INFECTION AND IMMUNITY, Nov. 1996, p. 4508–4513 Vol. 64, No. 11
0019-9567/96/$04.00+0

Identification of a Cross-Reactive Continuous B-Cell Epitope in Enterotoxigenic *Escherichia coli* Colonization Factor Antigen I

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Received 4 March 1996/Returned for modification 15 May 1996/Accepted 7 August 1996

Enterotoxigenic *Escherichia coli* (ETEC) colonizes the intestine by means of several antigenically distinct colonization factors (CFs). Several of these CFs have very significant amino acid sequence similarity or identity, particularly in the N-terminal end. We have previously shown that a monoclonal antibody (MAb) raised against the subunits of colonization factor antigen I (CFA/I) fimbriae, which reacts with a peptide corresponding to the 25 N-terminal amino acids of such subunits, can inhibit attachment to intestinal cells of ETEC expressing heterologous as well as homologous CFs, with related amino acid sequences. In this study we have, by means of Pepscan analysis, determined the sequence of the MAb-specific linear epitope to be ^{15}IDLLQ^{19}. Parenteral immunization of rabbits with an N-terminal 25-mer synthetic peptide of CFA/I fimbrial subunit, either covalently coupled to bovine serum albumin or uncoupled, induced high titers of specific antibodies against this peptide as well as against CFA/I fimbriae. Increased titers against several heterologous CF fimbriae with a related N-terminal sequence were also induced, whereas no increase was seen against fimbriae with an unrelated sequence. Neither antisera against the coupled peptide nor antisera against the uncoupled peptide inhibited binding of CF-expressing bacteria to the human intestinal cell line Caco-2 in spite of high titers. The difference in the inhibitory capabilities of the antipeptide sera and the MAb might be due to slightly different epitope specificities. Thus, whereas the antipeptide sera bound to several continuous epitopes in the N-terminal end, none of them reacted specifically with the epitope ^{15}IDLLQ^{19}.

Enterotoxigenic *Escherichia coli* (ETEC) is a common cause of diarrhea in children in developing countries and in travellers to these areas (4, 5). ETEC may produce different colonization factors (CFs), termed colonization factor antigens (CFAs), coli surface (CS) antigens, or putative colonization factors (PCFs), which usually are fimbriae and which are responsible for the attachment of the bacteria to the intestinal mucosa (12). The adhesion enables the bacteria to colonize the intestine and to deliver heat-labile and/or heat-stable enterotoxins, which bind to specific receptors on the enterocytes. Locally produced specific immunoglobulin A (IgA) antibodies against the CFs seem to be important for protection against ETEC disease, and an effective ETEC vaccine should probably contain protective CF epitopes as well as a toxoid and be given orally (38).

A wide variety of antigenically heterologous CFs have been described. The best-characterized one is CFA/I, which is a uniform fimbrial structure composed of one type of subunits (6, 11). Other well-characterized CFs include CS1 and CS2, which are expressed together with CS3, and CS4 and CS5, which are expressed together with CS6 (30, 37). Several additional CFs have also been characterized in the past decade, although usually in relatively low frequencies, e.g., CFA/III, PCFO159, PCFO166, CS7, CS17, and PCFO9 (17, 26, 27, 39). This antigenic heterogeneity among the CFs is a problem in the design of a broadly protective ETEC vaccine.

The ETEC fimbriae were originally shown to be antigenically distinct by testing antisera or monoclonal antibodies (MAbs) against fimbriated bacteria or purified intact fimbriae by using enzyme-linked immunosorbent assays (ELISAs) or immunodiffusion techniques (29). However, very significant amino acid sequence similarity or identity was later found in several of the ETEC fimbrial subunits; e.g., the N-terminal regions of CFA/I, CS1, CS2, CS4, PCFO166, and CS17 fimbrial subunits are very similar (13, 19, 20, 22, 32), and immunological cross-reactions have been shown between the fimbrial subunits of these CFs in immunoblotting experiments (28, 33). Furthermore, both CFA/I and CS4 have been shown to prime as well as to boost immune responses against the heterologous antigen in parenterally immunized mice (35).

In a previous study, we described MAbs that cross-react immunologically with several CFs. One of these MAbs, which was induced by immunizing mice with isolated subunits of CFA/I, reacted strongly with a peptide within the N-terminal 25-amino-acid region of CFA/I subunits, whereas the other anti-CFA/I subunit MAb did not react with this peptide. However, both MAbs inhibited hemagglutination as well as the binding of ETEC expressing different CFs to Caco-2 cells (33). In this study, by using Pepscan analysis, we have determined the continuous MAb-specific epitopes on CFA/I and compared the specificities of MAbs induced by immunization with subunits with those of MAbs raised against intact CFA/I fimbriae. In an attempt to produce antisera with an epitope specificity mimicking that of the peptide-binding MAb, we immunized rabbits with a synthetic peptide corresponding to the 25 amino acids of the N-terminal end of the CFA/I subunit and compared the binding specificities of the sera with that of the peptide-reactive MAb.

