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Purification and Characterization of the CFA/I Antigen of Enterotoxigenic *Escherichia coli*

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The fimbrial colonization factor antigen CFA/I of enterotoxigenic *Escherichia coli* was purified and characterized. The initial purification step was release of these fimbriae from the bacterial cells by homogenization with a Waring blender. Common fimbriae and flagellar antigen were avoided by careful control of growth conditions and the use of a nonmotile (H^-) mutant of the prototype strain H-10407 (O78:H11). The essential purification steps were membrane filtration (Millipore Corp.), ammonium sulfate fractionation, and negative diethylaminoethyl-Sephadex column chromatography. Yields were approximately 4.0 mg of CFA/I protein per g (wet weight) of bacteria. Purified CFA/I is a fimbrial molecule 7.0 nm in diameter and has an average molecular weight of 1.6×10^6, as determined by sedimentation equilibrium. CFA/I is a polymer of identical subunits of molecular weight 23,800 with an N-terminal valine, 37% hydrophobic amino acid residues, and 11 residues of proline per mol. The purified antigen retains its morphology, antigenicity, and biological activity. Purified CFA/I exhibits mannose-resistant hemagglutination of human group A, bovine, and chicken erythrocytes, as do CFA/I-positive bacteria. This was demonstrated by sensitizing latex microbeads with the purified antigen since cell-free CFA/I fimbriae do not hemagglutinate erythrocytes. Thus, CFA/I detached from the bacteria are monovalent; however, purified CFA/I antigen retains an affinity for the epithelial cells of rabbit small intestine and blocks adhesion of CFA/I-positive bacteria. These results demonstrate that purified CFA/I is a good candidate for use in an oral vaccine for immunoprotection against diarrhea caused by CFA/I-positive enterotoxigenic *E. coli*.

Many isolates of enterotoxigenic *Escherichia coli* (ETEC) associated with cases of acute watery diarrhea belong to a small number of distinct serotypes, including O78:H11, O78:H12, O25:H42, O15:H11, O6:H16, and O8:H9 (2, 14-18, 23, 25). Other *E. coli* serotypes have also been isolated as ETEC but with much less frequency (9, 15, 16, 20). Representative isolates which belong to the most frequently isolated ETEC serotypes produce either the CFA/I or the CFA/II colonization factor antigen (CFA), depending upon serotype, as we recently described (2, 4, 5). For example, ETEC of serotypes O78:H11 and O25:H42 produce only the CFA/I, antigen, and isolates belonging to the serotypes O6:H16 and O8:H9 produce only the CFA/II antigen (2). In addition to serotype, the production of the CFAs is interrelated with the production of enterotoxin(s). CFA-positive ETEC, whether associated with CFA/I or CFA/II, usually produce both the heat-stable enterotoxin (ST) and the heat-labile enterotoxin (LT) (2). ST-only and LT-only ETEC isolates are usually CFA negative, although a few interesting exceptions have been noted (2, 10, 19). The basis for this observation is indicated by experience with laboratory-passed cultures; ETEC exhibit the loss of enterotoxin production and the simultaneous loss of CFA production upon passage or storage under artificial laboratory conditions or both (2, 9). The genetic basis for such instability has not been fully elucidated but apparently lies in the fact that ST, LT, CFA/I, and CFA/II are all plasmid-determined characteristics in ETEC (3, 7; unpublished data). From these observations it is evident that the virulence factors of ETEC, both enterotoxins and CFAs, are a closely integrated system which is influenced and controlled by the nature of the virulence-associated plasmids, *E. coli* serotype, biotype, and selective pressures in the environment.

The well-documented role of the CFAs in the pathogenesis of ETEC diarrhea in humans (2, 6, 17-19, 21, 25) and the fact that the frequently isolated ETEC serotypes are associated with either CFA/I or CFA/II make it imperative that

the possible role of these antigens in acquired immunity to ETEC diarrhea be investigated. To this end, we are investigating the molecular characteristics of CFA/I and CFA/II and the effect of antibody prepared against purified preparations of these antigens on their biological activity. We previously reported on the purification of the CFA/I antigen by a method involving ultracentrifugation (5). The present report describes a different and relatively simple method for preparing large (milligram) quantities of pure CFA/I antigen and describes the characteristics of the molecule. Also, purified CFA/I is shown to retain both its fimbrial morphology and its biological activity as an adhesion.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. *E. coli* strain H-10407 (O78:H11) was isolated from an acute case of diarrhea in Dacca, Bangladesh (7). H-10407 produces both ST and LT and possesses the fimbrial CFA/I (3, 5, 7). *E. coli* strain PB-176 (O6:H16) was isolated from a case of travelers' diarrhea in Mexico (1a, 2). PB-176 produces both ST and LT and possesses fimbrial CFA/II (2). H-10407-P and PB-176-P are CFA-negative spontaneous derivatives of H-10407 and PB-176, respectively. Several ETEC isolates belonging to other serotypes, such as O78:H12 (19), were described previously and were used in the present work. A nonmotile (O78:H$^-$) spontaneous mutant of strain H-10407 was isolated for the production of CFA/I antigen (5). Stock cultures were stored at room temperature after lyophilization. All cultures were monitored for CFA production by testing 20 isolated colonies for CFA/I or CFA/II after 18 h of growth on CFA agar (4). CFA agar consists of 1.0% Casamino Acids (Difco), 0.15% yeast extract (Difco), 0.005% $MgSO_4$, and 0.0005% $MnCl_2$ plus 2.0% agar; the pH is approximately 7.4.

Large-scale production of CFA/I. Strain H-10407 does not produce common fimbriae (common pili) when grown on the surface of CFA agar medium; this has been shown by studies involving electron microscopy, tests with specific antisera (anti-CFA/I and antipili) and studies on the surface-associated hemagglutinins of H-10407 and other ETEC (3, 4, 6, 8; unpublished data). Thus, the following procedure was used to produce large amounts of CFA/I for purification. Cells obtained from the confluent growth of the nonflagellated strain of H-10407 on two CFA agar plates were suspended in sterile phosphate-buffered saline (PBS) in a total volume of 60 ml. This suspension was used to inoculate 37 large Roux bottles, each containing a layer of 150 ml of CFA agar. The agar surfaces were thoroughly dried by preincubation at 45°C before inoculation. The Roux bottle cultures were incubated at 37°C for 48 h in an inverted position.

