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Monoclonal Antibodies against Enterotoxigenic *Escherichia coli* Colonization Factor Antigen I (CFA/I) That Cross-React Immunologically with Heterologous CFAs

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Enterotoxigenic *Escherichia coli* binds to enterocytes in the small intestine by means of antigenically distinct colonization factors (CFs), usually termed colonization factor antigens (CFAs), coli surface antigens (CS), or putative colonization factor antigens (PCFs). To explore the immunological relationship between different CFs, we dissociated CFA/I fimbriae into subunits and produced monoclonal antibodies (MAbs) against these subunits. We selected three MAbs that cross-reacted immunologically with a number of different, whole purified CFs in a dot blot test and with the corresponding subunits in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One of the MAbs, i.e., subunit CFA/I 17:8 (S-CFA/I 17:8), reacted more strongly with subunits of CFA/I than with whole purified fimbriae. This MAb cross-reacted with whole purified fimbriae and subunits of CS4, PCFO166, CS1, and CS2. Moreover, it bound strongly to a peptide of 25 amino acids corresponding to the N-terminal end of CFA/I. The other two MAbs, i.e., S-CFA/I 5:6 and S-CFA/I 8:11, cross-reacted with CS1, CS2, CS4, PCFO166, and CS17 fimbriae but reacted only slightly or not at all with the CFA/I peptide. MAbs S-CFA/I 17:8 and S-CFA/I 5:6 were shown to inhibit hemagglutination by bacterial strains that express either CFA/I, CS1, or CS4. In addition, the binding of enterotoxigenic *E. coli* strains expressing CFA/I, CS2, CS4, and PCFO166 to enterocyte-like cell-line Caco-2 was inhibited by both MAbs. These results show that several antigenically different CFs have common epitopes and that among these at least one is located in the N-terminal end of the subunit protein. Moreover, antibodies against the common epitopes seem to block binding of the bacterial strains that express different CFs to both erythrocytes and Caco-2 cells.

Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of diarrhea in children in developing countries and in travellers to these areas (1, 2). It has been estimated that ETEC infections are responsible for at least 650 million cases of diarrhea, causing about 800,000 deaths each year in children below the age of 5 years (1).

ETEC strains produce colonization factors (CFs), usually termed colonization factor antigens (CFAs), coli surface antigens (CS), or putative colonization factors (PCFs), that are responsible for attachment of the bacteria to the intestinal mucosa (11). This binding is important to enable the bacteria to colonize and also to deliver heat-labile and/or heat-stable enterotoxins in close proximity to enterocytes. Production of specific secretory immunoglobulin A (IgA) antibodies against the CFs is very likely an important prerequisite for protection against ETEC disease (37).

Effective vaccines against ETEC should probably consist of the most prevalent CFs in combination with an appropriate toxoid (37). However, a major problem in the design of such a vaccine is the great number of antigenically heterogenic fimbrial adhesins. The best-characterized CFs are CFA/I (10), CFA/II (9), and CFA/IV (29). CFA/I has a uniform fimbrial structure and is composed of one type of subunit (3), whereas CFA/II-bearing strains may express one or two fimbriae, i.e., CS3 with or without CS1 or CS2 (36), and CFA/IV strains express CS6 alone or in combination with fimbrial antigen CS4 or CS5 (29). Several additional CFs have also recently been characterized, for example, CFA/III, PCFO159, PCFO166, CS7, CS17, PCFO9, and PCFO20 (13, 25, 26, 38, 40). Furthermore, epidemiological studies indicate that a considerable portion of pathogenic ETEC strains do not express any of the hitherto characterized fimbriae (39).

The ETEC fimbriae were originally shown to be antigenically distinct by using polyclonal antibodies and monoclonal antibodies (MAbs) against whole bacteria or purified whole fimbriae in, e.g., enzyme-linked immunosorbent assays (ELISA) and immunodiffusion techniques (28). However, amino acid sequencing has shown very significant homology among several of the ETEC fimbriae; i.e., the N-terminal regions of CFA/I, CS1, CS2, CS4, PCFO166, and CS17 are very similar (15, 17, 19, 33). In fact, immunological cross-reactions between CFA/I, CS1, CS2, CS4, and CS17 subunits have been revealed in immunoblotting experiments using polyclonal antisera raised against whole bacteria (27). Furthermore, it was found that the *cfaD* gene that regulates the expression of CFA/I fimbriae can substitute for *rns*, which is required for production of CS1 and CS2 (4), and it can also promote the production of CS4 fimbriae (41). Immunological cross-reactions between CS5 and CS7 have also been observed in immunoblotting experiments (13).

The different CFs are fimbriae, which may be either rod-like or helical structures about 6 to 8 nm in diameter or thinner, flexible, fibrillar structures (10, 21, 25, 29, 38, 42). They are composed of protein subunits, each consisting of 147 to 200 amino acids, which results in a molecular size varying from 15 to 27 kDa (10, 21, 25, 29, 38, 42). The structure of CFA/I fimbriae has been examined in detail, and it was found that only one type of CFA/I subunit exists (3). The subunit located at the fimbrial tip was shown to act as an adhesin, and a MAb against the CFA/I subunit proved to be a more effective inhibitor of hemagglutination (HA) than a MAb against whole

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CFA/I fimbriae (3). Mapping of the linear B-cell epitopes of the CFA/I subunit has located an immunodominant epitope at the N-terminal end (5).