MATERIALS AND METHODS

Bacterial strains. The following *E. coli* strains were used for studies of inhibition of binding to Caco-2 cells: 258909-3 (CFA/I, O128:H?, ST/LT) (15) and the corresponding CFA/I-negative mutant, 258909-3M (25) and 62R486 (CS4, O25:H42) (41) and the CS4-negative mutant E11881D (O25:H42, ST/LT) (30). The following strains were used for purification of CFs: H10407 (CFA/I, O78:H11, ST/LT) (11), 60R936 (CS1, O139:H28) (36), 58R957 (CS2, O6:H16) (36), E11881A (CS4, CS6, O25:H42, ST/LT) (40), E7476A (PCFO166, O166:H27, ST) (27), and E17018A (CS5, CS6, O167:H5, ST) (30).

All of the strains were grown on Casamino Acids-yeast extract agar (CFA agar) (11) or, when appropriate, on CFA agar with bile salts (17) at 37°C

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overnight. Most of the strains were kindly provided by D. Evans (Houston, Tex.) or B. Rowe (London, England).

Fimbrial preparations. Different CF fimbriae were purified as described previously (11, 21). Briefly, bacteria grown on CFA agar were homogenized with a blender and centrifuged at 12,000 × *g* for 20 min. The supernatant was ammonium sulfate precipitated (at 20 and 40% saturation), and after centrifugation at 12,000 × *g* and dialysis, the pellet was further purified by chromatography in a DEAE-Sephadex column or by ultracentrifugation in a CsCl gradient (density, 1.3 g/cm^3). The content and purity of the different fimbrial preparations were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining (PhastSystem; Pharmacia, Uppsala, Sweden) as well as by immunoblotting using antisera against whole bacteria expressing the homologous or heterologous fimbriae. The concentration of the fimbriae was determined by an inhibition ELISA with a highly purified lyophilized fimbrial preparation used as a reference (25).

Antibody preparations. Four previously described MAbs were used in these studies. MAbs S-CFA/I 17:8 (144 μg of IgG1 per ml) and S-CFA/I 5:6 (150 μg of IgG1 per ml) had been produced against guanidine hydrochloride-dissociated subunits of CFA/I fimbriae and had both been shown to cross-react with several CFs; in addition, MAb S-CFA/I 17:8 reacted with a peptide corresponding to the N-terminal 25 amino acids of CFA/I fimbrial subunits (33). MAb CFA/I 1:6 (44 μg of IgG1 per ml) and MAb 65D (56 μg of IgG1 per ml) had been raised against intact purified CFA/I fimbriae (25); the latter two MAbs cross-reacted with neither any heterologous CFs nor with the N-terminal peptide (data not shown). An antiserum against purified CFA/I fimbriae was produced in rabbits by repeated immunizations with purified CFA/I fimbriae and Freund's adjuvant, followed by absorption twice with live bacteria and twice with boiled bacteria, with a CFA/I-deficient mutant of strain H10407 (11).

Epitope analysis by Pepscan. Ninety-four continuous overlapping sextamer peptides corresponding to the N-terminal (amino acids [aa] 1 to 52), central (aa 85 to 114), and C-terminal (aa 121 to 147) parts of the CFA/I subunit protein were synthesized by the Geysen pin method (14). A Multipin noncleavable peptide kit and Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids were purchased from Chiron Mimotopes (Clayton, Australia). Activation of protected amino acids was done with diisopropylcarbodiimide (Merck, Schuchardt, Germany) and 1-hydroxybenzotriazole (Chiron Mimotopes). The solvents used were reagent grade from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Positive and negative control peptides, PLAQ and GLAQ, were synthesized simultaneously with the other peptides and found to react as expected with the control MAb from Chiron Mimotopes.

The capture ELISA of Geysen et al. (14) was utilized with minor modifications. After an initial blocking step with phosphate-buffered saline (PBS)–2% bovine serum albumin (BSA)–0.1% Tween 20 for 60 min, the peptides on the pins were incubated with the MAbs (1/20) or the antisera (1/1,000) diluted in blocking solution at room temperature for 2 h. The pins were then washed four times with PBS and incubated with secondary antibody, i.e., horseradish peroxidase-conjugated goat anti-mouse IgG (1/2,500) or goat anti-rabbit IgG (1/5,000; Jackson ImmunoResearch Laboratories, West Grove, Pa.), diluted in PBS–1% sheep serum–0.1% Tween 20 at room temperature for 60 min. After washing, the substrate-chromogen solution H_2O_2-ABTS (diammonium-2,2′-azino-bis[3-ethylbenz-thiazoline-6-sulfonate]) was added and the A_{405} was measured after 25 min on a Labsystems Multiscan PLUS.