The growth from 37 large Roux bottle cultures of strain H-10407, prepared as described above, was harvested with a total volume of approximately 400 ml of 0.1 M sodium phosphate buffer, pH 7.2, containing 0.05% sodium azide. The buffer was precooled in an ice bath, and the resultant cell suspension was kept in an ice bath for 30 min before homogenization to remove the CFA/I fimbriae. Homogenization was carried out in a precooled Waring blender for 15 min at 1-min intervals, with 1 min of cooling in an ice bath between each homogenization. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C, and the resultant supernatant fluid was filtered through a 0.65-μm membrane filter (Millipore Corp.). The filtrate was kept at 4°C for 3 days and then centrifuged again at 12,000 × *g* for 20 min. The pellet was discarded, and the supernatant fluid was again filtered as described above. This second filtrate was referred to as the crude extract. Solid ammonium sulfate (AMS) was added to the crude extract to achieve 20% saturation, and after equilibration for 30 min the precipitate was removed by centrifugation at 20,000 × *g* for 20 min at 4°C. The resultant pellet was resuspended in 2.0 ml of 0.05 M sodium phosphate buffer, pH 7.2, containing 0.001% *p*-chloromercuribenzoate as a preservative; this pellet material is called AMS preparation 1. To the supernatant was added solid AMS to achieve 40% saturation, and the resultant precipitate was recovered by centrifugation, as described above. The supernatant fluid was discarded, and the pellet material was resuspended in 10 ml of the 0.05 M sodium phosphate buffer; this product is referred to as AMS preparation 2. The crude extract and AMS preparations 1 and 2 were dialyzed for 24 h at 4°C against 1,000 volumes of the same buffer, with two buffer changes, to remove the residual salts and AMS.

Purification of CFA/I antigen contained in AMS preparation 2 was performed as follows. A diethylaminoethyl (DEAE)-Sephadex A-50 column (2.5 by 40 cm) was prepared and equilibrated with 0.05 M phosphate buffer, pH 7.2, and the dialyzed sample was applied. Fractions (5 ml) were collected at a flow rate of 0.8 ml/min. After collection of the first 150 ml of eluate, 1.0 M sodium chloride dissolved in the elution buffer was added to the column, and approximately 45 additional fractions were collected. The protein content of each fraction was determined by the method of Lowry et al. (12), and the CFA/I antigen was detected by agar immunodiffusion, using anti-CFA/I rabbit serum prepared by the H-10407/H-10407-P absorption technique described previously (2).

Analytical procedures. Slab gel electrophoresis was performed with 12% Cyanogum-41 by using as buffer 0.18 M tris(hydroxymethyl)aminomethane–ethylenediaminetetraacetate borate buffer, pH 8.6, containing 0.1% sodium dodecyl sulfate (SDS). TEMED (*N,N,N',N'*-tetramethylethylenediamine) and ammonium persulfate were used as catalysts for polymerization of the gel. Vertical electrophoresis was performed at 200 V with an E-C apparatus (E-C Corp., St. Petersburg, Fla.). Samples to be electrophoresed were pretreated by the addition of 1.5% SDS and 10% beta-mercaptoethanol (BME) and boiling for 60 min. A total of 100 to 150 μg of protein contained in a 30-μl volume was applied to each sample slot. After electrophoresis for 2 h at 14°C, each gel was removed from the apparatus, stained with amido black, destained, clarified, and photographed according to standard procedures.

The sedimentation coefficient of the purified anti-

gen was determined by ultracentrifugation with a Beckman model E analytical ultracentrifuge and schlieren optics. The samples were examined at a protein concentration of 2.5 mg/ml, and centrifugation was at 56,000 rpm for 48 h at 20°C.

Immunoelectrophoresis was performed with 1% agarose in tris(hydroxymethyl)aminomethane–sodium barbital buffer (pH 8.8) at a voltage of 3.5 to 5.0 V/cm of gel, using a Gelman instrument. Depending upon the experiment, the troughs were filled with rabbit serum prepared against either the crude antigen extract or purified CFA/I antigen.

Amino acid analysis was performed with a Beckman model 121 automated amino acid analyzer; samples were acid hydrolyzed in 6 N HCl for 24 h at 110°C. An antigen sample consisting of 132 μg of protein dissolved in 50 μl of acetic acid and water was used for the amino acid analysis; 50.15 mM norleucine was used as an internal standard. The same antigen sample (400 μl) was used for amino acid sequence analysis; N-terminal amino acid analysis was performed by using the Edman technique as modified by Hermodson et al. (11).

The molecular weight of the protein subunit of the CFA/I antigen was determined as follows. A column (1.5 by 24 cm) of Sephadex G-200 was equilibrated with 0.05 M sodium phosphate buffer containing 6 M guanidine-hydrochloride (highly pure; Bethesda Research Laboratories, Bethesda, Md.) plus 0.1 M BME, pH 6.5; 5 mg of the protein to be chromatographed was suspended in 0.6 ml of 6 M guanidine hydrochloride plus 0.1 M BME, and the pH was adjusted to 8.6 with 0.1 N NaOH. After incubation at room temperature for 3 days, the pH was readjusted to 6.5 with 0.1 N HCl, 250 μl of 0.6% blue dextran and 100 mg of sucrose were added, and 100 μl of the treated sample was applied to the column. Standard proteins with known molecular weights were chymotrypsinogen, cytochrome *c*, and ribonuclease. Fractions (0.05 ml each) were collected at a flow rate of 4.9 ml/h. The elutions of cytochrome *c* and blue dextran were monitored spectrophotometrically at wavelengths of 405 and 630 nm, respectively. Protein determinations were performed by the method of Mann and Fish (13), as follows. The protein standard curve was prepared by using alpha-chymosinogen A treated with guanidine hydrochloride–BME, as described above. Samples of each column fraction (0.3 ml each) were mixed with 1.2 ml of 4% trichloroacetic acid and incubated at room temperature for 30 min. The protein concentration was then determined as a function of optical density at a wavelength of 450 nm. The distribution coefficient (K_d) was thus determined for the CFA/I antigen protein subunit and for the protein standards, and the subunit molecular weight of the antigen was derived from a plot of K_d versus molecular weight.

