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Characterization of a Recombinant Fragment That Contains a Carbohydrate Recognition Domain of the Filamentous Hemagglutinin

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The filamentous hemagglutinin (FHA) of *Bordetella pertussis* plays an important role in establishing infection by attaching the bacteria to the ciliated respiratory epithelial cells. Expression of DNA encoding residues 1141 to 1279 of FHA in *Escherichia coli* yields a protein of 18,000 Da that exhibits some of the carbohydrate recognition properties of FHA (S. M. Prasad, Y. Yin, E. Rodzinski, E. I. Tuomanen, and H. R. Masure, Infect. Immun. 61:2780–2785, 1993). We have constructed an *E. coli* strain that expresses this protein, designated fragment A, in a soluble form at markedly elevated levels. Fragment A could be purified with high purity and yields and was immunogenic in mice. Both fragment A and anti-fragment A sera inhibited the binding of *B. pertussis* to asialo-GM_2 and to rabbit ciliated cells. These observations demonstrate that this fragment of FHA contains a cellular binding domain capable of eliciting functional antibodies.

Bordetella pertussis, the agent of whooping cough, establishes infection by attaching to the ciliated epithelial cells of the respiratory tract (1, 14, 22, 24, 25). The bacterial adherence is mediated largely by filamentous hemagglutinin (FHA) and pertussis toxin. FHA is able to bind lactosamines as well as the natural target ciliated cells (24) and to elicit antibodies which reduce lung and tracheal colonization by *B. pertussis* in the mouse aerosol challenge model (5). Acellular pertussis vaccines containing FHA, chemically inactivated pertussis toxin, and other *B. pertussis* proteins have routinely been used in Japan since 1981 (17), and many other acellular pertussis vaccines that are being developed also have FHA as a component (3, 12, 15).

FHA, a protein of ~220,000 Da, contains at least four distinct domains that exhibit specific affinities for different ligands or receptors (4, 8, 16). The amino-terminal end of the molecule is involved in the carbohydrate-dependent hemagglutination activity (7, 16). The region that contains the RGD triplet interacts with the leukocyte integrin CR3 (8, 13, 16, 18). A heparin binding site mediates the binding of FHA to the sulfated glycolipids on the epithelial cell surface (9). The carbohydrate recognition domain (CRD) is unique in its affinity for glycolipids and the natural targets, ciliated cells (11). Prasad et al. constructed an *Escherichia coli* strain that expressed an 18,000-Da polypeptide corresponding to residues 1141 to 1279 of FHA (11). Cell lysates of this *E. coli* strain bound to lactosylceramide in a manner identical to the binding to FHA. In addition, mutants of *B. pertussis* that contained an in-frame deletion of the coding sequence for this segment failed to bind effectively to rabbit ciliated epithelial cells, suggesting that the 18,000-Da polypeptide constitutes a CRD essential for bacterial adherence to host cells.

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We sought to determine if this recombinant protein, designated fragment A, would retain its carbohydrate recognition characteristics upon purification and elicit antibodies that could inhibit the binding of *B. pertussis* to ciliated cells. Such information would be useful in determining whether fragment A warrants further investigation for its potential as a component of future generations of acellular pertussis vaccines.

Expression of fragment A in *E. coli*. In this study, the plasmid pSMP2 and the expression vector pET3a (11) were isolated and transformed separately into the *lac* inducible expression system of *E. coli* BL21 (21). We found that expression of fragment A was significantly elevated in *E. coli* BL21 (data not shown). An overnight culture grown at 37°C in Luria-Bertani medium containing 50 μg of ampicillin per ml and 25 μg of chloramphenicol per ml was diluted 50-fold into fresh medium. Isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM was added when the culture reached an optical density of 0.6 to 0.8 at 600 nm. Two hours later, the cells were harvested and resuspended in 10 mM sodium phosphate buffer (pH 6.2) containing 1 mM EDTA. The cells were broken by French press treatment, and cellular debris was removed by centrifugation. The resulting cell lysate contained a prominent protein of ~18,000 Da by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (6), which was recognized in Western blots by polyclonal antibodies to FHA (data not shown). Upon fractionation of the cell lysate by centrifugation at 200,000 × *g* for 30 min, fragment A was detected exclusively in the supernatant fraction, indicating that the recombinant protein was made in a completely soluble form (data not shown).