The capture ELISAs were done sequentially with the same pins in all tests after effective removal of the antibodies by sonication (10 min in PBS–1% SDS–0.1% 2-mercaptoethanol; initial temperature, 65°C), hot water rinse, and boiling methanol treatment (65°C, 30 s). The different conjugates were tested repeatedly between the analyses and showed very low reactivity with the pins.

Peptide synthesis. On the basis of CFA/I sequence data (7, 19), solid-phase peptide synthesis was performed (Syntello AB, Göteborg, Sweden) with a model 430A peptide synthesizer (Applied Biosystems, Inc., Foster City, Calif.) and t-Boc (*tert*-butoxycarbonyl)-protected amino acids to produce a peptide consisting of the 25 N-terminal amino acids. A cysteine residue was introduced into the carboxy-terminal end to facilitate coupling to the carrier protein. The cleavage of the peptide from the resin and the removal of the protecting groups from the amino acids were done by acidic hydrolysis with hydrogen fluoride, with anisole and ethanedithiol as scavengers. The expected amino acid composition of the peptide was verified by amino acid analysis using an automated analyzer (Applied Biosystems model 473A). The peptide was purified by reverse-phase high-pressure liquid chromatography on a Bondapac C-18 column (3.9 by 300 mm; Waters) eluting isocratically with 70% acetonitrile in water containing 0.1% trifluoroacetic acid at 0.2 ml/min, because of the hydrophobicity of the peptide. The peptide content in the fractions was identified by using the MAb S-CFA/I 17:8, which is specific for a linear epitope in the N-terminal end of the CFA/I subunit, in dot blot analysis (33). The preparation was lyophilized and stored at 4°C until used.

Coupling of the peptide. *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP), a heterobifunctional coupling reagent, was used as described in the manufacturer's instruction for protein conjugation (Pharmacia). Briefly, SPDP was added to BSA (Sigma) in 0.2 M sodium phosphate buffer (pH 8.5) at ninefold molar excess which resulted in a molar ratio of SPDP/BSA of 4.6:1. About 30% of the peptides had available SH groups according to the Ellman reaction (10). After adding a 2:1 molar ratio of SH groups on the peptide to 2-pyridyl disulfide on BSA, virtually all reactive groups on BSA were coupled to an SH group of the peptide, as determined by measuring the released pyridin-2-thione spectrophotometrically. The conjugate was purified from unconjugated peptide and pyridin-2-thione by gel filtration on a Sephadex G-25 prepacked PD10 column (Pharmacia). The protein contents of the fractions were monitored by measuring the A_{280}, and the presence of the CFA/I peptide on BSA was verified by dot blot analysis using the peptide-specific MAb S-CFA/I 17:8. The accessibility of the peptide epitope on the conjugate was confirmed by an ELISA in which the conjugate was used as the coating agent (10 μg of peptide per ml) and the MAb S-CFA/I 17:8 was used as the primary antibody.

Immunizations and production of specific antisera. New Zealand White rabbits (weighing 2.5 kg) from a single breeder were used. After collection of preimmune serum, the rabbits were given four subcutaneous immunizations with 500 μg of either free CFA/I peptide or of the peptide coupled to BSA at 2- to 3-week intervals; the first immunization was given with Freund's complete adjuvant, and the second was given with Freund's incomplete adjuvant. Test bleedings were done after the second and third immunizations, and the final bleeding was done by heart puncture 1 to 2 weeks after the last immunization. The sera were absorbed with a CFA/I-negative mutant as described above to remove any preexisting antibodies that might react with the ETEC fimbriae or strains.

SDS-PAGE and immunoblotting. SDS-PAGE was carried out essentially by the method described by Laemmli (23). Purified fimbrial preparations were adjusted to a concentration of 0.5 mg/ml, applied to 16% polyacrylamide gels in 20-μl volumes, and run with a mini-Protean II vertical electrophoresis cell (Bio-Rad) at 150 V for 50 min. The gels were electroblotted onto nitrocellulose sheets at 100 V for 60 min, and after blocking the sheets with 1% BSA–PBS, they were reacted with the antisera diluted 1/500 in PBS–0.1% BSA–0.05% Tween. Antigen-antibody complexes were detected by reaction with horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) and then with hydrogen peroxide substrate and 4-chloro-1-naphthol chromogen.