Adherence of purified CFA/I antigen to rabbit small intestine. Three groups (three animals each) of 4-day-old New Zealand white rabbits were fasted for 24 h and then inoculated by the intraluminal route as follows. Groups 1 and 2 were given 1.0 mg of purified CFA/I contained in 1.0 ml of PBS; group 3 was given PBS alone. At 3 h after the administration of antigen or PBS, the rabbits in groups 1 and 3 were inoculated with 10^8 CFA/I-positive bacteria and those in group 2 received 1.0 ml of PBS. After another 3 h all animals were sacrificed with Nembutal, and the small intestines were removed and immediately frozen in liquid nitrogen. Intestinal cross sections, 5 μm each, were prepared by using an IEC cryomicrotome. Sections were fixed in acetone, air dried, and washed in PBS. Indirect immunofluorescence was used to identify the location of the bacteria or purified CFA/I antigen by the method previously reported (5). Antibody prepared in rabbits against the purified antigen was used to visualize CFA/I, and rabbit anti-O78 serum was used to visualize the bacteria. The fluorescein isothiocyanate-conjugated goat antirabbit serum was adsorbed with acetone-dried, powdered rabbit muscle in order to eliminate nonspecific fluorescence.

Sensitization of latex beads with CFA/I antigen. Latex microbeads (0.81-μm diameter; Difco) were washed with 0.1 M glycine–saline, pH 8.2, and 1.0 ml was diluted 1:10 with distilled water. A mixture was prepared to contain a 1:20 dilution of washed latex beads (1/200 of the original concentration) and 20 μg of CFA/I protein per ml in a final volume of approximately 10 ml. After incubation at 37°C for 60 min, the sensitized beads were washed twice by centrifugation and resuspension in glycine–saline buffer and finally resuspended in 2.5 ml of glycine–saline buffer, pH 8.2, containing 0.2% bovine serum albumin. CFA/I-sensitized latex microbeads agglutinated with anti-CFA/I serum but not with anti-O78 or anti-CFA/II antiserum.

Hemagglutination and hemagglutination inhibition tests. Human group A, bovine, and chicken erythrocytes were used to demonstrate the hemagglutinating properties of the CFA/I and CFA/II antigens by employing CFA-positive bacteria and CFA-sensitized latex microbeads. Mannose-resistant hemagglutination was demonstrated by using erythrocytes diluted 1:4 in PBS containing 1% mannose, as described previously (4, 8). Slide agglutination tests were performed by mixing equal volumes (25 μl) of either beads or bacterial cells and erythrocytes on the surface of a glass microscope slide.

Various carbohydrates and *N*-acetylamino sugars were tested for inhibition of hemagglutination of erythrocytes by CFA-positive bacteria and CFA-sensitized latex beads. Test compounds (D-fucose, D-mannose, D-galactose, fructose, sucrose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, and *N*-acetylneuraminic acid [NANA]) were prepared as 2% solutions in PBS and then mixed 1:1 with either human group A or bovine erythrocytes.

Preparation of antibody against crude and purified CFA/I antigen. Three rabbits were hyperimmunized with purified CFA/I antigen as follows. Each animal was injected with 300 μg of protein prepared in incomplete Freund adjuvant (0.2 ml per injection at four different axial and inguinal sites). The animals were given booster injections every 4 days with 50 μg of the respective antigen contained in a total volume of 0.5 ml of PBS. These booster injections were given subcutaneously into each of five sites (fore and hind legs and the dorsal-cervical region). The rabbits were bled at the end of a 14-day period after the last of six booster injections.

RESULTS

Purification of the CFA/I antigen. *E. coli* H-10407 was grown on the surface of CFA agar to avoid contamination of the CFA/I antigen preparations with common fimbriae (3, 4). Flagellar antigen was eliminated by using a nonmotile mutant of the test strain. AMS fractionation of a typical crude extract gave a product containing approximately 88% of the protein in the original extract (AMS preparation 2; Table 1). Passage of AMS preparation 2 through a column of DEAE-Sephadex in 0.05 M phosphate buffer, pH 7.2, removed all protein except CFA/I, which eluted as a single peak at the void volume of the column (Fig. 1). Protein analysis showed that CFA/I accounted for approximately 44% of the protein in AMS preparation 2. Subsequent passage of 1.0 M NaCl through this column removed protein which contained no CFA/I antigen.

Purification of CFA/I by the procedure described above was monitored by SDS slab gel electrophoresis, using a 12% gel (Fig. 2). The crude antigen preparation contained seven different proteins detectable as bands after staining with amido black, and the material recovered in the 20 to 40% AMS fraction (AMS preparation 2) contained two major proteins (Fig. 2, lane B). The first elution peak of the DEAE-Sephadex column contained only one protein (Fig. 2, lane C), which can be identified with protein band 7 in the crude extract (Fig. 2, lane A).

Analysis of purified CFA/I by immunoelectrophoresis. The purity of the CFA/I antigen preparation was demonstrated by immunoelectrophoretic analysis of the crude and final antigen preparations. After electrophoresis, rabbit antiserum prepared against the crude antigen produced only a single precipitin line with the final antigen preparation; in contrast, there were numerous precipitin lines with this antiserum and the crude antigen preparation (Fig. 3). Also, the native protein exhibited a low electrophoretic mobility (Fig. 3). Furthermore, the purified CFA/I produced a single line of identity when reacted against antibody prepared against the pure antigen (Fig. 4, trough A) and against antibody prepared against the crude antigen (Fig. 4, trough B). This demonstrates that only one of the antigens present in the crude extract was present in the final preparation.

Morphology and molecular weight of the CFA/I antigen. Purified CFA/I was examined by electron microscopy as previously described (5). The antigen retained its fimbrial morphology even after purification (Fig. 5). The rod-shaped molecules vary in length and have a width of approximately 7 nm; these tend to aggregate to form rodlike structures of varying widths.