Purification of fragment A. The supernatant fraction obtained from the high-speed centrifugation was loaded onto a carboxymethyl-Sepharose column (Pharmacia; fast flow) preequilibrated with 10 mM sodium phosphate buffer (pH 6.2). Fragment A was eluted with the same buffer at 0.1 M. As demonstrated in Fig. 1, the recombinant protein was pure; this gel was heavily loaded for the purpose of detecting contaminating proteins. The minor protein band below fragment A appears to be a degradation product since it was recognized by

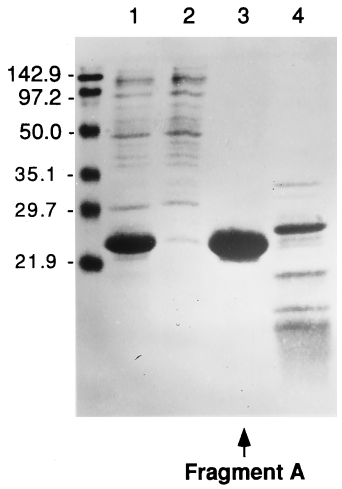

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the fractions obtained from the carboxymethyl-Sepharose column. A 16% gel was run under reducing and denaturing conditions and stained with Coomassie blue. Lanes: 1, the material loaded on the column (28 μg); 2, the proteins eluted with 10 mM sodium phosphate buffer (pH 6.2) (22 μg); 3, fragment A eluted with the same buffer at 0.1 M (36 μg); 4, the proteins eluted with the same buffer at 0.5 M (27 μg).

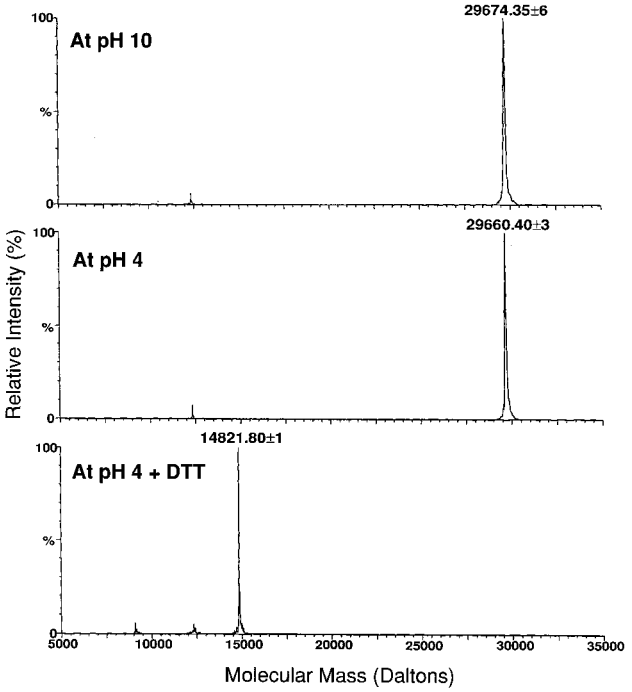

FIG. 2. Transformed electrospray ionization mass spectra of fragment A obtained at pH 10 and 4 and of the dithiothreitol-reduced fragment A (+DTT) obtained at pH 4. The purified protein was extensively dialyzed against 10 mM ammonium acetate buffer at pH 7 to remove buffer salts before mass analysis.

polyclonal antibodies to FHA (data not shown). Inclusion of EDTA in the buffer during cell lysis significantly diminishes the proteolysis of fragment A. When a purified fragment A preparation at 1.37 mg/ml (10) was tested in the *Limulus* amebocyte lysate assay, no lipooligosaccharide was detected at a sensitivity of 0.125 ng/ml for lipooligosaccharide. The overall yield of fragment A is about 160 mg of protein per liter of bacterial culture. The purified protein is stable when stored at −20°C.

