN106 described previously (5). DB117 is an unencapsulated, recombination-deficient derivative of a serotype 4 *H. influenzae* strain that contains a mutated *hap* gene and fails to express Hap (20). Strain DB117/Hap<sub>P860295</sub> produces on its surface plasmid-encoded wild-type Hap<sub>P860295</sub>, and strain DB117/Hap<sub>N187</sub> produces plasmid-encoded wild-type Hap<sub>N187</sub>. DB117/pGJB103 contains the plasmid vector pGJB103 and does not express Hap (23). *Escherichia coli* strain Top10 was purchased from Invitrogen (Carlsbad, Calif.), and strain BL21(DE3)/pLysS was purchased from Novagen (Madison, Wis.). Plasmids pET17b and pGEX-6P-1 were purchased from Novagen and Amersham Pharmacia Biotech (Piscataway, N.J.), respectively. Plasmid pGJB103 is an *H. influenzae*-*E. coli* shuttle vector described previously (26).

**Bacterial cultures.** NTHi cells were grown on brain-heart infusion agar plates supplemented with hemin and NAD (BHI-XV agar), on BBL CHOC II agar plates (Becton Dickinson & Co., Cockeysville, Md.), or in brain-heart infusion broth supplemented with hemin and NAD (BHI) as described previously (8). *E. coli* cells were grown on SOB agar or in SOB broth (14) containing the appropriate antibiotic.

**PCR primers, PCR amplification, and DNA sequencing.** PCR primer oligonucleotides were synthesized on a PerSeptive Biosystems oligonucleotide synthesizer (Applied Biosystems, Foster City, Calif.) using  $\beta$ -cyanoethylphosphoramidite chemistry. PCRs were performed in ReddyMix PCR Master Mix (ABgene House, Surrey, United Kingdom) on a Perkin-Elmer GeneAmp 2400 unit. A hot start of 5 min at 95°C prior to amplification was followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. DNA sequencing was carried out using BigDye terminator chemistry with specifically designed primers on an Applied Biosystems 377 automated DNA sequencer. Sequencher 4.0.5 software (Gene Codes, Corp., Ann Arbor, Mich.) was used to analyze the DNA sequences.

**Construction of plasmids for the expression of GST-rCBD fusion proteins.** GST-rCBD fusion proteins were made with plasmids that contain the *hap* gene from NTHi strain N187, 860295, or TN106 (5). PCR was performed to amplify the appropriate segment of the gene. The DNA segments include sequences encoding residues 726 to 1036 from Hap<sub>N187</sub>, residues 734 to 1040 from Hap<sub>P860295</sub>, and residues 728 to 1032 from Hap<sub>TN106</sub>. PCR primers were designed to incorporate a BamHI site at the 5' end and an XhoI site at the 3' end of each segment. Following amplification, PCR products were digested with BamHI and XhoI and then were ligated into BamHI-XhoI-digested pGEX-6P-1 (Amersham Pharmacia Biotech), juxtaposing the *hap* fragments downstream of the coding sequence for glutathione *S*-transferase and a PreScission protease cleavage site. The resulting plasmids pGEXN187C/311, pGEXP860295C/307, and pGEXTN106C/305 were transformed into *E. coli* BL21(DE3)pLysS by electroporation for expression of GST-rCBD<sub>N187</sub>, GST-rCBD<sub>P860295</sub>, and GST-rCBD<sub>TN106</sub> fusion proteins.

**Construction of a CBD<sub>N187</sub> expression vector.** Plasmid pJS106 (23), containing the *hap* gene from strain N187, was used as a template for cloning the DNA sequence encoding CBD<sub>N187</sub>. The PCR primers for amplifying the CBD coding region are oligonucleotides 5'-GATCGCTTAAGCTTCCCCAAAACAAATCAAT3' and 5'-CCTGTGTCAGCGTCGACTTATACAGGCTTTGATCAGG CAGGGTAT3'. The PCR products were purified and ligated to the T7 tag in the expression vector pET17b. The ligation reaction was transformed into TOP10 cells, and transformants were screened by PCR for the correct insert, yielding pWV1024. This plasmid was verified to have the correct nucleotide sequence and was then transformed into *E. coli* BL21(DE3)pLysS for expression of rCBD<sub>N187</sub>.

**Isolation of rCBD from GST fusion protein.** *E. coli* BL21(DE3)pLysS containing plasmid pGEX-N187C/311, pGEX-P860295C/307, or pGEX-TN106C/305 was grown overnight at 37°C with shaking in SOB broth supple-

mented with 1% glucose and ampicillin, and it was then diluted into fresh broth to an optical density at 600 nm (OD<sub>600</sub>) of 0.1. The culture was incubated at 37°C with shaking to an OD<sub>600</sub> of 1. To induce the cells, the temperature was shifted down to 28°C and 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) was added to the culture. Two hours later, cells were harvested by low-speed centrifugation. Cells from 1 liter of induced culture were resuspended in phosphate-buffered saline (PBS) containing 0.2% Triton X-100, 2 mM pefabloc (Boehringer Mannheim, Indianapolis, Ind.), and 1 mM EDTA, and then they were lysed by passing through a Microfluidizer (Microfluidics International Corp., Newton, Mass.). Cell lysate was centrifuged at 10,000  $\times$  g for 20 min to remove cell debris. The portion of GST fusion protein that remained in the supernatant was absorbed onto affinity matrix glutathione Sepharose 4B and cleaved with PreScission protease according to the manufacturer's guide (Amersham Pharmacia Biotech). Bound rCBD was eluted with 50 mM Tris buffer at pH 7.5 containing 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.2% Triton X-100. Eluted rCBD was concentrated to ~1 mg/ml with Triton X-100 adjusted to 0.5%. The resulting rCBD preparation was stored at -20°C. Protein concentrations were determined by using the modified method of Lowry (17).