In this study, we produced MAbs against CFA/I subunits and examined whether these antibodies cross-react immunologically with other CFs. We also evaluated whether such MAbs can bind to a peptide corresponding to the N-terminal region of CFA/I and if they have the capacity to inhibit HA, as well as the binding of ETEC bacteria expressing homologous and heterologous CFs to tissue-cultured intestinal epithelial (Caco-2) cells.

MATERIALS AND METHODS

Bacterial strains used in inhibition experiments. The following strains were used for HA inhibition (HAI) and inhibition of binding to Caco-2 cells: 258909-3 (CFA/I^+ O128:K-H? ST^+/LT^+) and the corresponding CFA/I^- mutant, 258909-3M (22); 60R936 ($CS1^+$ $CS3^-$ O139:H28 ST^-/LT^-) and the corresponding $CS1^-$ mutant, 58R602 (35); 58R957 ($CS2^+$ $CS3^-$ O6:H16 ST^-/LT^-) and the corresponding $CS2^-$ mutant, G176 (31); 62R486 ($CS4^+$ $CS6^-$ O25:H42 ST^-/LT^-) (41) and $CS4^-$ strain E11881D ($CS4^-$ $CS6^-$ O25:H42 ST^+/LT^+) (29); E7476A ($PCFO166^+$ O166:H27 ST^+/LT^-) and the corresponding $PCFO166^-$ mutant, E7476B (26); 334A ($CS7^+$ O15:H11 ST^+/LT^+) and the corresponding $CS7^-$ mutant, 334C (13).

All of the strains were grown on Casamino Acids-yeast extract agar (10) or, when appropriate, on Casamino Acids-yeast extract agar with bile salts (13) at 37°C overnight. Expression of type 1 fimbriae on the bacteria was excluded by testing for mannose-sensitive HA of guinea pig erythrocytes.

Fimbrial preparations. Purified CFA/I fimbriae were prepared from a flagellum-deficient mutant of strain H10407 (CFA/I^+ O78:H^- ST/LT) by homogenization with a blender (Waring, New Hartford, Conn.) followed by ammonium sulfate fractionation and negative DEAE-Sephadex column chromatography (10). We purified CS4 fimbriae from strain E11881A ($CS4^+$ $CS6^+$ O25:H42 ST/LT) (29), CS5 fimbriae from strain E17018A ($CS5^+$ $CS6^+$ O167:H5 ST^+/LT^-) (29), and PCF O166 fimbriae from strain E7476A by the methods described above. We prepared purified CS1 fimbriae from strain 60R936 and CS2 fimbriae from strain 58R957 by homogenization with an Ultra-Turrax T25 blender (Ica-Labortechnik) and then subjected them to ammonium sulfate fractionation and isopycnic cesium chloride gradient ultracentrifugation at 110,000 × *g* for 18 h (18). The different strains were kindly provided by D. Evans (Houston, Tex.) or B. Rowe (London, England). The purity of fimbrial preparations was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Coomassie staining (PhastSystem; Pharmacia, Uppsala, Sweden) and also by immunoblotting with antisera against whole bacteria expressing the homologous fimbriae. The concentration of the fimbriae was determined by using a combination of the weight of a highly purified lyophilized reference preparation and the spectrophotometric A_{280}-A_{310} value. New preparations were compared with the reference in an inhibition ELISA.

Dissociation of CFA/I into subunits. Purified CFA/I fimbriae were dissociated into subunits by incubation in 6 M guanidinium HCl–0.05 M Tris at 25°C for 2 h. Free subunits were then passed through an ultrafiltration membrane (XM 50; stirred cell; Amicon, Danvers, Mass.). After being washed in phosphate-buffered saline (PBS; pH 7.2), the filtrate was concentrated on a YM 10 filter (Amicon). The concentrated preparation was tested by polyacrylamide gel electrophoresis without SDS, followed by Coomassie staining (PhastSystem) to ascertain purity. The assay revealed the presence mainly of monomers, but a small number of dimers was also detected. The concentration of subunits was determined by comparing the intensity of staining of bands with different concentrations of separated whole fimbrial preparations in SDS.

Peptide synthesis. On the basis of CFA/I sequence data (5, 15), solid-phase peptide synthesis was performed with a 430A peptide synthesizer (Applied Biosystems Inc., Foster City, Calif.) and t-boc amino acids to produce a peptide consisting of the 25 N-terminal amino acids. A cysteine residue was introduced into the carboxyl-terminal end to facilitate coupling to the carrier protein. All solvents were from Applied Biosystems, and side chain-protected amino acids were from Nova Biochem. After synthesis, the peptide was cleaved from the resin and the protecting groups were removed from the amino acids by acidic hydrolysis using anisole and ethanedithiol (Merck, Darmstadt, Germany) as scavengers. Peptide composition was confirmed by amino acid analysis, and purity was assessed by reverse-phase high-pressure liquid chromatography.

Immunizations and production of specific antisera. Inbred female BALB/c mice, obtained from our breeding facilities, were used. After collection of preimmune serum, 6-week-old mice were immunized intraperitoneally with 5 μg of dissociated CFA/I or whole CFA/I fimbriae in complete Freund's adjuvant; 2 weeks later, 5 μg of the same antigen was given intraperitoneally in incomplete Freund's adjuvant. One week later, the mice were given 2.5 μg of the same antigen intravenously without adjuvant. Four days after the last immunization, the mice were killed by cervical dislocation, blood was drawn, and the spleens were excised. The antiserum was diluted 1/10 and absorbed twice with live bacteria and twice with boiled bacteria by using a CFA/I-deficient mutant of strain H10407, i.e., H10407P (CFA/I^- O78:H^- ST/LT).

