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Analysis of Colonization Factor Antigen I, an Adhesin of Enterotoxigenic *Escherichia coli* O78:H11: Fimbrial Morphology and Location of the Receptor-Binding Site

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Colonization factor antigen I (CFA/I) of enterotoxigenic *Escherichia coli* was dissociated into one type of subunit (15 kDa). The dissociation was achieved either by heating CFA/I in sodium dodecyl sulfate at 100°C or by heating it for 20 min in water. Heating in water to 100°C yielded only in the 15-kDa subunit, but heating to 85°C yielded small amounts of oligomers in addition. The monomeric subunits obtained after heating in water are stable, as demonstrated by gel permeation chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis without heating prior to the electrophoretic run. These subunits inhibited CFA/I-induced hemagglutination, indicating that they had maintained their receptor-binding properties. When the hybridoma technique was used, two types of monoclonal anti-CFA/I antibodies were obtained. Antibodies obtained by immunization with the purified subunits were more reactive with subunits than with fimbriae, as shown by enzyme-linked immunosorbent assay. These antibodies strongly inhibited CFA/I-induced hemagglutination. When examined by immunoelectron microscopy, these antibodies seemed to label the fimbrial tips. A similar labeling pattern was obtained with gold particles modified with the receptor ganglioside GM2. Antibodies obtained by immunization with fimbriae reacted in enzyme-linked immunosorbent assays equally well with fimbriae and subunits. They inhibited CFA/I-induced hemagglutination only slightly. Immunoelectron microscopy revealed that these antibodies labeled the fimbriae densely and regularly over their entire lengths. In a coagglutination experiment with *Staphylococcus aureus* and monoclonal antibodies, the subunits retained their receptor-binding properties. From these results, we conclude that CFA/I fimbriae consist entirely of one type of adhesive subunit, of which only the one at the tip is accessible to the receptor.

The adherence of pathogenic bacteria to epithelial surfaces is an important early event in the interaction of these bacteria with the host that leads to multiplication in situ and infection (27, 29). Adhesion is mediated by carbohydrate-specific bacterial recognition proteins (adhesins, hemagglutinins, lectins) which may or may not be associated with fimbriae (16). It was demonstrated that the mannose-specific type 1 fimbriae (13, 26), the Gal-Gal-specific P fimbriae (15, 23, 25), and the sialic acid-specific S fimbriae (12, 28) each consist of a supporting rodlike structure made up of one type of (major) subunit to whose tip several minor subunits are attached. One of these minor subunits is the adhesin. There is genetic evidence that the porcine-pathogenic K88 fibrillae lack the corresponding major subunit and thus seem to consist predominantly of adhesive subunits (2).

Enterotoxigenic *Escherichia coli* strains exhibit fimbrial adhesins, called colonization factor I (CFA/I) (4), which, together with the enterotoxin, are encoded on a 60-MDa plasmid (8). Both recognize sialic acid-containing gangliosides: the enterotoxin interacts with GM1, and CFA/I interacts with GM2 (9). CFA/I is made up of 15-kDa subunits (6, 17), and no distinct subunits to which the adhesive properties could be attributed have been found. We therefore wanted to investigate whether CFA/I contains a tip-located distinct adhesin, as found in the type 1, P, and S fimbriae (12, 13, 15, 28, 31), or whether it consists of adhesive subunits, as was suggested for the K88 fimbriae (2). In this communication, we describe the dissociation of CFA/I, the characterization of anti-CFA/I monoclonal antibodies (MAbs), and the use of these antibodies in functional analysis and immunogold electron microscopy.

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MATERIALS AND METHODS

Bacteria. *E. coli* 21569 (O78:H11) was grown on CFA agar (7). This strain, which exhibits CFA/I, the thermolabile enterotoxin LT, and the thermostable enterotoxin ST (8), has been described as *E. coli* H10407 (4). Prior to cultivation, the bacteria were selected for their ability to agglutinate human erythrocytes (RBC) and their failure to agglutinate *Saccharomayces cerevisiae* cells.

Preparation of fimbriae. After growth on CFA agar (18 h, 37°C), the bacteria were harvested in hal22f-concentrated phosphate-buffered saline (PBS), and this suspension (0.5 g of bacteria per ml) was agitated six times for 5 min each time with an Omnimixer (Sorvall; setting 4) with cooling in an ice bath. The bacteria were removed after centrifugation (8,000 × *g*, 30 min) as a pellet, and the supernatant was dialyzed against 20 mM Tris HCl, pH 8. The fimbriae were precipitated from the dialyzed solution with ammonium sulfate (20% saturation at pH 8 and 4°C). The precipitated fimbriae were collected by centrifugation (10,000 × *g*, 30 min), and the pellet was suspended in and dialyzed against deionized water. Insoluble material was removed by centrifugation (40,000 × *g*, 1 h), and the fimbriae were precipitated from the supernatant with ammonium sulfate (20% saturation at pH 8 and 4°C) and collected by centrifugation as described above. To remove contaminating lipopolysaccharide, the pellet was suspended in 20 mM Tris HCl (pH 8), and this solution (containing 10 mg of protein) was applied to a column of Q-Sepharose (fast flow, 5 ml; Pharmacia). After the column

was washed with 20 mM Tris HCl, pH 8 (25 ml), the fimbriae were eluted with 20 mM Tris HCl (pH 8) containing 0.5 mM NaCl.