As a negative control, *E. coli* BL21(DE3)/pLysS containing the plasmid vector pGEX-6P-1 was grown and induced and a cell lysate was prepared as described above. This induced *E. coli* cell lysate was subsequently used to generate a mouse polyclonal antiserum (see below).

**Isolation of rCBD<sub>N187</sub> from inclusion bodies.** *E. coli* BL21(DE3)/pLysS+pWV1024 was incubated at 37°C on SOB agar supplemented with 1% glucose and ampicillin. Cells were then grown in SOB broth overnight and were diluted into fresh SOB broth to an OD<sub>600</sub> of 0.1. When the culture reached an OD<sub>600</sub> of 1 to 1.5, 1 mM IPTG was added. Two hours after induction with IPTG, cells were harvested by low-speed centrifugation. Cells from 1 liter of induced culture were resuspended in PBS containing 1% Triton X-100, 5 mM pefabloc, 4 mM EDTA, 0.2 mg of lysozyme/ml, and 5- $\mu$ g/ml concentrations of each DNase and RNase. After incubation at 37°C for 1 h with gentle rocking, cells were lysed by passing through a Microfluidizer, and the resulting cell lysate was centrifuged at 10,000  $\times$  g for 20 min. The pellet composed mainly of rCBD<sub>N187</sub> inclusion bodies was resuspended in 0.1 M Tris buffer at pH 8 containing 6 M urea, 2 mM EDTA, and 5 mM pefabloc. The inclusion bodies were extracted with urea at 4°C overnight with rocking. The extract was then centrifuged at 36,000  $\times$  g for 20 min to remove insoluble material. The supernatant containing urea-solubilized rCBD<sub>N187</sub> was adjusted with the same buffer to ~6 mg/ml and was stored at -70°C.

Renaturation of urea-solubilized rCBD<sub>N187</sub> involves diluting the denatured protein into a buffer containing a high concentration of L-arginine (1, 2, 18). Briefly, urea-solubilized rCBD<sub>N187</sub> was first reduced with 0.3 M DTT for 2 h at room temperature. The reduced protein was diluted 100-fold into a buffer consisting of 0.1 M Tris at pH 8, 2 mM EDTA, 3 mM DTT, 5 mM oxidized glutathione (GSSG), 0.4 M L-arginine, 150 mM NaCl, 0.015% Triton X-100, and 2 mM pefabloc. Protein renaturation proceeded in this diluted state at 10°C for 4 days. Renatured rCBD<sub>N187</sub> was concentrated to 1 mg/ml in ~0.5% Triton X-100. Dialysis was carried out stepwise to reduce L-arginine from 0.4 M to 0.3 M to 0.2 M, and finally down to 0.1 M in a buffer consisting of 0.1 M Tris at pH 8, 150 mM NaCl, and 0.5% Triton X-100. The dialyzed protein solution was centrifuged at 36,000  $\times$  g for 20 min to remove insoluble material. The final renatured rCBD<sub>N187</sub> was stored at -20°C.

**Purification of native Hap<sub>S</sub> from *H. influenzae* strain P860295.** Native Hap<sub>S</sub> (nHap<sub>S</sub>-P860295) was purified from the culture supernatant as described previously (5), with minor modifications. Briefly, bacteria were grown in 10 liters of BHI broth for 18 h at 35°C with aeration. All the following steps were performed at 4°C. Bacterial cells were removed by centrifugation at 10,000  $\times$  g. The culture supernatant was concentrated 20-fold by using an Amicon stir cell and was fractionated overnight with ammonium sulfate at 60% saturation. After centrifugation at 17,000  $\times$  g for 1 h, the precipitate was dissolved in 20 mM Tris buffer at pH 7.4 containing 50 mM NaCl and 1 mM EDTA, dialyzed against the same buffer, and then centrifuged at 100,000  $\times$  g for 1 h to remove insoluble material. The resulting supernatant was loaded at a flow rate of 2 ml/min onto a 20-ml SP Sepharose column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The column was washed until the OD<sub>280</sub> reached the baseline, and nHap<sub>S</sub>-P860295 was eluted at a flow rate of 3 ml/min with a linear gradient of NaCl (from 55 to 500 mM) in 20 mM Tris at pH 7.5 with 1 mM EDTA. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, fractions containing nHap<sub>S</sub>-P860295 were pooled.

**SDS-PAGE and Western blot analysis.** SDS-PAGE was performed with 12% gels according to the method of Laemmli (13). For Western blot analysis, proteins were transferred to nitrocellulose (Schleicher & Schull, Keene, N.H.) (27) and probed with anti-GST antibody or MAb 314, a murine monoclonal antibody



that recognizes an epitope located in the C-terminal one-third of Hap<sub>S</sub> (6). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) and IgM antibodies (Biosource, Camarillo, Calif.).

**ELISA.** The antibody response of mice to rCBD was measured by using a standard enzyme-linked immunosorbent assay (ELISA) procedure. MaxiSorp surface Nunc-Immuno 96-well plates (Nalge Nunc International, Naperville, Ill.) were coated overnight at 37°C with purified nHap<sub>S-P860295</sub> diluted to 5 µg/ml in PBS. Antibodies bound to nHap<sub>S-P860295</sub> were detected with alkaline phosphatase-conjugated goat anti-mouse IgG and IgM antibodies. Antibody titers were expressed as the reciprocal of serum dilution with an absorbance of 0.1 extrapolated from a linear plot of the logarithm of absorbance versus the logarithm of serum sample dilution.