MAb production. Spleen lymphocytes were fused with cells of mouse myeloma cell-line P3x63-Ag8 653 (16) at a ratio of 1:2 by using polyethylene glycol (8). The culture medium for selection of stable hybridoma cell lines contained hypoxanthine-aminopterin-thymidine in Iscove's medium (Biochrom KG, Berlin, Germany) with 10% fetal calf serum (Biochrom). After 10 days, the hybridomas were tested for production of specific antibodies against CFA/I fimbriae and CFA/I subunits by analyzing culture supernatants in the ELISA described below. The hybridomas producing the highest titers were propagated, and the supernatants were tested for reaction with purified fimbriae of CS1, CS2, CS4, PCFO166, and CS5 by using the ELISA and a dot blot test as described below. The hybridomas that reacted in the highest titers with heterologous CFAs were cloned by limiting dilution (12), and stable hybridomas were expanded by cultivation first in 10 ml and then in 100 ml of 10% fetal calf serum–Iscove's medium devoid of antibiotics in tissue culture bottles (Nunc, Roskilde, Denmark). Culture fluids were harvested and frozen in aliquots at −30°C. In addition, MAb CS7 5:2, previously produced against whole purified fimbriae of CS7 (39), was used in the inhibition experiments (culture fluid containing 145 μg of IgG1 per ml).

Determination of isotype and concentration of MAbs. The isotypes of the different MAbs were determined by double diffusion in gel (32) with antisera specific for different isotypes of mouse immunoglobulins (Igs; Sigma, St. Louis, Mo.). The Ig concentration was determined by single radial immunodiffusion (24) with the isotype-specific anti-mouse Igs and, as standards, mouse sera of each Ig isotype with known concentrations (The Binding Site Ltd., Birmingham, England).

ELISA. Antisera or MAbs were tested for reactivity with CFA/I fimbriae; CFA/I subunits; the CFA/I peptide; and CS1,

CS2, CS4, PCFO166, and CS5 fimbriae. Polystyrene microtiter plates (Dynatech Laboratories Ltd., Billingshurst, United Kingdom) were coated with 1 μg of CFA fimbriae or subunits per ml in PBS at 37°C overnight. In the peptide ELISA, plates were coated with 25 μg of the CFA/I peptide per ml in carbonate buffer (0.05 M, pH 9.6) at 37°C overnight. After blocking with 0.1% bovine serum albumin (BSA)–PBS at 37°C for 30 min, antisera or MAbs were serially diluted in PBS–0.1% BSA–0.05% Tween and incubated at room temperature for 60 to 90 min. Bound antibody was then demonstrated by incubating the plates with horseradish peroxidase-conjugated goat anti-mouse Ig (Jackson Immuno Research Laboratories, West Grove, Pa.) and the enzyme substrate *o*-phenylenediamine–H_2O_2. Titers were determined as the reciprocal dilution giving an A_{450} of 0.4 above the background (Labsystems Multiscan PLUS) after a 5- to 20-min enzyme reaction. All titrations were performed in duplicate.

Dot blot tests. Two microliters of purified CFA or CS factors in PBS (0.5 mg/ml) and the CFA/I N-terminal peptide, coupled to BSA, were applied to nitrocellulose strips (Pure Nitrocellulose Membrane; 0.45-μm pore size; Bio-Rad Laboratories, Richmond, Calif.) soaked in PBS (23). After air drying, nonspecific binding was blocked by incubating the strips in 1% BSA–PBS and then antisera or culture fluids of MAbs diluted in PBS–0.1% BSA–0.05% Tween were added to the strips, which were incubated at room temperature overnight. The strips were then developed by incubation in horseradish peroxidase-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories) diluted in BSA-Tween-PBS for 2 h, followed by incubation in substrate solution (0.05% 4-chloro-1-naphthol (Bio-Rad)–0.01% H_2O_2 in 0.5 M Tris–0.15 M NaCl [pH 7.6]).

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were performed as described by Laemmli (20), with a MINI PROTEAN II vertical electrophoresis cell (Bio-Rad). Samples of purified fimbriae were adjusted to a concentration of 0.5 mg/ml and then boiled in equal amounts of sample buffer (0.625 M Tris [pH 6.8], 10% glycerol, 2% SDS, 5% mercaptoethanol in distilled water) for 5 min and then applied to 16% acrylamide gels in 15-μl volumes. Pyronin Y (Sigma) was used as the marker for the electrophoretic front. Samples separated at 200 V for 50 min were transferred to nitrocellulose sheets (Pure Nitrocellulose Membrane, 0.45-μm pore size) at 75 V for 60 min, and the immunoblotting procedure was then continued as with the dot blot tests.

HA and HAI. HA experiments were performed with round-bottom polystyrene microtiter plates (Nunc). Bacteria expressing the different CFs were tested in two-fold dilutions in saline containing 0.5% D-mannose (25 μl per well). Twenty-five microliters of washed human group A or bovine erythrocytes (1%) in the same diluent was added to each well, and the HA titer was recorded after incubation at room temperature for 30 min. Human erythrocytes from two different donors were used for the experiments with CFA/I-, CS4-, and CS7-expressing bacteria, and bovine erythrocytes from one donor were used for the experiments with CS1- and CS2-expressing bacteria. The lowest concentrations of bacteria giving complete HA in the wells were used in the HAI experiments.