Dissociation of CFA/I into subunits. The fimbriae were dissociated into their subunits by heating a suspension in deionized water at different temperatures. After cooling, the solution was subjected to gel filtration on Ultro-Pac TSK G-3000 SWG (21.5 by 600 mm; LKB, Bromma, Sweden) with 50 mM sodium phosphate buffer (pH 7.5) as eluant. The fraction (K_m = 0.7) containing protein was subjected to chromatography on MonoQ HR 5/5 (Pharmacia) with 20 mM Tris HCl (pH 8) (0 to 500 mM LiCl in the same buffer). Subunits of CFA/I were eluted at 70 mM LiCl, whereas intact CFA/I was eluted at 90 mM LiCl. The stability of the subunits in water was checked by chromatography on TSK SW2000 with Tris HCl (20 mM, pH 7.0) as eluant at a flow rate of 0.25 ml/min.

Biotinylation of MAbs. The antibodies used in this reaction were purified by cation-exchange chromatography and dialyzed against sodium carbonate buffer (0.1 M, pH 8.2), and the solution was clarified by centrifugation (10,000 × g, 20 min). To a portion of the solution (1 mg of protein in 1 ml), 50 μl of a freshly prepared solution of 1.63 mg of Biotin-X-NHS (Calbiochem) in 1 ml of dimethyl sulfoxide was added at 37°C. After 1 h, glycine (500 mM) and ethanolamine (50 mM) were added, and the mixture was kept at room temperature for 2 h and then chromatographed on a column of PD10 with Tris buffer (50 mM; containing 3 M NaCl, pH 8.1). The biotinylated antibodies were kept in this buffer at 7°C.

MAbs. Female BALB/c mice (6 weeks old) were immunized subcutaneously with a mixture of 0.1 ml of 20 μg of protein in PBS, 0.1 ml of Alu-Gel S (Serva), and 0.1 ml of complete Freund adjuvant. On day 8, the injection was repeated with incomplete Freund adjuvant. After 15, 41, 42, and 43 days, injections with the same amount of protein lacking Freund adjuvant were given, and 2 days later, the mice were killed and spleen lymphocytes (10^8) were fused with PAI-0 myeloma cells (2×10^7) (33) by using polyethylene glycol 1500 (18). Myeloma cells were selected and screened by enzyme-linked immunosorbent assay (ELISA) as previously described (35). Complete CFA/I fimbriae as well as subunits were used as immunogens and as antigens in the ELISAs. Positive hybridoma cells were subcloned by limiting dilution, and cells producing anti-CFA/I antibodies were frozen in fetal calf serum containing 10% (by volume) dimethyl sulfoxide. For the production of MAbs, hybridomas were grown in Dulbecco modified Eagle's medium (GIBCO, Karlsruhe, Germany) with 10% CLEX (Pansystems, Aidenbach, Germany), and MAbs were precipitated from the supernatants with ammonium sulfate (50% saturation). Two independent immunizations were performed with (i) complete CFA/I fimbriae and (ii) subunits as obtained by heat dissociation and chromatographic purification.

ELISA and inhibition. ELISAs of MAbs with purified fimbriae (50 μg/ml) as antigens were performed in flexible multititer plates with peroxidase-conjugated goat anti-mouse immunoglobulin (Medac, Hamburg, Germany) as second antibody and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma, Deisenhofen, Germany) as substrate. After 1 h of incubation, extinction at 405 nm was read. Coating of the microtiter wells was performed in 50 mM carbonate buffer (pH 9.6) at 4°C. All washing steps were done with PBS containing 0.05% Tween 20. In inhibition experiments, one of the gangliosides GM1 and GM2 (100 μg/ml of PBS each) was added to the fimbria-coated microtiter wells prior to the addition of MAbs.

Hemagglutination and inhibition. Hemagglutinations were performed with dilutions of CFA/I fimbriae in PBS (50 μl), a suspension (5%) of human RBC in PBS (15 μl), and PBS (50 μl) in polystyrene round-bottom microtiter plates at pH 6.0. For inhibitions, solutions of the putative inhibitor (in 50 μl of PBS) and CFA/I fimbriae (in 50 μl of PBS) were incubated for 10 min at 21°C. Then, a suspension of RBC (5%, 15 μl) was added. The results were read after 90 min on ice.