**Antibody inhibition of adherence.** Mouse antisera against rCBD were tested for their ability to block *H. influenzae* Hap-mediated adherence to human epithelial cells. In performing these assays, bacteria were grown in BHI broth to late log phase, pelleted in a microcentrifuge, and resuspended in plain BHI broth containing an antiserum with a 1:100 dilution. After incubation for 30 min at room temperature,  $1 \times 10^7$  to  $2 \times 10^7$  CFU were inoculated onto monolayers of Chang conjunctival epithelial cells (Wong-Kilbourne derivative, clone 1-5c-4 [human conjunctiva]), and adherence was measured as described previously (22). Percent adherence was calculated by dividing the number of adherent CFU per monolayer by the number of inoculated CFU. Preimmune serum and antiserum against an induced *E. coli* BL21(DE23)/pLysS/pGEX-6P-1 cell lysate were tested as negative controls.

**Subcutaneous immunization.** Groups of 10 female, 6- to 8-week-old Swiss Webster or BALB/c mice (Taconic Farms, Germantown, N.Y.) were immunized subcutaneously at weeks 0 and 4 with protein antigens. To prepare the protein antigens for vaccination, 10 µg of rCBD or 2 µg of an induced *E. coli* BL21(DE23)/pLysS/pGEX-6P-1 cell lysate was absorbed onto 100 µg of aluminum phosphate at 37°C for 1 h, and then 50 µg of 3-O-deacylated monophosphoryl lipid A (MPL; Corixa Corp., Hamilton, Mont.) was added. Sera collected at weeks 0, 4, and 6 were pooled for analysis.

**Intranasal immunization.** Groups of 10 female, 6-week-old BALB/c mice were immunized intranasally with protein antigens as described previously (5) at weeks 0, 1, 3, and 5. To prepare the protein antigens for vaccination, 15 µg of rCBD was diluted in Dulbecco's PBS (D-PBS) to a final volume of 20 to 40 µl with or without 0.1 µg of CT-E29H (a mutant cholera toxin) as an adjuvant (24). Control mice received D-PBS alone or D-PBS with 0.1 µg of CT-E29H. Sera collected at weeks 0, 3, 5, and 8 were pooled for analysis. Prior to immunization, mice were anesthetized with a mixture of ketamine (80 mg per kg of body weight) and xylazine (7 mg per kg of body weight), a dosage that maintains a state of anesthesia for 15 to 20 min. Vaccines were delivered by pipette in a volume of 20 µl per nostril. The pipette was positioned so that the tip touched the opening of the nostril, allowing the liquid to be drawn into the nasopharynx with breathing. Immediately following immunization, mice were placed in a supine position for 3 to 5 min.

**Intranasal challenge.** Three weeks after the final immunization, mice were challenged intranasally with approximately  $\times 10^6$  CFU of strain TN106.P2 as described previously (5). To determine the actual bacterial count of the inoculum, an aliquot of the bacterial suspension was diluted in D-PBS and plated on BHI-XV plates. Three days after challenge, mice were sacrificed and the nasal tissue was harvested, weighed, homogenized, and plated on BHI-XV plates containing 100 µg of streptomycin/ml. The plates were incubated overnight, and the colonies were counted. The data were expressed as log CFU per gram of nasal tissue. The variability of the standard errors of the means between the groups in the present study violates the assumption of equal variance between groups, making it more appropriate to first rank the data and then use an analysis of variance. Accordingly, statistical differences among groups were analyzed by use of the Tukey-Kramer test, a nonparametric method applied when data points are not normally distributed (3, 4).

## RESULTS

**Protein sequence alignment of CBD of *H. influenzae*.** In an earlier work, Fink et al. (6) established that the C-terminal 311 amino acids of Hap<sub>S-N187</sub> harbor the adhesive activity of the protein. To determine the level of conservation of this region, we used the AlignX program of VectorNTI, version 6.0, software to align the C-terminal end of Hap<sub>S</sub> from three NTHi strains. The analysis indicates that the CBD fragments from

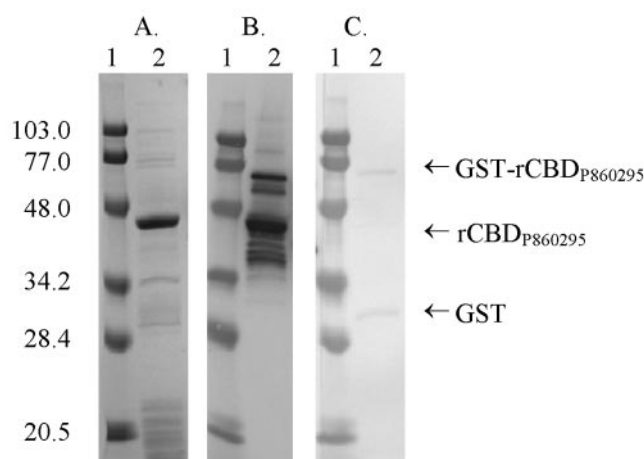


FIG. 1. SDS-PAGE and Western blot analysis of rCBD<sub>P860295</sub> generated from GST-rCBD<sub>P860295</sub> fusion protein. (A) Coomassie-stained SDS gel loaded with 5 µg of rCBD<sub>P860295</sub> (lane 2); lane 1 shows Bio-Rad prestained low-molecular-mass markers (in kilodaltons). (B) Western blot probed with MAb 314, showing GST-rCBD<sub>P860295</sub> fusion protein and rCBD<sub>P860295</sub>. (C) Western blot probed with anti-GST antibody, showing GST and GST-rCBD<sub>P860295</sub> fusion protein.

strains P860295, TN106, and N187 are 97.1% similar and 81% identical.