Analytical ultracentrifugation of the purified CFA/I antigen produced a schlieren pattern with an S value of 24 and a single peak indicating homogeneity (Fig. 6). The average molecular weight of the native molecule as estimated by sedimentation equilibrium was thus found to be 1.6×10^6, and this result is consistent with

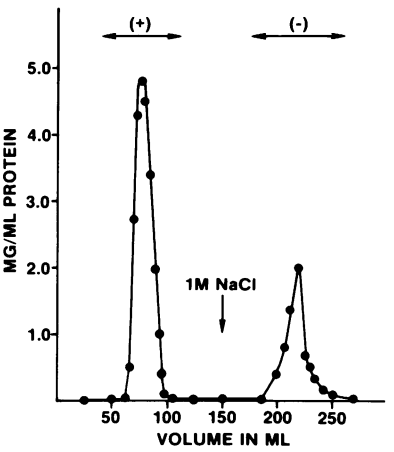

FIG. 1. *DEAE-Sephadex A-50 column chromatography of AMS preparation 2 (CFA/I-containing AMS fraction). Column and sample were first equilibrated with 0.05 M phosphate buffer, pH 7.2. After elution of the void volume peak, which contained the CFA/I protein (+), buffer containing 1.0 M NaCl was used to elute the remaining protein, which was found not to contain the antigen (-).*

TABLE 1. *Recovery of protein and CFA/I antigen during purification*

Procedure	Product	Vol (ml)	Protein concn (mg/ml)	Amt of total protein	Approx % of CFA/I
Homogenization, Millipore filtration, dialysis	Crude extract	285	1.0	285	38
AMS precipitation (0 to 20%)	AMS preparation 1	2	7.0	14	0
AMS precipitation (20 to 40%)	AMS preparation 2	10	25.1	251	43.7
DEAE-Sephadex A50 column chromatography	Purified CFA/I	45.7	2.4	109.6[a]	100

[a] The final yield of CFA/I protein was 4.0 mg of antigen per g (wet weight) of bacteria.

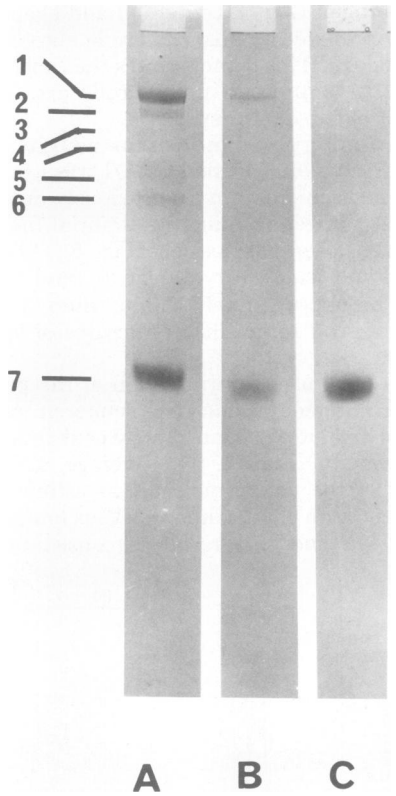

FIG. 2. *SDS slab gel electrophoresis of crude extract (lane A), AMS preparation 2 (lane B), and purified CFA/I antigen after DEAE-Sephadex chromatography (lane C).*

observations made by electron microscopy.

Subunit protein and amino acid analysis of CFA/I. As shown above, the CFA/I antigen is a rod-shaped molecule with a high molecular weight. Analysis of the subunit structure of CFA/I was carried out by SDS gel electrophoresis. Denaturation of the sample with SDS produced a single protein band of high electrophoretic mobility. This indicated that the intact molecule of CFA/I is a polymer made up of identical subunits of much lower molecular weight. Furthermore, two different types of analysis (see below) produced results consistent with this conclusion.

Passage of 6 M guanidine-hydrochloride-treated CFA/I through a column of Sephadex G-200 resulted in the elution of a single protein peak identifiable serologically as the subunit protein of CFA/I. The molecular weight of CFA/I subunit protein was estimated by comparing values obtained for this protein and for proteins of known molecular weight (alpha-chymotrypsinogen, ribonuclease, and cytochrome *c*) (Fig. 7). The molecular weight of the CFA/I subunit protein was calculated to be 24,000. This is in close agreement with the weight calculated by the technique of N-terminal amino acid analysis.

Analyses by both high-pressure liquid chromatography and gas-liquid chromatography showed that valine is the N-terminal amino acid of the CFA/I subunit protein. Analysis of the first five N-terminal amino acid residues indicated that only one amino acid was present at the N-terminus of the polypeptide chain. Although this method cannot evaluate blocked or partially blocked polypeptide chains, the fact that the molecular weight of the subunit as determined by this method was 23,800 strongly supports the conclusion that all of the subunits of the antigen are identical.

The amino acid composition of CFA/I is shown in Table 2. Since this was an acid hydrolysis technique, the concentration of serine was increased by 10% and that of threonine was increased by 5% to compensate for destruction by the acid. The minimum molecular weight determined by amino acid analysis was 24,000, and three different determinations produced results in close agreement. The subunit has a high content of hydrophobic amino acids, and alanine, leucine, isoleucine, phenylalanine, and va-

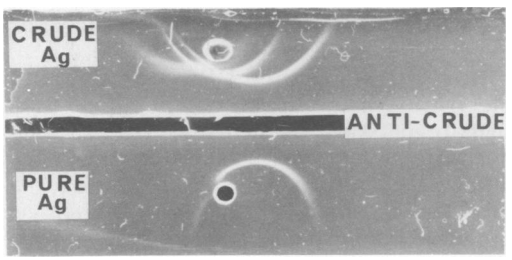

FIG. 3. *Immunoelectrophoresis of crude antigen preparation (crude Ag) and purified CFA/I antigen (pure Ag). The trough contained rabbit antiserum prepared against the crude extract preparation.*

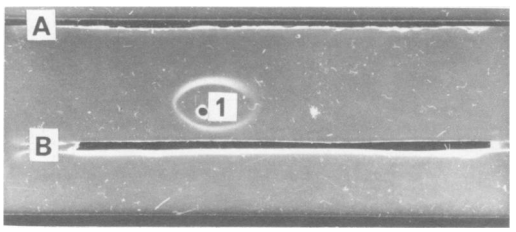

FIG. 4. *Immunoelectrophoresis of purified CFA/I antigen demonstrating purity. Trough A contained antibody prepared against the purified antigen, and trough B contained antibody prepared against the original crude extract.*