ELISA. Antisera were tested for reactivity with the CFA/I peptide and purified preparations of CFA/I, CS1, CS2, CS4, PCFO166, CS5, and CS7 fimbriae. Polystyrene microtiter plates (Dynatech Laboratories Ltd., Billingshurst, United Kingdom) were coated with CFA fimbriae in PBS, 1 μg/ml, at 37°C overnight. In the peptide ELISA, plates were coated with 10 μg of CFA/I peptide per ml in carbonate buffer (0.05 M, pH 9.6) with 1% dimethyl sulfoxide at 37°C overnight. After blocking with 0.1% BSA–PBS at 37°C for 30 min, antisera were serially diluted in PBS–0.1% BSA–0.05% Tween and incubated at room temperature for 60 min. The presence of bound antibody was then demonstrated by incubating the plates with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Jackson ImmunoResearch) and *o*-phenylenediamine–H_2O_2. Titers were determined as the reciprocal dilution giving an A_{450} of 0.4 above background (Labsystems Multiscan PLUS) after 10 to 20 min of enzyme reaction. All titrations were performed in duplicate.

Inhibition of adhesion to human colon carcinoma cell line Caco-2. Caco-2 cells were grown for 14 to 16 days 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. A suspension of 10^7 bacteria per ml in culture medium containing 0.5% D-mannose was mixed with an equal amount of diluted serum or MAb and incubated at room temperature for 20 min. The mixture was then added to the washed tissue culture cells and incubated at 37°C for 3 h. After five washes, the 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 adhering bacteria was determined by counting 10 randomly chosen microscopic fields with approximately 100 cells per field. Each strain was tested at least in duplicate, and all assays were performed in a blind manner.

Statistical analysis. Binding of the bacteria to the cells was expressed as the mean percentage of cells with bound bacteria ± 1 standard deviation. Results were obtained from three to six different experiments performed in duplicate. The 95% confidence intervals for the percentages of cells with adherent bacteria were calculated.

RESULTS

Pepscan analysis of antibodies against CFA/I subunits and fimbriae. MAbs raised against CFA/I subunits or against intact purified CFA/I fimbriae were examined for possible continuous epitope specificities by Pepscan analysis. To obtain the highest resolution in the determination of the continuous epitopes, hexamer peptides with an overlap of five amino acids were synthesized. Three regions of the CFA/I subunit sequence were focused on, the N-terminal (aa 1 to 52), the central (aa 85 to 114), and the C-terminal (aa 121 to 147) regions, which encompassed 94 of the 147 amino acids. The reason for focusing on the N-terminal amino acids was that this region is a relatively conserved part of the subunit protein of several of the CFs and because the MAb S-CFA/I 17:8 had

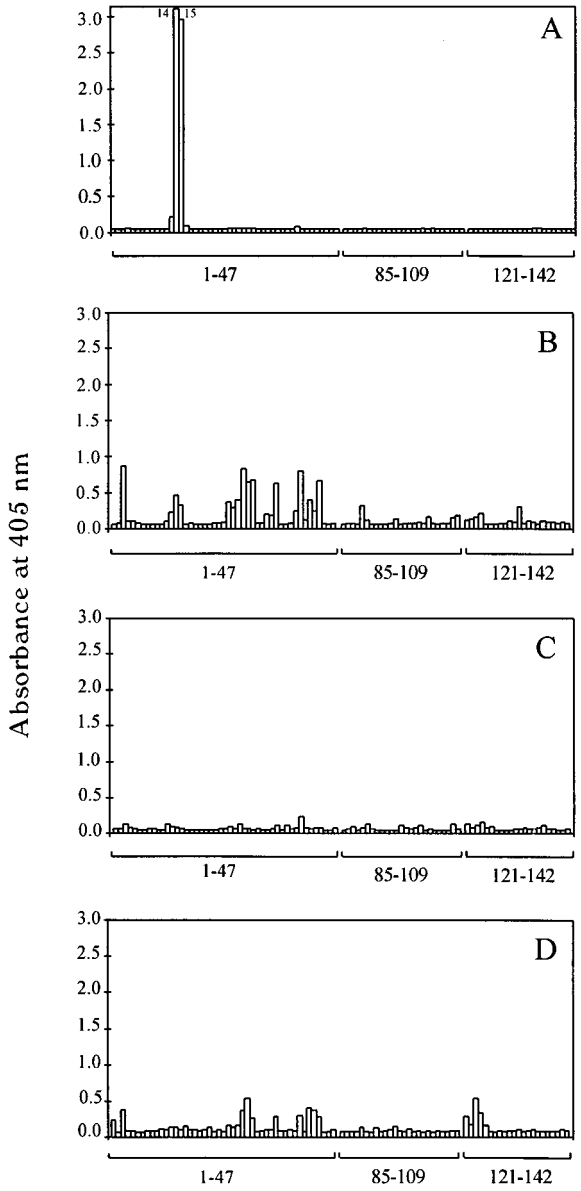

FIG. 1. ELISA results (expressed as A_{405} values) from testing different antibody preparations against 94 sextamer peptides corresponding to the N-terminal, central, and C-terminal part of the CFA/I subunit primary structure. Each peptide number refers to the first amino acid in the sequence of each sextapeptide on each pin. MAb S-CFA/I 17:8 (A) and MAb S-CFA/I 5:6 (B) were both raised against CFA/I fimbrial subunits; MAb CFA/I 1:6 (C) and rabbit hyperimmune antiserum (D) were both raised against purified CFA/I fimbriae. MAbs were diluted 1/20, and the antiserum was diluted 1/1,000.

previously been shown to bind to a CFA/I peptide consisting of the 25 N-terminal amino acids. The basis for choosing the other two regions is that a previous study of continuous epitopes in a CFA/I subunit showed that specific anti-CFA/I subunit antibodies also react with these regions (7).