**Antibody response elicited by rCBD generated from GST fusion protein.** rCBD<sub>P860295</sub>, rCBD<sub>TN106</sub>, and rCBD<sub>N187</sub> were generated from the soluble fraction of the respective GST-rCBD fusion proteins. As shown in Fig. 1A, rCBD<sub>P860295</sub> is the major protein in the affinity-purified preparation, with a purity of ~60%. In Western blot analysis, rCBD<sub>P860295</sub> reacts with MAb 314 (Fig. 1B) but not with anti-GST antibody (Fig. 1C). MAb 314 also detects small amounts of uncleaved GST-rCBD<sub>P860295</sub> fusion protein cleaved at alternate sites, and degradation products of rCBD<sub>P860295</sub> (Fig. 1B). Some of the minor bands in Fig. 1A not recognized by MAb 314 or anti-GST antibody probably represent contaminating *E. coli* proteins. Similar patterns of reactivity are observed with rCBD<sub>TN106</sub> and rCBD<sub>N187</sub> preparations derived from GST fusions (data not shown).

Mouse antisera against the three rCBD fragments derived from soluble GST fusion proteins were tested for antibody response to nHap<sub>S</sub> purified from strain P860295. After one immunization, the antisera had low titers toward nHap<sub>S-P860295</sub> (data not shown). After a second immunization, high antibody titers were detected in mice that received rCBD<sub>N187</sub> and rCBD<sub>P860295</sub> and a relatively high titer was detected in mice that received rCBD<sub>TN106</sub> (Table 1), suggesting that rCBD<sub>N187</sub> and rCBD<sub>TN106</sub> are able to elicit antibodies that cross-react with nHap<sub>S-P860295</sub>. Preimmune sera had antibody titers to nHap<sub>S-P860295</sub> of less than 100. Because the rCBD preparations obtained from GST fusion proteins contain small amounts of GST and possibly *E. coli* proteins, we generated a control antiserum against an induced *E. coli* BL21(DE3)/pLysS/pGEX-6P-1 cell lysate. Like the preimmune sera, this control antiserum exhibited an antibody titer toward nHap<sub>S-P860295</sub> of less than 100.

**Antibody inhibition of adherence to Chang epithelial cells.** To assess functional characteristics of antisera raised against

TABLE 1. Antibody response<sup>a</sup> elicited by rCBD via subcutaneous immunization

Antiserum against rCBD <sup>b</sup> derived from	Antibody titer to nHap <sub>S-P860295</sub>
GST-rCBD <sub>N187</sub> FP.....	58,050
GST-rCBD <sub>P860295</sub> FP.....	97,739
GST-rCBD <sub>TN106</sub> FP.....	14,833
rCBD <sub>N187</sub> IB.....	32,440
Preimmune sera .....	<100
Control antiserum against <i>E. coli</i> lysate <sup>c</sup> .....	<100

<sup>a</sup> Mice were immunized subcutaneously with rCBD mixed with MPL and aluminum phosphate. Preimmune era and week 6 sera pooled from 10 mice were analyzed for antibody titer to purified nHap<sub>S-P860295</sub>.  
<sup>b</sup> rCBD was generated from soluble GST-rCBD fusion protein (FP) or urea-solubilized inclusion bodies (IB).  
<sup>c</sup> Control antiserum against an induced BL21 (DE3)/pLysS/pGEX-6P-1 cell lysate.

rCBD derived from GST fusion protein, we examined their effect on *H. influenzae* Hap-mediated adherence. Both strains DB117/Hap<sub>P860295</sub> (Fig. 2A) and DB117/Hap<sub>N187</sub> (Fig. 2B) exhibited appreciable adherence to Chang cells, well above the background level of adherence by the DB117/pGJB103 control. Adherence by DB117/Hap<sub>P860295</sub> was reduced significantly by preincubation with anti-nHap<sub>S-P860295</sub> serum (Fig. 2A). Similar decreases in adherence were observed with DB117/Hap<sub>P860295</sub> preincubated with anti-rCBD<sub>P860295</sub> serum (Fig. 2A) and with DB117/Hap<sub>N187</sub> preincubated with anti-rCBD<sub>N187</sub> serum (Fig. 2B). In contrast, adherence by DB117/Hap<sub>P860295</sub> or DB117/Hap<sub>N187</sub> was unaffected by preincubation with preimmune serum (Fig. 2A and B). Additional control experiments using antiserum against an induced *E. coli* BL21(DE3)/pLysS/pGEX-6P-1 cell lysate also showed no effects on adherence of DB117/Hap<sub>P860295</sub> or DB117/Hap<sub>N187</sub> (data not shown). These findings suggest that nHap<sub>S-P860295</sub>, rCBD<sub>P860295</sub>, and rCBD<sub>N187</sub> elicit antibodies capable of blocking Hap-mediated adherence to human epithelial cells.

**Effect of immunization with rCBD on nasopharyngeal colonization.** To investigate if immunization with rCBD would affect nasopharyngeal colonization by *H. influenzae*, rCBD<sub>N187</sub>, rCBD<sub>P860295</sub>, and rCBD<sub>TN106</sub> generated from GST fusion proteins were administered intranasally to mice. Upon challenge with strain TN106, a significant reduction in nasal colonization was observed in mice immunized with rCBD<sub>P860295</sub> compared to that of the control group D-PBS (Fig. 3A). Further reduction in nasal colonization was detected in mice immunized with rCBD<sub>P860295</sub> together with CT-E29H adjuvant compared to that of the control groups D-PBS plus CT-E29H and D-PBS plus vaccine buffer. The same pattern was noted for mice immunized with either rCBD<sub>N187</sub> (Fig. 3B) or rCBD<sub>TN106</sub> (Fig. 3C). These results demonstrate that rCBD derived from strains P860295, N187, and TN106 can elicit antibodies capable of reducing nasopharyngeal colonization by strain TN106.