In the HAI experiments, twofold serial dilutions of the MAbs (initial concentration, 150 μg of Ig per ml) in saline with 0.5% D-mannose (25 μl) were preincubated with a fixed number of CF-expressing bacteria (25 μl) or the corresponding CF-negative mutant bacteria at room temperature for 10 min. A suspension of the erythrocytes used in the HA experiments (1%, 25 μl) was then added, and results were read after incubation at room temperature for 30 to 45 min. The HAI titer was expressed as the highest dilution of the MAb that completely inhibited HA. All analyses were done in duplicate on the same day and repeated twice on separate days.

TABLE 1. Comparison of reciprocal ELISA IgG titers[a] of antisera against CFA/I fimbriae and subunits

Antigen	Anti-CFA/I fimbria titer		Anti-CFA/I subunit titer	
	Mouse 1	Mouse 2	Mouse 3	Mouse 4
CFA/I fimbriae	15,000	14,000	100,000	100,000
CFA/I subunits	15,000	25,000	>100,000	>100,000

[a] Mean ELISA titers calculated from duplicate determinations are shown.

Inhibition of adhesion to human colon carcinoma cell line Caco-2. Caco-2 cells were grown in Dulbecco's modified Eagle's medium containing fetal calf serum (10%) and glutamine (1%) in eight-well chamber slides (Nunc Inc., Naperville, Ill.) in 7% CO_2 at 37°C. We used cells that were past confluence after 15 days of culture and washed them in Eagle's medium. A suspension of 10^7 bacteria per ml (in one experiment, 10^8 bacteria per ml) in culture medium containing 0.5% D-mannose was mixed with an equal amount of a MAb (150 μg/ml) and incubated at room temperature for 20 min. The mixture was then added to the tissue culture cells and incubated at 37°C for 3 h. After five washes, cells were fixed in methanol, stained with 10% Giemsa, and examined by oil immersion light microscopy to determine bacterial adherence. The percentage of epithelial cells with adherent bacteria was determined by counting 10 randomly chosen microscopic fields with approximately 100 cells per field. Each strain was tested in duplicate, and assays were performed blind.

Statistical analysis. Binding of CFA/I-positive bacteria to Caco-2 cells was expressed as the mean percentage of cells with bound bacteria ± 1 standard deviation. Results were obtained from three different experiments performed in duplicate. The statistical significance of the difference between the proportions of cells with bound bacteria in the control groups and in the groups with inhibitors (MAbs) was analyzed and expressed as a *P* value. The results of the three experiments performed on different occasions were analyzed separately and sequentially in a fixed test order.

RESULTS

Production and reactivity of polyclonal antibodies. Sera from mice immunized with whole purified CFA/I fimbriae and isolated CFA/I subunits, respectively, were tested in an ELISA for reactivity with CFA/I fimbriae and subunits. Sera of mice immunized with CFA/I subunits had, as a mean, sevenfold higher antibody titers against both CFA/I subunits and fimbriae than did mice immunized with whole purified CFA/I fimbriae (Table 1). In immunoblotting experiments, antisera from the different groups of mice were diluted to contain similar numbers of ELISA units against CFA/I fimbriae, and the abilities of the two different antisera to bind to the different CFs, which were separated into subunits by SDS-PAGE, were compared. These analyses showed that serum raised against CFA/I subunits reacted more strongly with homologous, and also with heterologous, CFA subunits than did serum against whole purified CFA/I fimbriae (Fig. 1). As shown, antiserum against CFA/I subunits cross-reacted strongly with CS4 monomers and dimers and also, although less strongly, with CS1, PCFO166, CS17, and CS2. No reactivity with CS5 (Fig. 1), which lacks homology with the amino acid sequence of CFA/I (6, 15), was seen.

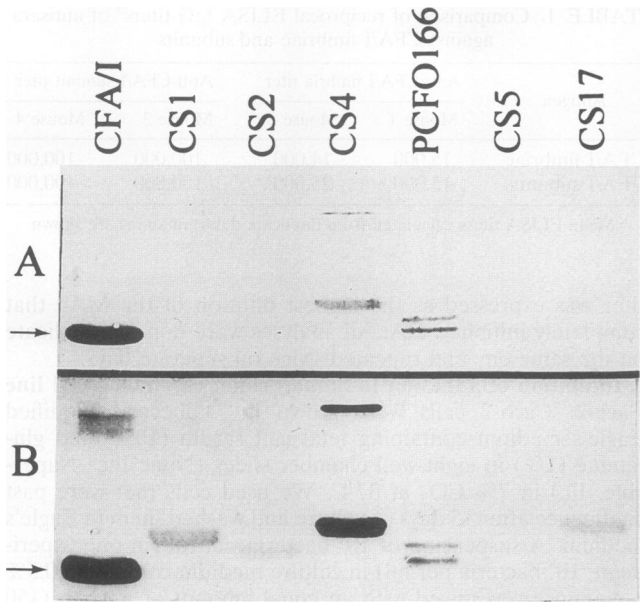

FIG. 1. Antigenic cross-reactivities between different CFs as demonstrated by immunoblotting with mouse antisera against whole fimbriae of CFA/I diluted 1/1,500 (A) and subunits of CFA/I diluted 1/7,500 (B) (dilutions were based on the ELISA titers of the sera). The fimbrial preparations were applied to the gel as purified fimbriae (4 μg per well). Each arrow indicates the position of a 15-kDa protein.

When the different antisera were tested for reactivity with a synthetic peptide consisting of the 25 N-terminal amino acids of CFA/I in a dot blot test, we found that antiserum against whole CFA/I fimbriae did not react, while antiserum against subunits of CFA/I reacted strongly (results not shown). The specificity of the reaction of the anti-subunit serum with the CFA/I peptide was supported by the findings that the preimmune serum did not react with the CFA/I peptide and that the anti-subunit serum did not react with an unrelated peptide similar in size, i.e., *E. coli* heat-stable toxin consisting of 19 amino acids.