The reactivities of the different MAbs with the hexamer peptides are shown in Fig. 1. The MAb S-CFA/I 17:8 bound very strongly to two of the peptides, i.e., peptides 14 and 15, having the sequence ^{15}IDLLQ^{19} in common, indicating that this epitope is continuous (Fig. 1A). We also tested the reactivity of another CFA/I subunit MAb, i.e., S-CFA/I 5:6, which also cross-reacts with several CFs (Fig. 1B). This MAb reacted

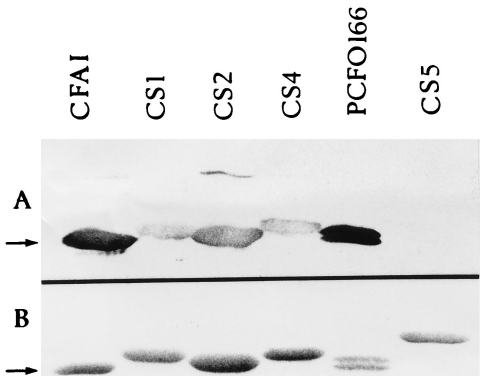

FIG. 2. Immunoblot analysis showing cross-reactivity of an antiserum against unconjugated 25-mer synthetic CFA/I peptide with different CF subunits (A) and the corresponding protein staining with Ponceau S (Sigma) (B). Ten micrograms of each fimbrial preparation was applied to the gel, and antiserum was diluted 1/500.

in a weaker fashion with several peptides in the N-terminal region. Two MAbs that had been produced against intact purified CFA/I fimbriae, i.e., MAbs 1:6 and 65D (25), did not react with any of the peptides included in the Pepscan analysis (Fig. 1C; data not shown for MAb 65D). A rabbit antiserum against intact purified CFA/I fimbriae reacted very weakly with some of the peptides (Fig. 1D).

Antisera against CFA/I peptide. To evaluate the immunogenicity of the N-terminal sequence of the CFA/I subunit, we produced a synthetic peptide consisting of the N-terminal 25 amino acids and used it in a nonconjugated form as well as covalently coupled to BSA for immunization of rabbits. When testing two different antisera raised against the nonconjugated peptide (Fig. 2) and the BSA-coupled peptide (data not shown), in immunoblotting, they both reacted in a similar fashion with subunits of the homologous CFA/I fimbriae as well as with subunits of the heterologous fimbriae CS1, CS2, CS4, and PCFO166. However, no reaction was seen with CS5 subunits, which have a completely different amino acid sequence (17). Both antisera also reacted with purified homologous as well as heterologous whole fimbriae in an ELISA (Fig. 3), although the responses against the heterologous fimbriae were considerably lower than those against CFA/I fimbriae.

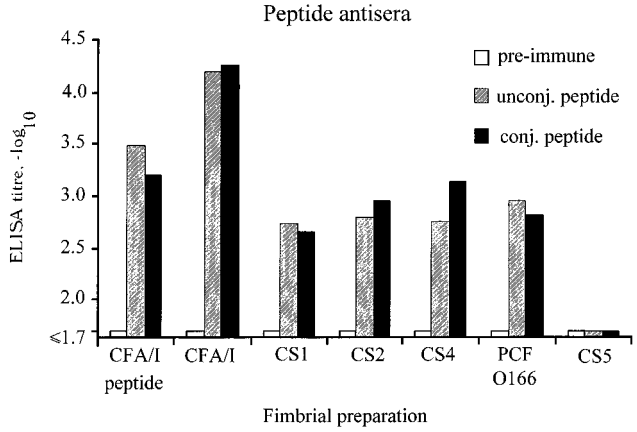

FIG. 3. ELISA titers against homologous and heterologous fimbrial preparations in antisera raised against unconjugated and BSA-conjugated N-terminal CFA/I peptides, respectively. Sera were tested in an initial dilution of 1/50.