Antisera from intranasally immunized mice were tested for antibody response to nHap<sub>S</sub> purified from strain P860295. After four immunizations, mice that received either rCBD<sub>N187</sub> or rCBD<sub>P860295</sub> had high antibody titers to nHap<sub>S-P860295</sub> (Table 2). The anti-nHap<sub>S-P860295</sub> antibody titer was increased by threefold when rCBD<sub>N187</sub> was delivered with CT-E29H adjuvant. In contrast, no such adjuvant effect was observed with

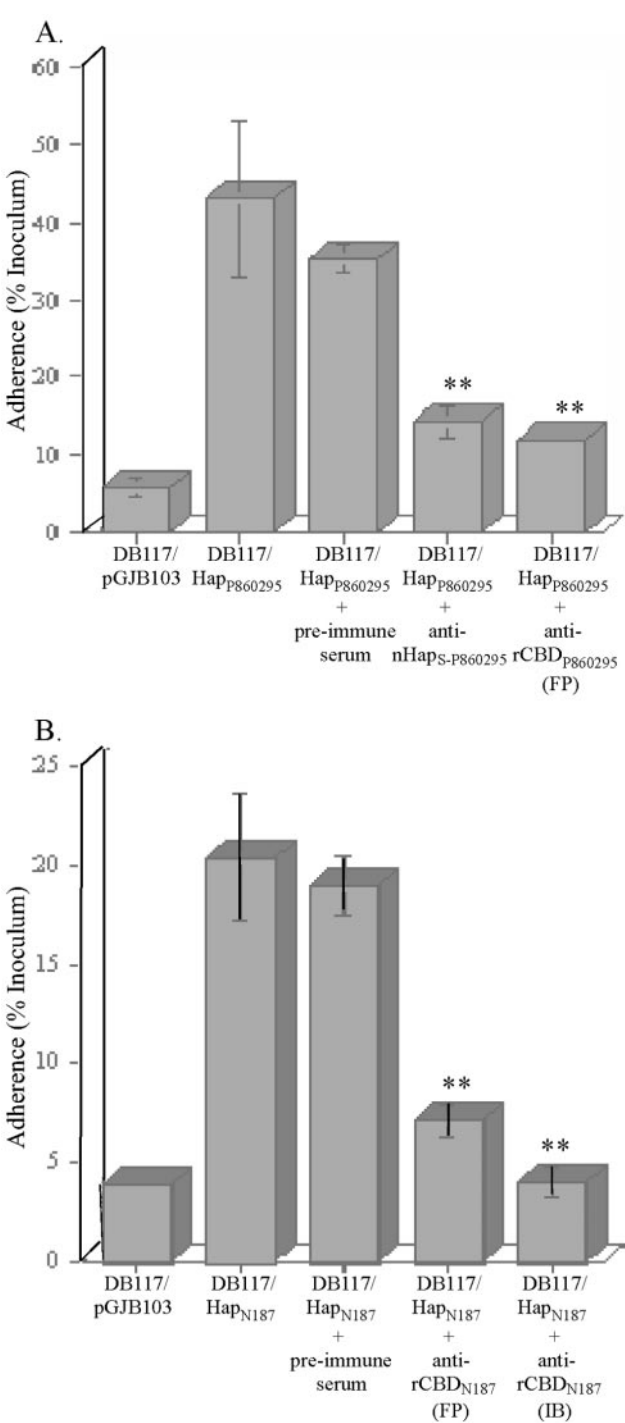


FIG. 2. (A) Adherence to monolayers of Chang conjunctival epithelial cells by strains DB117/pGJB103 (vector), DB117/Hap<sub>P860295</sub>, and DB117/Hap<sub>P860295</sub> that had been preincubated with either preimmune serum, antiserum against nHap<sub>S-P860295</sub>, or antiserum against rCBD<sub>P860295</sub> derived from GST fusion protein (FP). \*\*,  $P < 0.05$  compared to DB117/Hap<sub>P860295</sub> plus preimmune serum. (B) Adherence to monolayers of Chang conjunctival epithelial cells by strains DB117/pGJB103, DB117/Hap<sub>N187</sub>, and DB117/Hap<sub>N187</sub> that had been preincubated with either preimmune serum, antiserum against rCBD<sub>N187</sub> derived from GST fusion protein (FP), or antiserum against rCBD<sub>N187</sub> isolated from inclusion bodies (IB). \*\*,  $P < 0.05$  compared to DB117/Hap<sub>N187</sub> plus preimmune serum. In both panels, each bar represents the means  $\pm$  standard error of the means of measurements made in triplicate from a representative experiment.