Physiochemical properties of fragment A. DNA sequencing was performed to confirm that the insert in pSMP2 encodes amino acid residues 1141 to 1278 of FHA with an additional methionine at the N terminus and a tail of Gly-Ser-Gly-Cys at the C terminus. The presence of a methionine residue at the N terminus was corroborated by protein sequencing of the purified fragment A with an Applied Biosystems model 477A protein/peptide sequencer. The first 50 amino acid residues of fragment A identified are in complete agreement with the protein sequence predicted by the DNA sequence. Furthermore, the amino acid composition of fragment A was determined as described previously (20). A good agreement was found between the theoretical value and the experimental value determined for each residue (data not shown).

The molecular weight of fragment A was investigated by mass spectrometry as described previously (19). A mass of 29,670 ± 20 Da was observed, an average value obtained from the analyses carried out at pH 4 and pH 10 (Fig. 2). This molecular mass is twice that predicted by the DNA sequence. However, if the protein was treated with 50 mM dithiothreitol before being subjected to the mass analysis, a molecular mass of 14,821.8 ± 1 Da was observed (Fig. 2). As mentioned above, the DNA sequence of fragment A reveals that the recombinant protein contains a cysteine residue at the C terminus and that this is the only cysteine residue present in the molecule. It is conceivable that the purified fragment A is a dimer resulting from a disulfide bond formation. The subsequent experiments were conducted with purified fragment A under nonreducing conditions.

Comparison of the immunogenicity of fragment A to that of FHA. Six-week-old Swiss Webster mice (Taconic Farm, Germantown, N.Y.) were immunized subcutaneously at weeks 0 and 4 with different amounts of fragment A or 1 μg of FHA adjuvanted with 25 μg of 3-O-deacylated monophosphorylated lipid A (Ribi Immunochem Research, Inc., Hamilton, Mont.). The mice were bled at weeks 0, 4, and 6. As illustrated in Table 1, the sera raised by fragment A contained antibodies that

TABLE 1. Antibody response of mice immunized with fragment A or FHA[a]

Antigen and amt (μg)	Antibody titer[b] to:			
	Fragment A		FHA	
	Wk 4	Wk 6	Wk 4	Wk 6
Fragment A				
0.3	<50	5,957	<50	2,274
1.0	1,061	71,194	557	38,708
10.0	3,340	200,340	1,714	148,941
FHA				
1.0	145	929	21,513	297,865
E. coli proteins[c]				
5.0	<50	<50	<50	<50

[a] The antibody titer of total immunoglobulins of the pooled sera obtained from 13 to 15 mice was measured by the standard enzyme-linked immunosorbent assay procedure. In these assays, 96-well flat-bottom medium binding plates (Costar, Cambridge, Mass.) were coated with either 0.6 μg of fragment A per ml or 10 μg of FHA per ml diluted in carbonate buffer at pH 9.6.

[b] The antibody titer is expressed as the reciprocal of the serum dilution that produced an absorbance of 0.1 for fragment A and 0.3 for FHA, calculated from a linear plot of log absorbance versus log sample dilution. The weak 0 sera to either fragment A or FHA exhibited a titer of <50.

[c] The control group of mice received *E. coli* proteins. This material was generated by the same procedure for generating the purified fragment A, except that the *E. coli* strain carries the expression vector pET3a plasmid.