TABLE 1. Inhibition of binding of ETEC bacteria expressing CFA/I or CS4 to Caco-2 cells by antipeptide antisera and different anti-CFA/I MAbs

Strain expressing:	Inhibitor	Mean % Of cells with adhering bacteria (±SD)	95% Confidence intervals[a]
CFA/I	Culture medium	30.8 (±7.2)	25.1–36.5
	Anti-peptide[b]	27.2 (±5.2)	20.1–34.3
	Anti-peptide conjugate[b]	35.4 (±1.2)	33.7–37.1
	Anti-CFA/I fimbriae[b]	2.3 (±1.0)	0.9–3.7
	MAb S-CFA/I 17:8[c]	5.7 (±0.9)	4.8–6.5
	MAb S-CFA/I 5:6[c]	2.2 (±1.1)	0.8–3.7
CS4	Culture medium	24.2 (±3.4)	19.5–28.9
	Anti-peptide[b]	22.7 (±1.3)	20.9–24.5
	Anti-peptide conjugate[b]	25.8 (±5.9)	17.7–34.0
	Anti-CS4 fimbriae[b]	2.7 (±1.8)	0.2–5.2
	MAb S-CFA/I 17:8[c]	8.5 (±4.8)	3.0–13.9
	MAb S-CFA/I 5:6[c]	11.3 (±1.6)	9.1–13.6

[a] Confidence intervals (95%) of percentages of cells with adhering bacteria.
[b] Rabbit antisera were tested at a final dilution of 1/20.
[c] MAbs were tested at a final concentration of 75 μg of IgG1 per ml.

Neither the unconjugated nor the conjugated peptide induced increased titers against the unrelated CS5 fimbriae. A control antiserum against an *E. coli* ST peptide conjugated to BSA by the SPDP method reacted in at least sixfold-lower titers with the fimbriae in the CFA/I group than did the antiserum against the relevant peptide conjugate.

Inhibition of binding of bacteria to Caco-2 cells. The capacity of the antipeptide sera to inhibit the binding of ETEC expressing homologous CFA/I fimbriae as well as heterologous CS4 fimbriae to Caco-2 cells was tested by comparing the percentages of cells with adhering bacteria after preincubation in antisera or culture medium only. As shown in Table 1, there was no reduction in the percentage of cells with adhering CFA/I- or CS4-expressing bacteria after preincubation with either antipeptide serum or culture medium. In contrast, preincubation in MAb S-CFA/I 17:8 or MAb S-CFA/I 5:6 has been shown to effectively inhibit the binding of CFA/I- as well as CS4-expressing bacteria to Caco-2 cells (33), and preincubation in rabbit antisera against intact CFA/I or CS4 fimbriae inhibited the binding of bacteria expressing the homologous fimbriae to Caco-2 cells. Inhibition of binding of bacteria was never seen when the different rabbit preimmune sera were tested (data not shown).

Pepscan analysis of antisera against the CFA/I peptide. To evaluate whether the different capabilities of the antipeptide sera and the MAb S-CFA/I 17:8 to inhibit binding of the bacteria to the cells was due to different epitope specificities of the various antibody preparations, we also undertook Pepscan analysis of the antisera against unconjugated and conjugated peptides, respectively. The antiserum raised against the unconjugated N-terminal 25-amino-acid peptide reacted in a weaker fashion with the peptides in Pepscan than did MAb S-CFA/I 17:8; the strongest reactivity was with peptides 13 and 14 having the shared sequence ^{14}VIDLL^{18} (Fig. 4A). In contrast, the antiserum against the BSA-coupled peptide showed the strongest reactivity with peptide 14, but it also reacted with several other peptides in the N-terminal 25-amino-acid region as well as with peptides in the region between amino acids 25 and 50 (Fig. 4B). The reactivity with peptides in the latter region suggests that these antibodies were induced by BSA or by new determinants created by the coupling procedure. None of the preimmune rabbit sera reacted with any peptides in the Pepscan analysis.

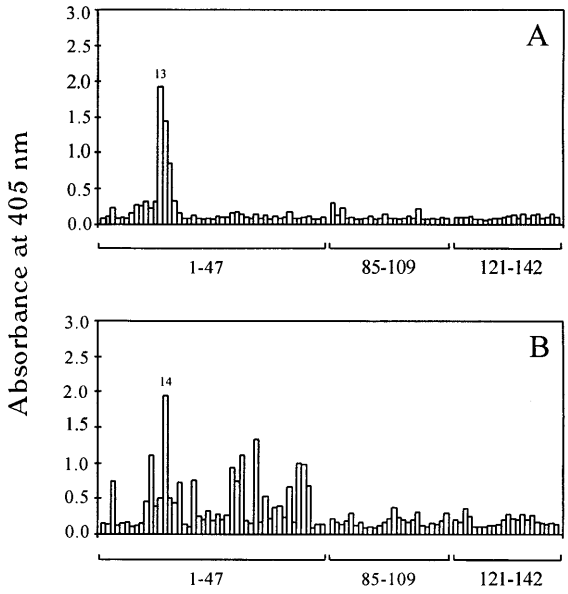

FIG. 4. ELISA results (expressed as A_{405} values) from testing rabbit antisera against 94 sextamer peptides corresponding to the N-terminal, central, and C-terminal part of the CFA/I subunit primary structure. Each peptide number refers to the first amino acid in the sequence of each sextapeptide on each pin. (A) Rabbit serum against an unconjugated 25-mer CFA/I peptide corresponding to the N-terminal part of the CFA/I fimbrial subunit; (B) rabbit serum against BSA-conjugated CFA/I peptide. The antisera were diluted 1/1,000.