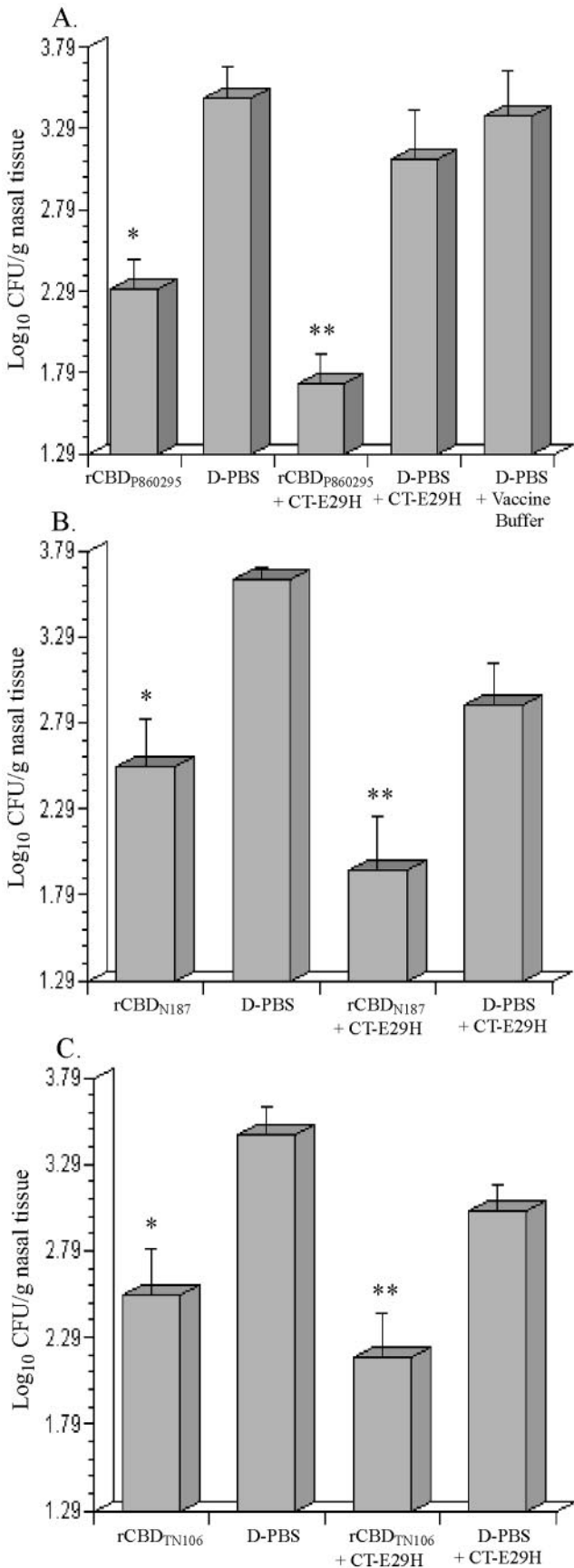


TABLE 2. Antibody response<sup>a</sup> elicited by rCBD via intranasal immunization

Antiserum against rCBD <sup>b</sup> derived from	Buffer/adjuvant	Antibody titer to nHap <sub>S</sub> -P860295
GST-rCBD <sub>N187</sub> FP	D-PBS/none	8,120
	D-PBS/CT-E29H	27,240
GST-rCBD <sub>P860295</sub> FP	D-PBS/none	21,175
	D-PBS/CT-E29H	20,659
None	D-PBS/none	<100
None	D-PBS/CT-E29H	<100
Preimmune sera	NA <sup>c</sup>	<100

<sup>a</sup> Mice were immunized intranasally with rCBD mixed with D-PBS or adjuvant CT-E29H. Preimmune sera and week 8 sera pooled from 15 mice were analyzed for antibody titer toward purified nHap<sub>S</sub>-P860295.  
<sup>b</sup> rCBD was generated from soluble GST-rCBD fusion protein (FP).  
<sup>c</sup> NA, not applicable.

rCBD<sub>P860295</sub>. These results demonstrate that intranasal immunization with rCBD elicits antibodies that cross-react with nHap<sub>S</sub>-P860295.

**Antibody response elicited by rCBD<sub>N187</sub> derived from inclusion bodies.** Because it is impractical to produce large quantities of pure rCBD from GST fusion protein, we constructed an *E. coli* strain that overproduces rCBD<sub>N187</sub> under the control of an IPTG-inducible T7 expression system. The amount of rCBD<sub>N187</sub> represented 43% of the total protein of induced cells (Fig. 4, lane 2). The bulk of rCBD<sub>N187</sub> formed inclusion bodies with a purity of greater than 92% (Fig. 4, lane 4). The inclusion bodies were extracted with urea, and urea-solubilized rCBD<sub>N187</sub> was renatured in a dilute state at 10°C in the presence of 0.4 M L-arginine. Renatured rCBD<sub>N187</sub> required 0.1 M L-arginine and 0.5% of Triton X-100 to remain soluble upon repeated cycles of freeze and thaw from storage at -20°C.

Mice immunized once with rCBD<sub>N187</sub> derived from inclusion bodies had an anti-nHap<sub>S</sub>-P860295 antibody titer of 2,053, which was boosted almost 16-fold by a second immunization (Table 1). This antibody response demonstrates that renatured rCBD<sub>N187</sub> resembles rCBD<sub>N187</sub> generated from soluble GST fusion protein, capable of eliciting antibodies that cross-react with nHap<sub>S</sub>-P860295. The antiserum against renatured rCBD<sub>N187</sub> also exhibited moderate titers toward whole cells of NTHi strains N187, P860295, TN106, SR7332, and P861454 while the preimmune sera showed titers of less than 100 (data not shown), suggesting that antibodies elicited by renatured

FIG. 3. Nasopharyngeal colonization by strain TN106.P2 in BALB/c mice immunized with rCBD derived from GST fusion proteins. (A) Bacterial colonization in mice vaccinated with rCBD<sub>P860295</sub>. (B) Bacterial colonization in mice vaccinated with rCBD<sub>N187</sub>. (C) Bacterial colonization in mice vaccinated with rCBD<sub>TN106</sub>. In panel A, an asterisk indicates significant difference from D-PBS control and two asterisks indicates significant difference from D-PBS plus CT-E29H and D-PBS plus vaccine buffer controls. In panels B and C, an asterisk indicates significant difference from D-PBS control and two asterisks indicates significant difference from D-PBS plus CT-E29H control. The x axis represents the detection limit of the assay, and each bar represents the standard error of the means.