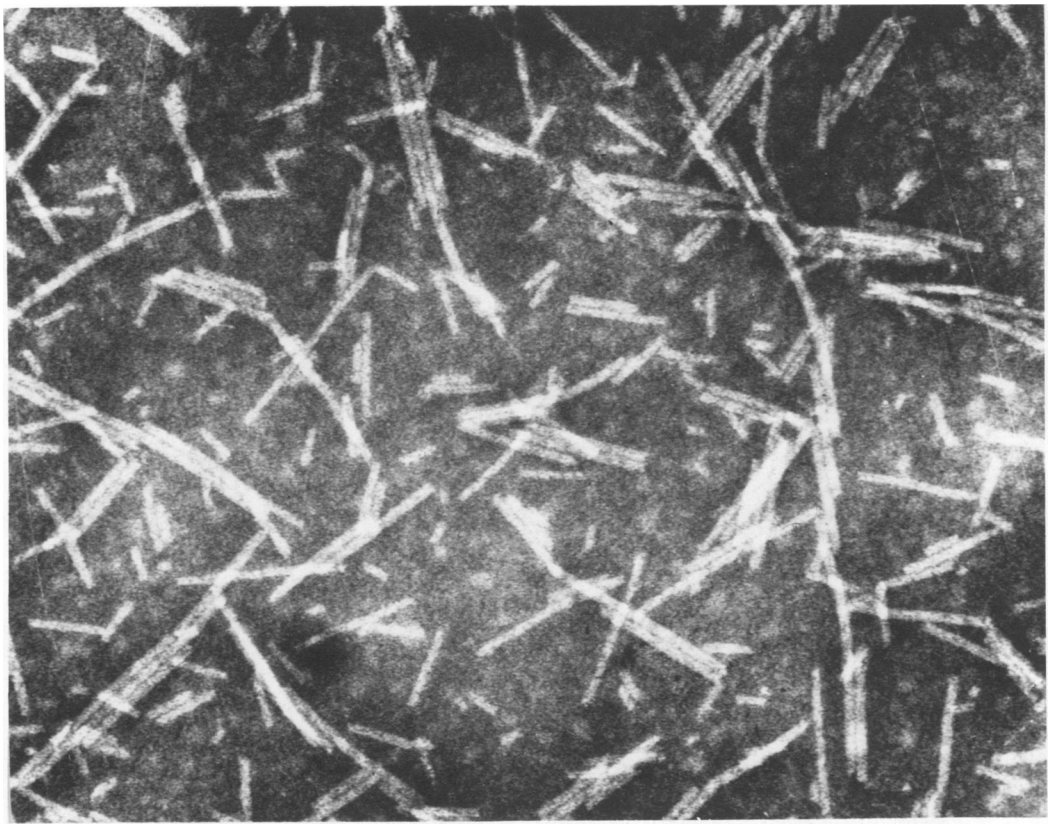

FIG. 5. *Electron micrograph of purified CFA/I antigen. Note rod-shaped morphology and side-to-side aggregation. ×60,000.*

line constitute 37% of the amino acid residues in CFA/I. Eleven residues of proline per subunit were identified. Neutral carbohydrate analysis of the purified antigen demonstrated a concentration of less than 10 μg/mg of protein.

Biological activity of purified CFA/I. ETEC which possess the CFA/I antigen adhered to the intestinal mucosa of infant rabbits (Fig. 8A). The purified antigen also adhered to the small intestines (Fig. 9). Note that the antigen is evenly distributed along the length of the microvilli; this is visualized by the indirect immunofluorescence technique employing antibody prepared against the purified antigen. When rabbits pre-exposed to the purified CFA/I antigen were challenged with whole bacteria, no adhesion of the bacteria could be demonstrated (Fig. 8B), but the bacteria could be seen free in the lumen of the intestine.

Hemagglutinating property of purified CFA/I. CFA/I-positive bacteria agglutinate human, bovine, and chicken erythrocytes in the presence of mannose (4, 8). After purification, CFA/I did not exhibit hemagglutination even in the absence of mannose and at high protein concentrations. However, aggregates of the purified CFA/I antigen which were induced by low pH could be demonstrated to possess mannose-resistant hemagglutination activity. Control experiments demonstrated that this result was not due solely to a reduction of pH, but occurred in the presence of aggregated CFA/I.

Latex microbeads sensitized with CFA/I did exhibit mannose-resistant hemagglutination of human, bovine, and chicken erythrocytes (Fig. 10). Latex beads sensitized with the CFA/II antigen (a crude extract preparation) served as a control in this experiment since CFA/II does not hemagglutinate human erythrocytes (2, 8). CFA/II-sensitized latex beads did exhibit mannose-resistant hemagglutination of bovine erythrocytes, as did intact CFA/II-positive bacteria (Table 3).

Both bacteria possessing the CFA/I antigen and latex beads sensitized with CFA/I agglutinated human group A erythrocytes in the pres-

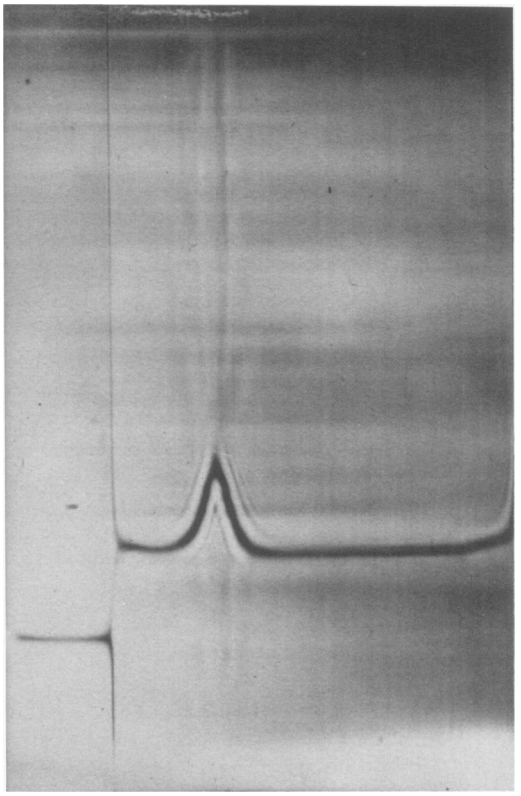

FIG. 6. *Photograph of schlieren pattern obtained with purified CFA/I protein sample (2.5 mg of protein). Note homogeneity of peak; S value = 24.*