DISCUSSION

The CFs are important targets for protective immunity against ETEC in humans (24). A problem in vaccine development is the existence of many antigenically different CFs. Until recently, only whole CF-positive bacteria or purified CF fimbriae have been employed to induce anti-CF immune responses. In both humans and animals, such immunization has induced protection against challenge with ETEC expressing only homologous, not heterologous, fimbriae (3, 24). However, considering that many of the CFs have a high degree of amino acid sequence similarity, particularly in the N-terminal end (Fig. 5), we have attempted to induce anti-CFA/I antibodies that cross-react immunologically with several CFs (33). By immunizing with CFA/I fimbrial subunits, we were able to

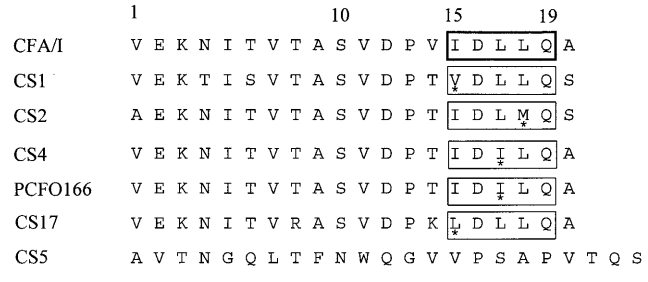

FIG. 5. Alignment of the N-terminal sequences of different CF fimbrial subunit proteins. The boxed amino acids of the CFA/I sequence show the MAb S-CFA/I 17:8-specific epitope. The boxed amino acids of the other CFs indicate the corresponding sequences in these subunit proteins. All amino acids in the boxes are identical to the CFA/I sequence except the residues indicated by an asterisk (which are all functionally identical to the CFA/I residue in the corresponding position). The sequences of the CF subunits are published in the following references: CFA/I, CS1, CS4, PCFO166, and CS17 in reference 22, CS2 in reference 13, and CS5 in reference 9.

produce several MAbs that have cross-reactive properties, i.e., they also reacted with fimbriae and subunits of CS1, CS2, CS4, and PCFO166. Indeed, two of these MAbs were also capable of inhibiting adhesion of ETEC expressing homologous and heterologous CFs to human enterocytes and of mediating passive cross-protection in rabbit ileal loops (34).

In this study, we have shown that one of the two cross-protective MAbs, S-CFA/I 17:8, bound strongly to a continuous epitope of five amino acids, namely, ^{15}IDLLQ^{19}, whereas the other MAb, S-CFA/I 5:6, did not appear to bind to a continuous epitope. The fact that MAb S-CFA/I 5:6 did react, although weakly, with some peptides suggests that the epitope is made up of short continuous sequences that are close to each other in the tertiary structure. We compared the N-terminal amino acid sequences of several heterologous CF subunits with that of the CFA/I subunit (Fig. 5). The subunits of CS1, CS2, CS4, PCFO166, and CS17 have four identical amino acids and one functionally identical amino acid residue in the region corresponding to the MAb S-CFA/I 17:8-reactive epitope. The high degree of sequence homology between CFA/I, CS1, CS2, CS4, and PCFO166 in this region probably explains our previous results, which showed inhibition of adhesion of homologous as well as heterologous strains, with the MAb S-CFA/I 17:8. Surprisingly, however, we have not previously found any reactivity of the MAb S-CFA/I 17:8 with CS17 fimbriae despite the homology of this subunit in the sequence corresponding to the continuous epitope. This might be explained by the functional difference of the amino acid lysine, located adjacent to the N-terminal side of the ^{15}LDLLQ^{19} sequence in CS17, compared with valine or threonine, which are present in a corresponding position in the other CFA/I-like fimbriae. In contrast to the similarities in sequences within the CFA/I-like group discussed above, the sequence of the N terminus of CS5 subunits shows no homology. When the MAb S-CFA/I 17:8 was tested by immunoblot and ELISA, it reacted neither with the CS5 fimbrial subunits nor with the intact CS5 fimbriae.