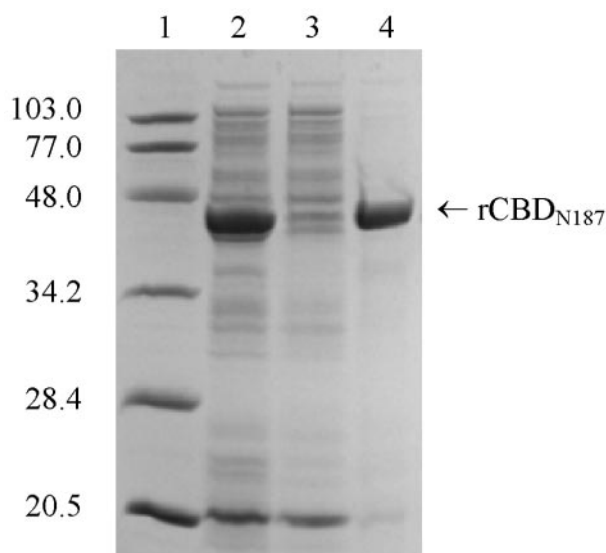


FIG. 4. Subcellular location of rCBD<sub>N187</sub> produced by *E. coli* BL21(DE3)/pWV1024. Coomassie-stained gel showing cell lysate (lane 2) of an IPTG-induced culture, cytosolic and membrane proteins (lane 3), and urea-solubilized rCBD<sub>N187</sub> inclusion bodies (lane 4). The amount of sample loaded in each lane is equivalent to 20  $\mu$ l of culture. Lane 1 shows Bio-Rad prestained low-molecular-mass markers (in kilodaltons).

rCBD<sub>N187</sub> are cross-reactive with surface-exposed Hap of heterologous strains.

**Inhibition of adherence to Chang epithelial cells by antibodies against rCBD<sub>N187</sub> derived from inclusion bodies.** The antiserum raised against rCBD<sub>N187</sub> isolated from inclusion bodies was tested in the *in vitro* adherence assay. As shown in Fig. 2B, strain DB117/Hap<sub>N187</sub> readily adhered to Chang cells, and this adherence was markedly reduced by preincubation with anti-rCBD<sub>N187</sub> serum, indicating that, similar to rCBD generated from soluble GST fusion protein, rCBD<sub>N187</sub> derived from inclusion bodies was also able to elicit functional antibodies that block Hap<sub>N187</sub>-mediated adherence of *H. influenzae* to human epithelial cells.

**Effect of immunization with rCBD<sub>N187</sub> derived from inclusion bodies on nasopharyngeal colonization.** Mice immunized intranasally with rCBD<sub>N187</sub> isolated from inclusion bodies were challenged with strain TN106 as described in Materials and Methods. A significant reduction in nasal colonization was observed in mice that received renatured rCBD<sub>N187</sub> compared to that of the control group D-PBS (Fig. 5). This level of colonization was further reduced to the limit of detection when renatured rCBD<sub>N187</sub> was administered with CT-E29H compared to that of the control groups D-PBS plus CT-E29H and D-PBS plus vaccine buffer. These results demonstrate that rCBD<sub>N187</sub> derived from inclusion bodies, just like rCBD generated from soluble GST fusion protein elicits cross-reacting antibodies capable of reducing nasopharyngeal colonization by strain TN106.

## DISCUSSION

To establish infection, *H. influenzae* utilizes adhesive molecules to interact with tissues of the respiratory tract, a process

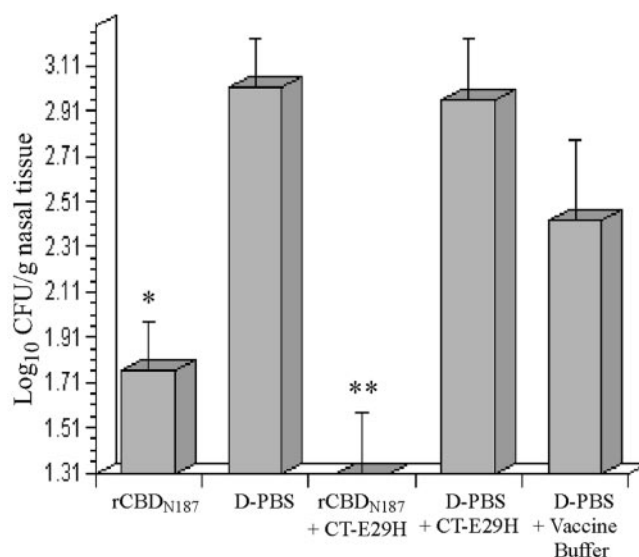


FIG. 5. Nasopharyngeal colonization by strain TN106.P2 in BALB/c mice immunized with rCBD<sub>N187</sub> renatured from urea-solubilized inclusion bodies. \*, significant difference from D-PBS control; \*\*, significant difference from D-PBS plus CT-E29H and D-PBS plus vaccine buffer controls. The *x* axis represents the detection limit of assay, and each bar represents the standard error of the means.

that facilitates bacterial colonization. The Hap protein is one such adhesin, promoting bacterial aggregation, microcolony formation, and adherence to human respiratory epithelial cells and extracellular matrix proteins. The Hap<sub>S</sub> portion of Hap harbors all adhesive activities, making Hap<sub>S</sub> an attractive vaccine candidate. Furthermore, antibodies against Hap<sub>S</sub> have been shown to impede colonization by *H. influenzae* in an animal model (5). The recent finding that the C-terminal 311 amino acid residues of Hap<sub>S</sub> retain the functional domain essential for adherence to human epithelial cells and formation of microcolonies (6) prompted us to examine this fragment for its vaccine potential.