ence of a wide variety of carbohydrates. Only NANA inhibited the hemagglutinating activity of CFA/I-positive bacteria (Table 3). This inhibition may be nonspecific since this compound inhibited hemagglutination by both CFA/I-positive and CFA/II-positive bacteria. However, inhibition by NANA was not due to the effect of low pH since hemagglutination occurred even at a pH of 3.0 in the absence of NANA but not in its presence.

Antibody-mediated hemagglutination inhibition. Inhibition of hemagglutination by antibody prepared against the purified antigen was used to demonstrate that the CFA/I antigen present on whole bacteria is in fact responsible for the hemagglutinating activity of such bacteria. Hemagglutination inhibition tests with antisera prepared against the purified antigen were performed as previously described (4). Briefly, a bacterial suspension was mixed with twofold dilutions of anti-CFA/I serum at 37°C, and equal volumes of these mixtures were then mixed with human group A erythrocytes in mannose to test for mannose-resistant hemagglutination. The hemagglutination inhibition titer was that dilution of serum completely inhibiting hemagglutination. Antiserum prepared against the purified antigen agglutinated CFA/I-positive bacterial cells, and this occurred at titers which closely correlated with the hemagglutination inhibition titers of the serum.

DISCUSSION

We previously described a method for the purification of the CFA/I antigen in which acid

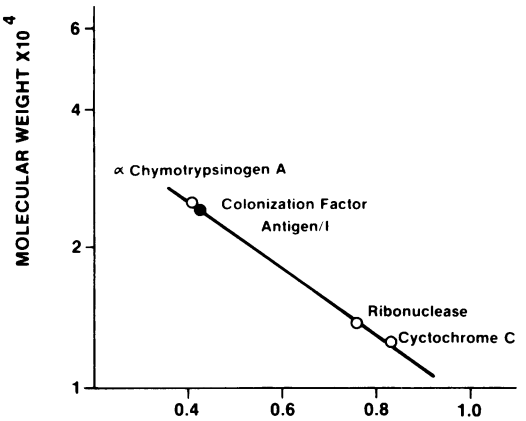

FIG. 7. *Results of Sephadex G-200 column chromatography of CFA/I antigen and protein standards pretreated with 6 M guanidine hydrochloride and 0.1 M BME, pH 6.5. Samples and elution buffer were adjusted to pH 8.6. The lower axis is the distribution coefficient (K_d). These results indicate that the subunit protein of CFA/I has a molecular weight of approximately 24,000.*

TABLE 2. *Amino acid composition of CFA/I antigen of E. coli strain H-10407*[a]

Amino acid	Amt (μmol/mg of protein)	Approx no. of residues per mol
Alanine	1.260	30
Arginine	0.049	1
Aspartic acid	0.902	22
Glutamic acid	0.765	18
Glycine	0.698	17
Histidine	0.056	2
Isoleucine	0.273	7
Leucine	0.811	20
Lysine	0.539	13
Methionine	0.170	4
Phenylalanine	0.114	3
Proline	0.437	11
Serine	1.326	32
Threonine	1.076	26
Tyrosine	0.252	6
Valine	1.167	28

[a] The molecular weight of the CFA/I subunit protein was calculated to be 23,800; the N-terminal amino acid was found to be valine.