MAb production. The hybridomas obtained after immunization with isolated CFA/I subunits were initially screened for reactivity with CFA/I fimbriae and subunits, respectively. Ten of the hybridomas with the highest ELISA titers against these antigens were selected and tested for reactivity in an ELISA and a dot blot test with whole fimbriae of CS4, PCFO166, CS1, CS2, CS17, and CS5, as well as the CFA/I N-terminal peptide. These fimbrial antigens were selected because all of them, except CS5, have been shown to have very similar N-terminal ends. The three hybrids that showed the highest degree of reactivity with heterologous fimbriae in the ELISA and the dot blot test were selected for subcloning. One subclone from each hybridoma that was stable after repeated testing in the ELISA and the dot blot test was used in the continued studies. The three MAbs selected, which were all of the IgG1 isotype, were designated subunit CFA/I 17:8 (S-CFA/I 17:8), S-CFA/I 5:6, and S-CFA/I 8:11. The Ig concentrations of these MAbs in culture fluids were 150, 144, and 116 μg/ml, respectively.

MAb reactivity. The specific titers of the three different MAbs against CFA/I fimbriae, CFA/I subunits, and the synthetic CFA/I N-terminal peptide were determined in ELISAs. As shown in Table 2, MAb S-CFA/I 17:8 reacted in a threefold higher titer with CFA/I subunits than with whole CFA/I fimbriae. This MAb also reacted strongly with the CFA/I peptide. MAb S-CFA/I 5:6 reacted somewhat better with CFA/I subunits than with CFA/I fimbriae but did not react with the CFA/I peptide in the ELISA. MAb S-CFA/I 8:11, which reacted equally well with CFA/I fimbriae and CFA/I subunits, did not react with the peptide either.

TABLE 2. Comparison of reciprocal ELISA IgG titers[a] of three different anti-CFA/I subunit MAbs against CFA/I fimbriae, CFA/I subunits, and a CFA/I peptide

Antigen	ELISA IgG titer		
	MAb S-CFA/I 17:8	MAb S-CFA/I 5:6	MAb S-CFA/I 8:11
CFA/I fimbriae	4,800	18,200	13,200
CFA/I subunits	13,300	27,600	17,000
CFA/I peptide[b]	6,900	<100	<100

[a] Mean ELISA titers calculated from duplicate determinations are shown.
[b] Synthetic CFA/I peptide corresponding to the 25 N-terminal amino acids.

The three different MAbs were also tested for reactivity with nitrocellulose-bound CF fimbriae, CF subunits, and the CFA/I peptide in dot blot experiments. As shown in Fig. 2A, MAb S-CFA/I 17:8 bound to CFA/I fimbriae as well as to CFA/I subunits. On the original dot blot, a stronger reaction with CFA/I subunits than with CFA/I fimbriae was seen, confirming the ELISA results. Cross-reactions with whole fimbriae of CS4, PCFO166, CS1, and CS2 were observed, and a strong cross-reaction with the CFA/I peptide coupled to BSA was also detected. Surprisingly, however, this MAb did not react with

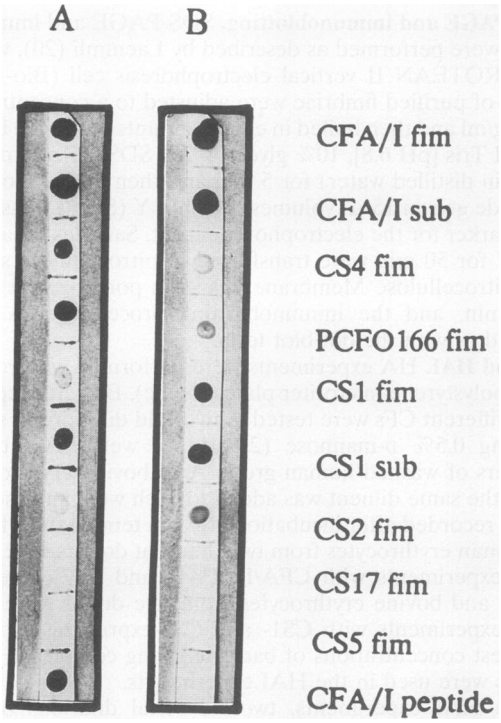

FIG. 2. Immunological reactivities of MAbs against CFA/I subunits, i.e., MAb S-CFA/I 17:8 (A) and MAb S-CFA/I 5:6 (B) (culture fluids of MAbs were diluted 1/5) with different CFs (purified CF fimbriae [fim], CF subunits [sub], and the CFA/I peptide corresponding to the 25 N-terminal amino acids) as demonstrated by a dot blot test. The different antigen preparations were tested at similar protein concentrations.