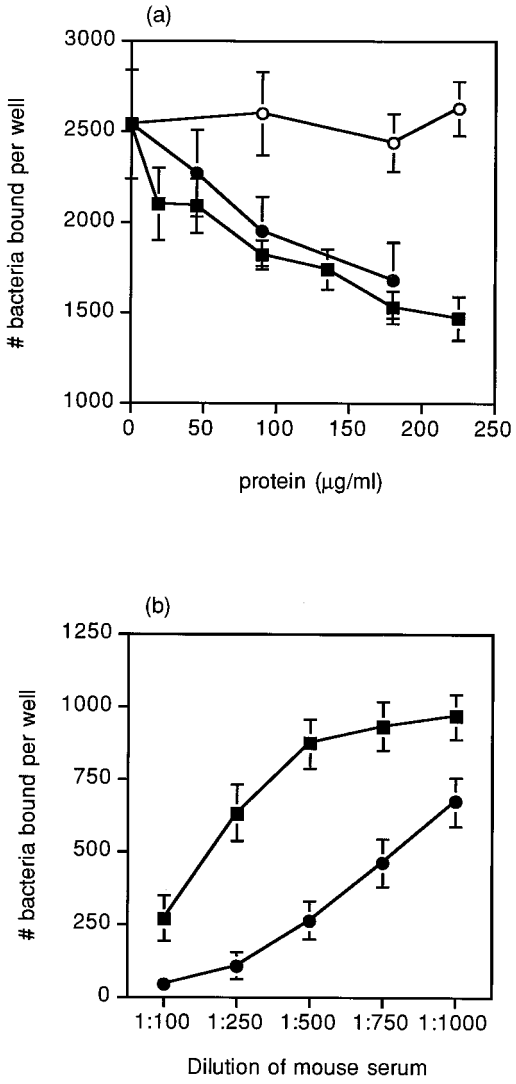

FIG. 3. Inhibition of *B. pertussis* adherence to immobilized asialo-GM_2 by fragment A (a) or anti-fragment A serum (b). Fluorescein-labeled bacteria were preincubated with either protein antigens at specified concentrations or mouse immune sera at various dilutions and then added to tissue culture plates that were coated with asialo-GM_2. The number of bacterial cells bound to the plates was counted. The background binding of bacteria to plates without a glycolipid coating is 470 ± 30. Each condition was tested in triplicate, and the results are expressed as means and standard deviations of three experiments. (a) Bacteria were pretreated with fragment A (■), FHA (●), or BSA (○). (b) Bacteria were pretreated with anti-fragment A serum (●) or anti-tetanus toxoid serum (■).

recognized both fragment A and FHA. The antibody titers to the recombinant protein were dose dependent and boostable. This immune response was specifically elicited by fragment A since no anti-fragment A or anti-FHA antibody was detected in the sera raised by the *E. coli* proteins. On the other hand, the sera raised by FHA contained high titers of antibodies that recognized mainly FHA but not fragment A. These results suggest that fragment A may offer a unique benefit due to its ability to elicit antibodies specific for the CRD of FHA. If anti-fragment A antibodies are functional, immunization with fragment A may lead to disease prevention by interfering with bacterial adherence to host cells, which is a critical step in establishing infection (1, 14, 22, 24, 25).

Effect of fragment A and anti-fragment A serum on *B. pertussis* adherence to glycolipids or to ciliated cells. *B. pertussis* and FHA both have the ability to bind glycolipids and isolated respiratory ciliated cells (1, 14, 22–25). Fragment A carrying the CRD of FHA should in theory exhibit similar binding properties. Two in vitro bacterial adherence assays were performed to further characterize the function of fragment A as well as the antibodies elicited by the recombinant protein. To conduct these experiments, mouse immune sera containing high antibody titers were produced. Mouse immune sera to a tetanus toxoid were also generated to serve as a negative control. Six-week-old Swiss Webster mice were immunized subcutaneously at weeks 0, 3, and 5 with 10 μg of fragment A, 1 μg of FHA, or 5 μg of the tetanus toxoid with 50 μg of aluminum phosphate adjuvant. The individual sera obtained from 15 mice