While many surface-exposed proteins of NTHi are highly variable, Hap<sub>S</sub> appears to be similar among strains, based on the homology of available *hap* gene sequences (5). Protein sequence alignment of the C-terminal binding domain of Hap<sub>S</sub> from strains P860295, N187, and TN106 indicates that the CBD region is highly conserved. However, it was unclear whether this degree of homology would be sufficient for CBD from one strain to elicit functional antibodies that are cross-reactive with Hap<sub>S</sub> of other strains. To determine if CBD derived from more than one strain would be required for generating protective immunity against multiple strains, we examined the immune response of mice to rCBD from these three strains.

Initially we constructed *E. coli* strains to express CBD as GST fusion protein, taking advantage of the commercially available protease and affinity matrix for purifying target proteins from their GST fusion counterparts. To avoid complications due to insolubility, rCBD was isolated from GST fusion protein that remained soluble. Although rCBD is the major species in these affinity-purified preparations, high purity was not achieved. Nevertheless, the small amounts of GST and

presumed *E. coli* proteins did not contribute to the immune response elicited by rCBD, because the antiserum against an induced *E. coli* BL21(DE3)/pLysS/pGEX-6P-1 cell lysate, resembling the preimmune sera, exhibited an antibody titer of less than 100 toward nHap<sub>S-P860295</sub>. Therefore, the significant titers observed with the antisera against rCBD<sub>P860295</sub>, rCBD<sub>N187</sub>, and rCBD<sub>TN106</sub> derived from soluble GST fusion protein demonstrate that, indeed, anti-rCBD antibodies are cross-reactive with nHap<sub>S-P860295</sub>.

Using in-frame deletion derivatives of Hap expressed by DB117, Fink et al. established that the C-terminal 311 amino acids of Hap<sub>S</sub> mediate *H. influenzae* adherence to human epithelial cells (6). In addition, they reported that MAb 314 inhibits Hap<sub>S</sub>-mediated bacterial adherence to both A549 cells and Chang cells, suggesting that the epitope recognized by MAb 314 is located nearby the adhesive domain. In this study we found that MAb 314 is reactive with rCBD from strains P860295 (Fig. 1B), N187, and TN106 (data not shown), indicating that the 314 epitope is in a conserved region of CBD. More importantly, antibodies elicited by rCBD<sub>P860295</sub> and rCBD<sub>N187</sub> block Hap-mediated adherence of DB117/Hap to Chang cells (Fig. 2), while antiserum against an induced *E. coli* cell lysate had no effects on adherence. These results imply that the adherence-blocking activity of anti-nHap sera previously reported (7) is attributed to anti-rCBD antibodies.

Intranasal immunization has been utilized to demonstrate the potential of vaccine candidates against *H. influenzae* infections (11, 12). Because otitis media is a mucosal surface disease, intranasal immunization may be the preferred route for humans. While mice immunized intranasally with native Hap<sub>S</sub> purified from strain P860295 or N187 showed significant reductions in nasopharyngeal colonization by strain TN106 (5), it was unclear which portion(s) of Hap<sub>S</sub> was responsible for generating such cross-reactive immune responses. In this investigation, we used the same murine model to test rCBD. Intranasal immunization with either rCBD<sub>N187</sub> or rCBD<sub>P860295</sub> elicited antibodies that were reactive with purified nHap<sub>S-P860295</sub> (Table 2). Furthermore, mice immunized intranasally with rCBD<sub>P860295</sub>, rCBD<sub>N187</sub>, or rCBD<sub>TN106</sub> exhibited significant decreases in nasopharyngeal colonization by TN106, and the reduction was lowered still further when rCBD was administered with CT-E29H (Fig. 3). These results suggest that an rCBD generated from soluble GST fusion protein, regardless of the strain, assumes a proper conformation to elicit antibodies that are cross-reactive and functional in the in vitro adherence assay as well as in the in vivo challenge model.

Obstacles to developing a Hap<sub>S</sub>-based vaccine are twofold. At the cellular level, *H. influenzae* does not produce large amounts of Hap<sub>S</sub>. Thus, it is impractical to purify native Hap<sub>S</sub> for vaccine use. At the protein level, Hap<sub>S</sub> tends to form aggregates at even moderate concentrations, making handling of the protein a challenge. Although protein solubility is substantially improved by expressing only the N-terminal 311 amino acids of Hap<sub>S</sub> as a GST fusion protein, producing rCBD from GST fusion protein as a vaccine component is not a desirable approach because of processing issues. Moreover, rCBD purified by affinity chromatography contains small amounts of uncleaved fusion protein, rCBD breakdown products, GST, and possibly *E. coli* proteins. Therefore, we sought to generate a non-fusion-derived rCBD of much greater purity.

CBD from HapN187 was selected for further investigation based on good expression levels in pilot experiments. rCBD<sub>N187</sub> under the control of T7 promoter was overproduced as inclusion bodies. The inclusion bodies were readily solubilized by urea with a purity of greater than 92% (Fig. 4). Utilizing L-arginine to assist protein renaturation, renatured rCBD<sub>N187</sub> remained soluble above 1 mg/ml. Just like rCBD derived from GST-fusion proteins, renatured rCBD<sub>N187</sub> elicited antibodies that are not only cross-reactive with purified nHap<sub>S-P860295</sub> but also are capable of blocking Hap-mediated adherence of strain DB117/Hap<sub>N187</sub> to Chang cells (Fig. 2B). Most importantly, intranasal immunization of mice with renatured rCBD<sub>N187</sub> significantly reduced nasopharyngeal colonization by strain TN106 (Fig. 5). The high purity of rCBD<sub>N187</sub> preparations shows that the immune response to rCBD is responsible for these functional activities both in vitro and in vivo. These results clearly demonstrate that rCBD<sub>N187</sub> solubilized and renatured from inclusion bodies is a potential vaccine candidate against *H. influenzae* infection.

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