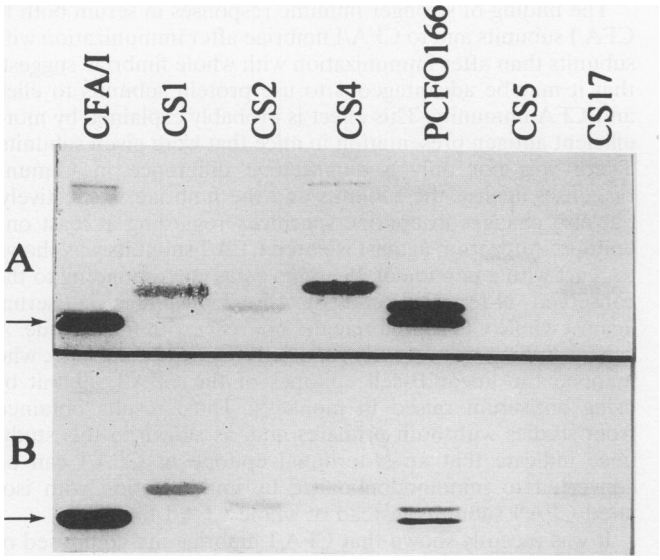

FIG. 3. Immunological reactivities of two MAbs against subunits of CFA/I, i.e., MAb S-CFA/I 17:8 (A) and MAb S-CFA/I 5:6 (B) (culture fluids of MAbs were diluted 1/10) with different CFs as demonstrated by immunoblotting. The fimbrial preparations indicated were applied to the gel as purified fimbriae (4 μg per well). Each arrow indicates the position of a 15-kDa protein.

CS17, which has an N-terminal end similar to that of CFA/I (19). The patterns of MAb S-CFA/I 5:6 and S-CFA/I 8:11 binding to the different CFs in the dot blot test were very similar; hence, only the dot blot results obtained with MAb S-CFA/I 5:6 are shown (Fig. 2B). Both MAbs S-CFA/I 5:6 and S-CFA/I 8:11 bound more strongly to whole CFA/I fimbriae than did MAb S-CFA/I 17:8. They also cross-reacted with CS1, CS2, PCFO166, and CS4 and cross-reacted weakly with CS17. None of the MAbs reacted with a CS5 fimbrial preparation which was used as a negative control.

The reactivities of MAbs S-CFA/I 17:8 and S-CFA/I 5:6 were also studied in immunoblotting experiments. As shown in Fig. 3A, MAb S-CFA/I 17:8 cross-reacted with subunits of PCFO166, CS4, CS1, and CS2. MAb S-CFA/I 5:6 cross-reacted with subunits of PCFO166, CS1, CS2, CS4, and CS17 (Fig. 3B). None of the MAbs reacted with subunits of CS5. Although the fimbriae were dissociated into subunits, there was a clear agreement between the binding patterns of the two MAbs with the different CFs in the immunoblotting test and the dot blot test.

HAI. Although peptide-binding MAb S-CFA/I 17:8 bound in a weaker fashion to whole CFA/I fimbriae than did the other two MAbs, it was the most effective of the MAbs at inhibiting HA by CFA/I-expressing bacteria (Fig. 4). MAb S-CFA/I 5:6 was almost as effective at inhibiting CFA/I-positive bacteria, whereas MAb S-CFA/I 8:11 had a lower inhibitory titer. HAI by heterologous bacteria, especially CS1- and CS4-expressing strains, was almost as effective as that of homologous bacteria for both MAbs S-CFA/I 17:8 and S-CFA/I 5:6, whereas neither of these MAbs inhibited HA by CS7-expressing bacteria. However, MAb S-CFA/I 8:11 did not inhibit HA of the heterologous strain expressing CS4. Neither did a MAb raised against CS7 fimbriae inhibit HA of CFA/I-positive bacteria. In the HAI experiments, we used a CS7-expressing strain and a MAb raised against purified CS7 fimbriae as negative controls, since we did not have access to a CS5-only strain. None of the three MAbs used agglutinated CFA/I-expressing bacteria.

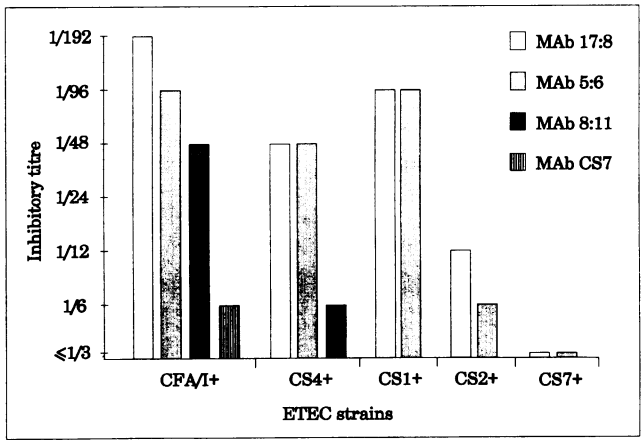

FIG. 4. Inhibition of HA by bacteria expressing different CFs by MAbs against CFA/I subunits (S-CFA/I 17:8, S-CFA/I 5:6, and S-CFA/I 8:11) and CS7 fimbriae. The bacteria were used at the lowest concentration giving complete HA. Human group A erythrocytes were used for HA of CFA/I-, CS4-, and CS7-expressing strains, whereas bovine erythrocytes were used for CS1- and CS2-expressing strains. MAbs were tested at similar Ig concentrations, i.e., about 150 μg/ml in the first well. The columns indicate reciprocal inhibitory titers.