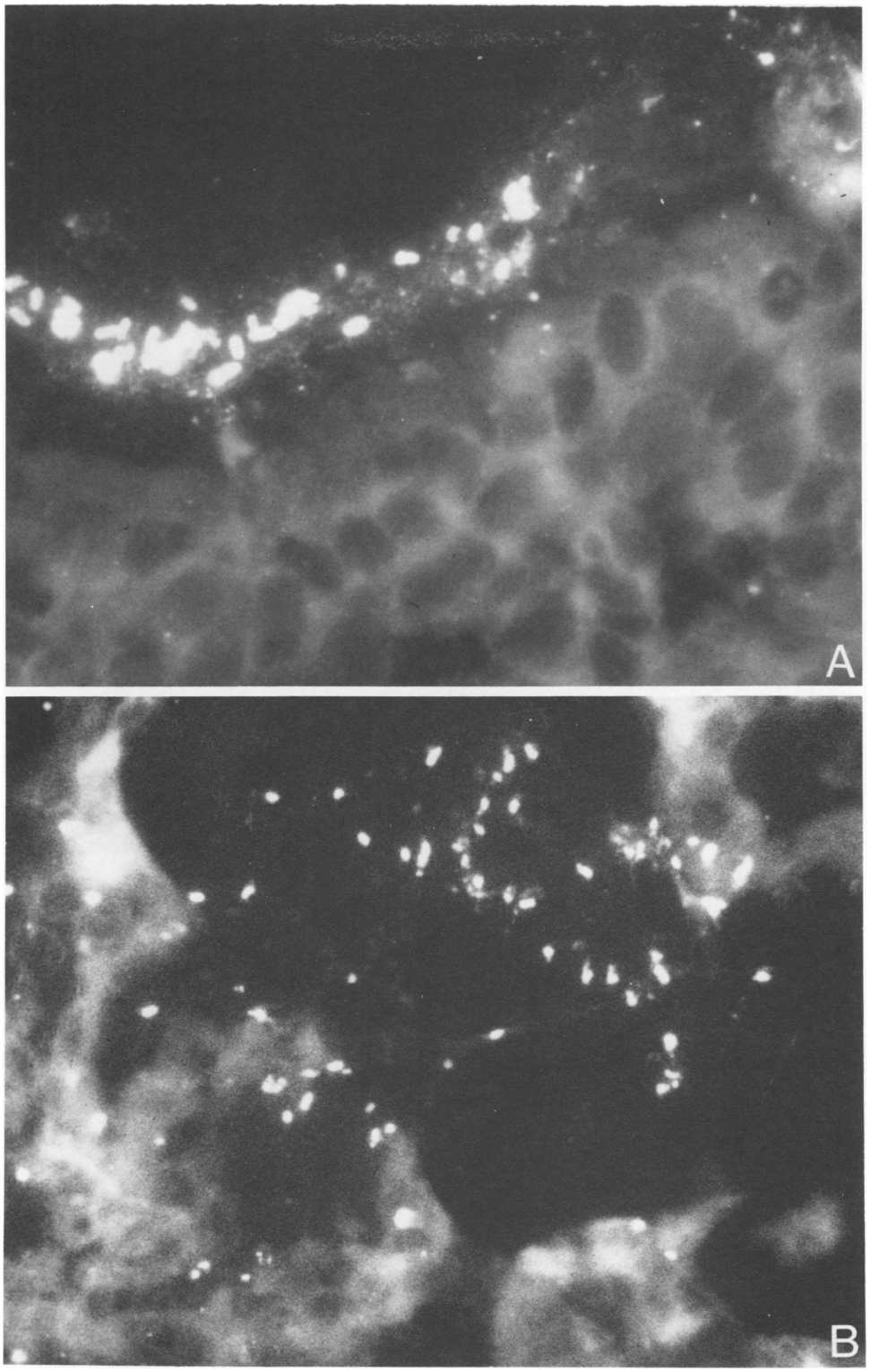

FIG. 8. *(A) CFA/I-positive E. coli (O78:H11) attached to the intestinal mucosa of an infant rabbit, as demonstrated by indirect immunofluorescence by using rabbit anti-O78 serum. ×250. (B) Infant rabbit intestine exposed to CFA/I-positive E. coli (O78:H11) after pre-exposure to purified CFA/I antigen, as in Fig. 9. Indirect immunofluorescence was carried out as in Fig. 8A. Note that the bacteria failed to adhere but are visible in the luminal space. ×250.*

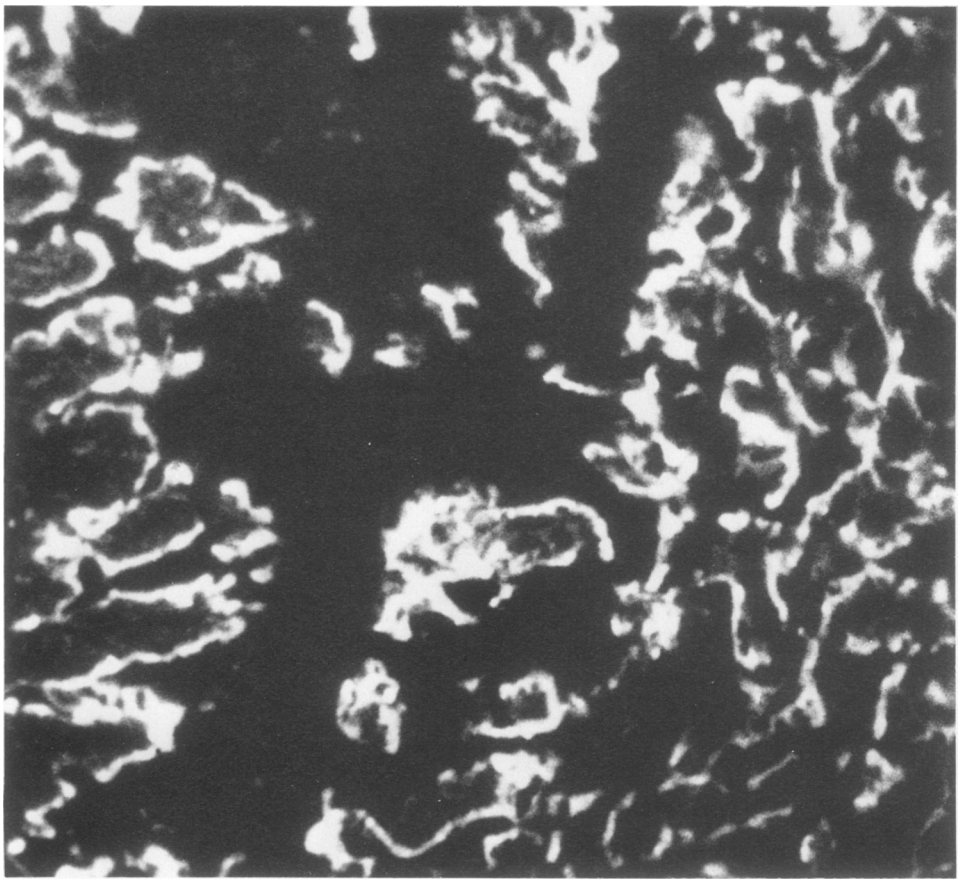

FIG. 9. *Purified CFA/I antigen attached to the intestinal mucosa of an infant rabbit, as demonstrated by indirect immunofluorescence by using rabbit anti-CFA/I serum. ×75.*

TABLE 3. *Demonstration of mannose-resistant, NANA-sensitive hemagglutination by purified CFA/I antigen and by cell-free CFA/II antigen*

Carbohydrate mixed with erythrocytes	Hemagglutination of human (or bovine) erythrocytes by:			
	CFA/I-positive bacteria	CFA/II-positive bacteria	Latex beads sensitized with:	
			Purified CFA/I	Cell-free CFA/II
N-Acetyl-D-galactosamine	+ (+)[a]	− (+)	+ (+)	− (+)
N-Acetyl-D-glucosamine	+ (+)	− (+)	+ (+)	− (+)
NANA	− (−)	− (−)	− (−)[b]	− (−)
N-Acetyl-D-mannosamine	+ (+)	− (+)	+ (+)	− (+)
D-Fucose	+ (+)	− (+)	+ (+)	− (+)
D-Mannose	+ (+)	− (+)	+ (+)	− (+)
D-Galactose	+ (+)	− (+)	+ (+)	− (+)
D-Fructose	+ (+)	− (+)	+ (+)	− (+)
Sucrose	+ (+)	− (+)	+ (+)	− (+)
Mixed gangliosides	+ (+)	− (+)	+ (+)	− (+)

[a] Parentheses indicate results obtained with bovine erythrocytes. Purified CFA/I alone, cell-free CFA/II alone, or latex beads alone did not produce hemagglutination of either human or bovine erythrocytes.