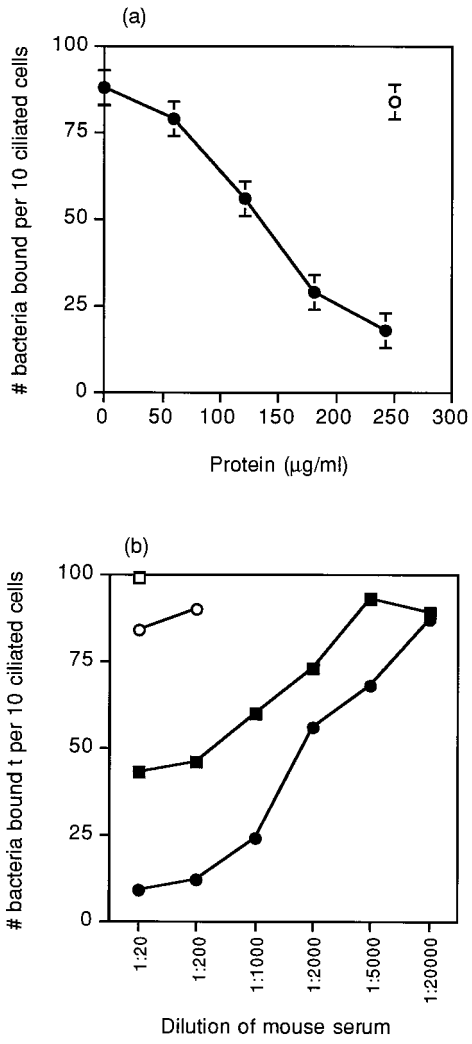

FIG. 4. Inhibition of *B. pertussis* adherence to ciliated cells by fragment A (a) or anti-fragment A serum (b). *B. pertussis* was pretreated with fragment A or BSA at specific concentrations or with mouse sera at various dilutions and then incubated with ciliated cells. The number of bacteria that adhered to the ciliated cells was quantitated by staining with fluorescent antibodies to *B. pertussis*. The results are expressed as the mean number of bacteria per 10 ciliated cells with a standard deviation of approximately ±5. All the values obtained are statistically different at $P < 0.01$. Each assay was performed in triplicate on three occasions. (a) Bacteria were pretreated with fragment A (●) or BSA (○). (b) Bacteria were pretreated with anti-fragment A sera (●), anti-FHA serum (■), anti-tetanus toxoid serum (○), or the preimmune serum (□).

at week 7 were pooled and analyzed in the in vitro functional assays. To ensure that the differences between groups observed in these assays were statistically significant, the data were subjected to the Wilcoxon signed-rank test.

One assay measures bacterial adherence to asialo-GM_2 immobilized on tissue culture plates (2). Just like FHA, fragment A blocked the attachment of *B. pertussis* to asialo-GM2 in a concentration-dependent fashion, while bovine serum albumin (BSA) exerted no effect (Fig. 3a). In the same assay system, the anti-fragment A mouse serum also inhibited *B. pertussis* binding to the glycolipids (Fig. 3b). The extent of inhibition diminished proportionally as the serum was diluted. In comparison, the anti-tetanus toxoid mouse serum exhibited significantly less effect on the bacterial adherence to asialo-GM_2.

In the second assay, bacterial adherence to ciliated cells isolated from rabbits was observed (11). After preincubation with fragment A, the ability of *B. pertussis* to bind ciliated cells decreased steadily as the concentration of fragment A increased (Fig. 4a). Preexposure of *B. pertussis* to BSA did not affect bacterial adherence to the ciliated cells. Various mouse immune sera were tested in the assay as well. As demonstrated in Fig. 4b, the anti-fragment A serum significantly inhibited the attachment of the bacteria to the ciliated cells, and the degree of inhibition was mitigated as the serum was diluted gradually. The anti-FHA serum also blocked the bacterial adherence process but to a lesser extent. Neither the preimmune serum nor the anti-tetanus toxoid serum at the lowest dilution of 1:20 interfered with the binding of *B. pertussis* to the ciliated cells.

These results suggest that fragment A, a dimeric form of the CRD, made by *E. coli* shares the carbohydrate recognition characteristics of FHA of *B. pertussis*. We did not evaluate if a reduced monomeric form of fragment A would retain receptor recognition properties. Fragment A appears to be an attractive candidate as a component of future acellular pertussis vaccines. The protein is overproduced by *E. coli*, is soluble, and is easy to purify with high yields. The purified fragment A is stable, active in its binding activities, and able to elicit antibodies that are functional in in vitro assays. To further investigate its vaccine potential, it would be important to determine whether active immunization with the recombinant protein could promote the clearance of *B. pertussis* from the respiratory tract upon respiratory challenge. In animal models, FHA, a component of most acellular pertussis vaccines (3, 12, 15), stimulates an immune response that promotes the respiratory clearance of *B. pertussis* (5). To replace FHA with fragment A as a vaccine component, it is reasonable to require that fragment A be capable of evoking such a functional immune response in similar animal models of infection. The availability of procedures to obtain large amounts of the purified fragment A makes these investigations possible.

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