Inhibition of binding of bacteria to Caco-2 cells. In the experiments testing inhibition of bacterial binding to Caco-2 cells, the percentage of cells with adhering bacteria was determined after preincubation with MAbs S-CFA/I 17:8 and S-CFA/I 5:6 and, as a negative control, MAb CS7 5:2 or tissue culture medium. As shown in Fig. 5, as many as 40% ± 9.9% (mean ± standard deviation) of the cells bound CFA/I-expressing bacteria preincubated in tissue culture medium, whereas only 3.6% ± 5.4% of the cells bound the CFA/I-negative mutant. Preincubation of CFA/I-positive bacteria

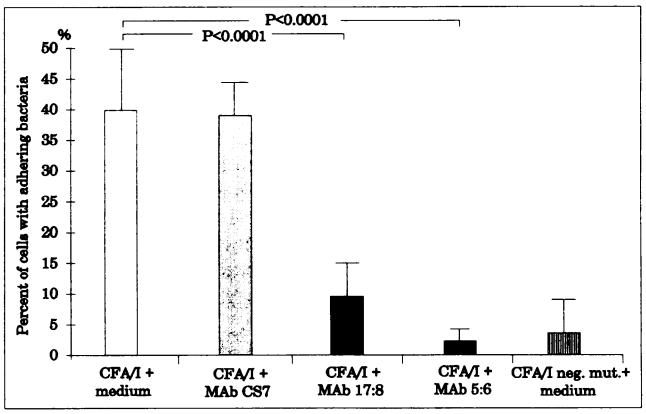

FIG. 5. Inhibition of binding of CFA/I-expressing bacteria to Caco-2 cells by MAbs produced against CFA/I subunits and a MAb produced against CS7 fimbriae. MAbs were tested at similar Ig concentrations. The columns indicate mean percentages of cells with adhering bacteria. The standard deviations of results obtained in duplicate tests from three experiments are shown. The binding of a CFA/I-negative mutant was determined to obtain a baseline level. The statistical significance of the difference between the control groups and the groups with inhibitors (MAbs) was determined by using a hypothesis test of proportions performed separately and sequentially on results from the three different experiments.

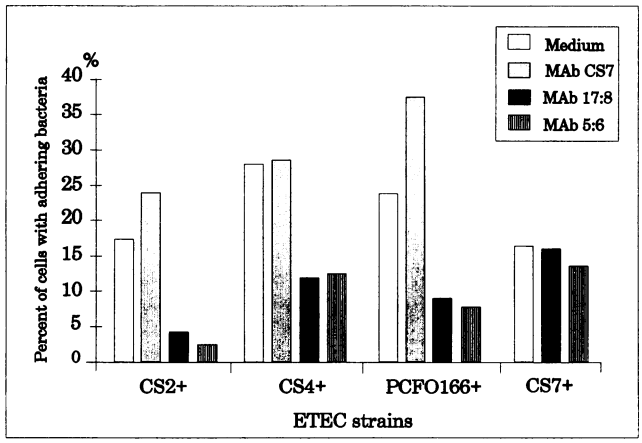

FIG. 6. Inhibition of binding of bacteria expressing different CFs to Caco-2 cells by MAbs against CFA/I subunits and a MAb against CS7 fimbriae. MAbs were used at similar Ig concentrations. The columns indicate mean percentages of cells with adhering bacteria. The percentage of cells with bound bacteria in the absence of any MAb was also tested by adding only medium. MAb CS7 could not be used to test for inhibitions of CS7-expressing bacteria because it agglutinated such bacteria.

with MAb S-CFA/I 17:8 reduced binding to 9.6% ± 5.4% of the cells ($P < 0.0001$ [comparison of proportions]), and preincubation with MAb S-CFA/I 5:6 reduced binding to 2.3% ± 5.4% of the cells ($P < 0.0001$), whereas the MAb raised against CS7 fimbriae did not reduce the binding of CFA/I-expressing bacteria to the cells.

The anti-CFA/I subunit MAbs were also tested for the capacity to inhibit binding of bacteria expressing heterologous fimbriae. Inhibition of bacterial binding to Caco-2 cells was tested with bacteria expressing CS2, CS4, PCFO166, CS5, and CS7, since all of these fimbriae bind strongly to this cell line, whereas inhibition of binding of bacteria expressing CS1 and CS17 could not be tested, since these fimbriae do not bind to Caco-2 cells (38a). As shown in Fig. 6, both MAbs S-CFA/I 17:8 and S-CFA/I 5:6 were able to inhibit almost completely the binding of bacteria expressing CS2 or PCFO166 fimbriae. The binding of CS4-expressing bacteria was only partially inhibited by either MAb, maybe because of the strong spontaneous agglutination of the CS4-expressing strains. The percentage of binding of the corresponding CFA-negative mutants was between 2 and 9%; the highest value was obtained with the PCFO166-negative mutant. In control experiments, the MAb against CS7 fimbriae did not inhibit binding of CS2-, CS4-, and PCFO166-expressing bacteria, nor were bacteria expressing CS7 fimbriae inhibited by MAbs S-CFA/I 17:8 and S-CFA/I 5:6.

DISCUSSION

In the present study, we raised antisera against whole purified CFA/I fimbriae, as well as isolated subunits of CFA/I, and compared their reactivities with heterologous CFs. Three different MAbs against isolated subunits of CFA/I that cross-reacted immunologically with several ETEC CFs were also produced. One of these MAbs reacted strongly with a synthetic peptide corresponding to the N-terminal end of CFA/I. We also found that two of the MAbs could inhibit the binding of ETEC bacteria expressing different CFs to erythrocytes, as well as to Caco-2 cells.

The finding of stronger immune responses in serum both to CFA/I subunits and to CFA/I fimbriae after immunization with subunits than after immunization with whole fimbriae suggests that it may be advantageous to use protein subunits to elicit anti-CFA immunity. This effect is probably explained by more efficient antigen presentation in mice that were given subunits. There was not only a quantitative difference in immune responses against the subunits and the fimbriae, respectively, but also changes in epitope specificity regarding at least one epitope. Antiserum against isolated CFA/I subunits was shown to react with a peptide of 25 amino acids corresponding to the conserved N-terminal end of CFA/I, whereas antiserum against whole CFA/I fimbriae did not react with the peptide. A similar finding was recently reported by Cassels et al. (5), who mapped the linear B-cell epitopes of the CFA/I subunit by using antiserum raised in monkeys. Thus, results obtained from studies with both primates and, as shown in this study, mice indicate that an N-terminal epitope of CFA/I can be converted to immunodominance by immunization with isolated CFA/I subunits instead of whole CFA/I fimbriae.