Using MAbs raised against CFA/I subunits, Bühler et al. (6) have previously shown that the receptor-binding site of CFA/I is located on each subunit but only exposed at the tip of the fimbriae. Although the anti-CFA/I subunit MAbs produced in that study reacted less well with intact fimbriae than MAbs against intact CFA/I fimbriae, the anti-subunit MAbs were shown to be much more effective in inhibiting hemagglutination. The characteristics of our MAb S-CFA/I 17:8 are similar to those of the anti-CFA/I subunit MAbs of Bühler et al. Thus, MAb S-CFA/I 17:8 was the most effective one among the different cross-reactive MAbs in inhibiting hemagglutination, although it reacted less well with intact fimbriae than the other two MAbs (33). Thus, it seems likely that the anti-CFA/I subunit MAbs produced by Bühler et al. also bind to a continuous epitope in the N-terminal region. This is supported by results from Cassels et al. (7) showing that the immunodominant continuous epitopes of the CFA/I subunit are located in the N-terminal end of the protein. We have, however, no direct evidence that the sequence ^{15}IDLLQ^{19} is part of the receptor-binding domain, since the effect of antibodies specific for an epitope located close to the receptor-binding site of the subunits could be to sterically hinder the binding of the fimbriae to the cells. To obtain evidence of the role of the sequence ^{15}IDLLQ^{19} in binding, inhibition experiments should be performed with, as inhibitors, short soluble peptides that contain the sequence.

Our findings that neither an antiserum nor two different MAbs produced against intact CFA/I fimbriae (MAbs CFA/I 1:6 and 65D) reacted significantly with any peptides, including the N-terminal region of CFA/I, indicate that this region is immunorecessive on intact fimbriae. This could at least partly explain the lack of cross-protective immunity against CFs induced when using bacteria or purified intact fimbriae as immunogens. However, antibodies to the N-terminal region seem to be protective since they are capable of blocking CF-mediated binding to the epithelial cells (34). In analogy with our results, Cassels et al. (7) did not observe any reactions with any of their 140 octamer peptides spanning the whole sequence of the CFA/I subunit, following immunization with intact CFA/I fimbriae.

In contrast to CFA/I fimbriae, in which there is only one subunit protein, the adhesins of uropathogenic S and P fimbriae and of the ubiquitious type 1 fimbriae are specialized proteins located at the tip of the fimbrial shaft (16, 18, 31). It has been shown that MAbs raised against the specific adhesin proteins inhibit binding of these bacteria much more effectively than MAbs against the fimbrial subunit proteins; however, very low amounts of specific antibodies are produced against the adhesins after immunization with intact fimbriae. Thus, it seems as if the parts of the fimbriae that are important for binding are immunorecessive by being exposed only at the tips of intact fimbriae.

To produce specific antibodies against cross-reactive peptides in CF subunits, we immunized rabbits with a synthetic peptide consisting of the N-terminal 25 amino acids of the CFA/I subunit. The reason for choosing this peptide was that it contains the sequence ^{15}IDLLQ^{19} and that it has previously been shown to bind MAb S-CFA/I 17:8. We chose to immunize with the peptide that was not only covalently linked to a carrier protein, i.e., BSA, but also uncoupled, since it has been shown that relatively short peptides can be immunogenic. For example, Chong et al. (8) demonstrated that the majority of thirteen 24- to 37-mer peptides corresponding to the sequence of the outer membrane protein P1 of *Haemophilus influenzae* were equally immunogenic, or even more so, if injected in the unconjugated than in the conjugated form. Our results confirm that an unconjugated peptide can be as efficient as a conjugated peptide in eliciting antipeptide and antifimbrial immune responses. Explanations for this could be that the relatively long peptide used was immunogenic in itself, e.g., that it contains a T-cell epitope, or that the peptide had polymerized as a result of its relatively high content of hydrophobic amino acids (1).

Surprisingly, although the peptide sera had high titers of antibodies against intact fimbriae as well as against the 25-mer peptide, they did not block adhesion. The explanation for this lack of inhibitory capacity of the different sera may be that none of them reacted specifically with the B-cell epitope ^{15}IDLLQ^{19}. This probably reflects the difficulty in mimicking the natural conformation of a continuous epitope by immunizing with a peptide containing the sequence. However, a 25-mer peptide corresponding to the adhesin FimH of type 1 fimbriae was shown to inhibit the binding of type 1-expressing strains of *E. coli* to human buccal epithelial cells, in spite of having a low titer against intact fimbriae (2). In future studies, we will try to design different peptides that express the epitope ^{15}IDLLQ^{19} more efficiently, for example, by producing multimers of a shorter CFA/I peptide, to evaluate the possibility of inducing specific immune responses against this cross-protective epitope.

ACKNOWLEDGMENTS

This work was supported by the Swedish Medical Research Council (grant 16-X09085); the Faculty of Medicine, Göteborg University; the Swedish Agency for Research Cooperation with Developing Countries; and the Göteborg Medical Society.

We gratefully acknowledge Marianne Lindblad for giving instructions and advice on peptide coupling and Moyra M. McConnell for critically reading the manuscript.

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Editor: J. R. McGhee