[b] Hemagglutination inhibition with sensitized latex beads by NANA was difficult to demonstrate because NANA mixed with the latex beads caused hemagglutination of the erythrocytes. However, hemagglutination inhibition could be demonstrated by neutralizing the pH of the NANA solution.

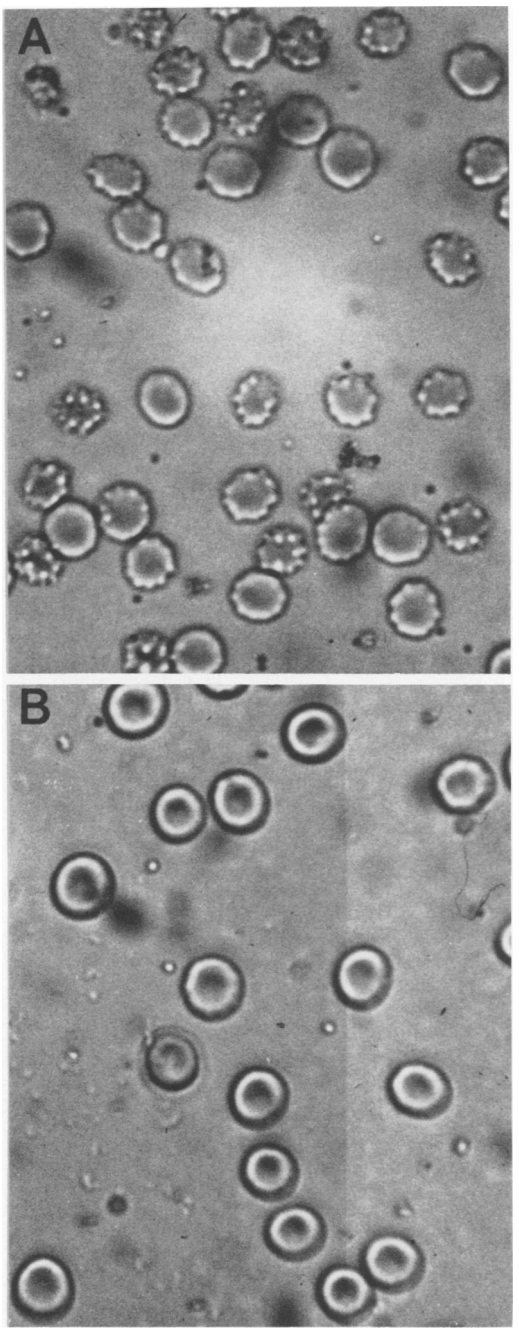

FIG. 10. *(A) Adherence of CFA/I-sensitized latex microbeads to human erythrocytes. (B) Nonadherence of CFA/II-sensitized latex microbeads to human erythrocytes.*

II are common to many of the frequently isolated precipitation, Millipore filtration, and ultracentrifugation are used (5). The method described here is superior in that large quantities of antigen can be purified in high yield without ultracentrifugation. The purity of the antigen was confirmed by physical and biochemical, as well as immunological, analyses. CFA/I is a rod-shaped, fimbrial molecule which retains its morphology throughout the purification procedure. CFA/I is a polymer (average molecular weight, 1.6×10^6) of a single polypeptide chain with a molecular weight of approximately 24,000. The high percentage of hydrophobic amino acids and the proline content are not unusual for such a highly polymerized molecule. The possible role of the hydrophobicity of CFA/I (and also K88 and K99) in the attachment of these adhesins to intestinal epithelial cells, as suggested by Smyth et al. (24), remains to be determined. On the other hand, the host specificity of ETEC possessing these various colonization factors and evidence that specific receptors play a role in attachment of K88-positive ETEC in swine intestines (23), as well as the binding studies reported here with CFA/I, argue against the importance of hydrophobicity as a mechanism of attachment.

Biologically, the CFA/I antigen retains its function even after purification; i.e., the purified antigen is an adhesion which adheres to receptors in the infant rabbit small intestine and blocks adherence of CFA/I-positive bacteria to these receptors. The binding is similar to that exhibited by the family of glycoproteins called lectins but is not identical because CFA/I released from the bacterial cell behaves in a monovalent fashion. Purified CFA/I does not hemagglutinate erythrocytes, a reaction which depends upon lattice formation. However, the adherence of purified CFA/I to human and bovine erythrocytes can be easily demonstrated by artificially creating multivalent CFA/I as in (i) aggregation induced by low pH and (ii) sensitization of latex microbeads with the monovalent antigen. It is significant that CFA/I-sensitized latex microbeads bind to erythrocytes in a mannose-resistant reaction, as do CFA/I-positive bacterial cells. Also, the CFA/II fimbrial antigen retains its biological specificity in the cell-free state, as shown by the fact that latex microbeads sensitized with cell-free CFA/II antigen adhere to bovine but not human erythrocytes in a mannose-resistant fashion, as do CFA/II-positive bacterial cells. NANA blocks the reaction of both CFA/I and CFA/II with erythrocytes, but the significance of this observation is not yet clear. The inhibitory effect of NANA is apparently not due to low pH. NANA blocks the reaction of CFA/I-positive bacteria with human intestinal epithelial cells in tissue culture but also blocks the attachment of CFA-negative *E. coli* which apparently bind to the same cells via common fimbriae.

Finally, it is important that CFA/I and CFA/ serotypes of ETEC which cause diarrhea and travelers' diarrhea throughout the world (2, 4, 5, 14-18, 22, 25). Moreover, purified CFA/I is antigenic and retains the ability to bind to intestinal epithelial cells; this opens the possibility that purified CFAs may be highly effective for artificially inducing immunoprotection against the major causative agent of travelers' diarrhea, i.e., ETEC. We are currently investigating the nature of both humoral and local (secretory) immunoglobulin responses to CFA/I to determine the best approach to a vaccine based on the use of purified ETEC CFAs.

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