It was recently shown that CFA/I fimbriae are composed of multiple identical protein subunits, each containing a receptor-binding site which is only exposed at the tip of the fimbria (3). This finding suggested that the probability of obtaining antibodies directed against epitopes close to the receptor-binding site might be improved by immunization with CFA/I subunits rather than with whole purified fimbriae. Moreover, immunologically cross-reactive antibodies might be more readily produced by immunization with CFA/I subunits rather than with whole purified fimbriae because of the exposure of conserved epitopes hidden in the quaternary structure. Although the immunodominant epitopes of the quaternary conformations of the various CFs are very different (28), we have previously shown that CFA/I fimbriae can both prime and boost the immune responses to CS4 and vice versa (34), which indicates that conserved epitopes are exposed during degradation of the fimbriae in vivo. In the immunoblotting experiments of the present study, we found that an antiserum against the CFA/I subunits reacted more strongly with electrophoretically separated heterologous subunits than did an antiserum against whole fimbriae. Similarly, detailed studies of the fimbrial adhesins of uropathogenic *E. coli* species, e.g., those of P and S fimbriae, have shown that regions conserved among antigenically different fimbriae are often immunorecessive with regard to the polymeric structure of the intact fimbriae (14, 30). Nevertheless, these regions can be important because of involvement in the receptor-binding site.

The finding that one of the MAbs against CFA/I subunits, i.e., MAb S-CFA/I 17:8, cross-reacted strongly with the peptide corresponding to the N-terminal end of the CFA/I subunits was not unexpected, since the N-terminal regions of the subunit proteins of CFA/I, CS1, CS2, CS4, and PCFO166 have been shown to be very similar (19). However, although the subunit protein of CS17 also appears to share considerable homology in the N-terminal end with these subunit proteins (19), MAb S-CFA/I 17:8 did not bind to CS17 fimbriae. One possible explanation for this lack of reactivity may be that the minor differences in amino acid composition between CFA/I and CS17 result in different tertiary configurations of the two fimbriae or their subunits.

It was apparent from the ELISA and dot blot test results that MAb S-CFA/I 17:8 bound more strongly to CFA/I subunits than to whole CFA/I fimbriae. Furthermore, this MAb was the most effective one at inhibiting HA by bacteria which express CFA/I. This observation confirms an earlier finding by Bühler et al. (3) that a MAb produced against heat-dissociated CFA/I

subunits is more effective at HAI than a MAb produced against whole CFA/I fimbriae. However, in those experiments, neither cross-reactions with other CFs nor the binding specificity of the strongly inhibitory MAb was studied. Importantly, our MAbs against CFA/I subunits were able to inhibit HA of bacteria which express either CS1 or CS4 but to a lower degree than HAI of bacteria which express the homologous antigen. The inhibitory titers of the different MAbs were lower when we studied the HAI of bacteria expressing CS2 than when we studied that of bacteria expressing CS1 or CS4; this was probably due to lower binding affinities of the MAbs to CS2 than to CS1 or CS4.

We also evaluated whether the MAbs against CFA/I subunits were capable of inhibiting binding to human intestinal cells. Since Caco-2 cells are highly differentiated and have a brush border very similar to that of small intestinal enterocytes, they were chosen for these experiments. Even though the Caco-2 cell-binding properties are not identical to those of enterocytes from the human small intestine, it is probably the best available cell line for studying the inhibition of binding. Since the bacteria are not randomly distributed over the entire Caco-2 monolayer (7), we found it important to count a large number of cells. Adhesion was quantified by determining the percentage of cells with adhering bacteria and not by counting the number of bacteria per cell, owing to the large number of cells counted. We think that this way to quantify adhesion is correct, because in wells with a high percentage of cells with adhering bacteria, many bacteria were bound to each cell, whereas in wells with a lower percentage, fewer bacteria were bound to each cell. The finding that the MAbs against the CFA/I subunits not only inhibited the binding of bacteria expressing the homologous CF but also some heterologous CFs suggests that epitopes common to several CFs are expressed on the surface of the fimbriae at or close to the binding region. As antibodies can inhibit adherence with mechanisms other than direct interference with the binding part of the protein, e.g., steric hindrance, our results do not prove that the N-terminal sequence is part of this region. The lower capacity of the MAbs to inhibit binding of CS4-expressing bacteria than CS2- or PCFO166-expressing bacteria may be due to the autoagglutinating properties of CS4-expressing bacteria. The discrepancy in the capacity to inhibit binding of bacteria which express CS2 in the Caco-2 cell assay and the HAI assay, respectively, suggests that the two types of cells have different binding specificities.

In conclusion, our results suggest that it is possible to induce specific antibodies against ETEC CFs that may inhibit binding of both homologous and heterologous CFs to epithelial cell receptors in the small intestine. This finding may have important implications in the design of a simplified ETEC vaccine. Recently developed prototype vaccines consist of whole ETEC bacteria expressing the most prevalent CFs in combination with a toxoid. The results of this study suggest the possibility of preparing a vaccine that contains bacteria expressing a few different CF subunits that may confer protection against a wide range of different ETEC strains.

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