Coagglutination of subunit-modified *Staphylococcus aureus* and RBC. To a suspension of formaldehyde-fixed *S. aureus* (0.5 ml; Pansorbin; Calbiochem, La Jolla, Calif.), 10 mM Tris HCl buffer (1 ml, 10 mM, pH 8) was added, and the suspension was centrifuged (12,000 × g, 2 min). The sediment was suspended in 1 ml of the same buffer, and a solution (200 μl) of a MAb which did not inhibit CFA/I-induced hemagglutination (200 times concentrated from the tissue culture supernatant) was added. After 2 h at room temperature, the cells with the adsorbed antibody were centrifuged and washed three times with 10 mM Tris HCl, pH 8. CFA/I subunits (10 mg/ml; separated by gel filtration from the fimbriae) were added. After 2 h at room temperature, antibody-modified *S. aureus* cells were washed five times. The final suspension (20 μl) was mixed with a 50% RBC suspension (10 μl) and PBS (pH 7.4, 10 μl) on an ice-cooled microscopic slide, and agglutination was detected microscopically. CFA/I fimbriae and their subunits were used in this test.

Preparation of receptor-gold conjugates. Gold colloid (15-nm particle diameter) was prepared by the reduction of gold chloride with sodium citrate (14), and the particle size was checked with the electron microscope. The gold particles were coated with receptor ganglioside GM2 (Pallmann KG, Munich, Germany) in distilled water. The modified gold particles were stabilized with 1% polyethylene glycol 20000 and 1% bovine serum albumin (BSA).

Electron microscopy and immunoelectron microscopy. Formvar-coated nickel grids were incubated with a bacterial suspension (10^9 cells per ml of PBS) or with a fimbrial suspension (1 mg/ml of PBS). For electron microscopic inspection, the grids were stained with phosphotungstic acid (1%, pH 7.0). Prior to immunoelectron microscopy, the grids were incubated with a solution of skimmed-milk powder (0.25%) and BSA (0.1%) in PBS. After incubations with receptor-coated gold particles suspended in the same milk powder-BSA solution for 45 min, grids were washed with water and stained with phosphotungstic acid (1%, pH 7). To study antibody binding, the grids were incubated with hemagglutination-inhibiting and noninhibiting antibodies (concentrated from hybridoma supernatants) and then with gold particles (15 nm) modified with antimouse antibodies (Janssen, Olen, Belgium). The grids were then washed with water and stained with phosphotungstic acid (1%). Electron microscopy of dried grids was performed with a Phillips EM T/ST-400 microscope at 80 kV.

Chromatofocusing. Solutions of CFA/I fimbriae or their subunits were applied to a MonoP HR 5/20 column (Pharmacia, Freiburg, Germany) in equilibration buffer (20 mM Tris HCl, pH 8). The proteins were eluted with elution buffer PB74 (Pharmacia; adjusted to pH 4; 10% in water) and monitored at 275 nm. The pH was determined with a pH electrode (Ingold, Urdorf, Switzerland).

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with appropriate molecular weight markers was performed according to Laemmli (22). For immunoblot analysis, samples were transferred after the electrophoretic run to Immo-

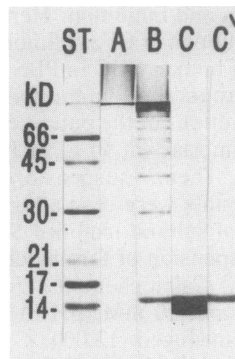

FIG. 1. SDS-PAGE analysis of CFA/I after disaggregation. After heating, samples were cooled to room temperature before the sample buffer was added. Lanes: ST, molecular weight references; A, undissociated fimbriae; B, oligomeric CFA/I obtained by heating fimbriae in water for 20 min at 85°C; C and C', single subunits obtained by heating fimbriae in water for 20 min at 100°C. In lanes A, B, and C, 20 μg of protein was applied, and in lane C', 2 μg of protein was applied. The separation was on a 13% gel.

bilon-P sheets (Millipore, Bedford, Mass.) by using the following transblot buffer system: anode buffer I, 0.3 M Tris HCl, pH 9; anode buffer II, 0.025 M Tris HCl, pH 9.4; cathode buffer, 0.025 M Tris HCl containing ε-aminocaproic acid (0.04 M) and SDS (0.1%), pH 8.4. After the transfer, the remaining active sites on the Immobilon-P sheets were blocked with 0.5% BSA in PBS (pH 7.4) for 1 h at 37°C, washed three times with PBS, and incubated with MAbs diluted in the blocking solution. Bound antibodies were detected with an antimouse immunoglobulin-alkaline phosphatase complex (Medac), and a mixture of 35 μl of Nitro Blue Tetrazolium in 70% dimethyl formamide (50 mg/ml) and 17 μl of bromo-chloro-indolylphosphate in 100% dimethyl formamide (50 mg/ml), all in diethanolamine buffer (pH 9.8), as described previously (20).

Protein determinations. The concentrations of proteins were determined with the bicinchoninic acid reagent (32).

RESULTS

Characterization of CFA/I and its subunit. The hemagglutination of human and bovine RBC with purified CFA/I fimbriae proved to be pH dependent. Although no agglutination was observed at pH 7.4, agglutination of RBC was distinct at pH 6.0. The same pH dependence of hemagglutination capacity has been described for isolated P and S fimbriae (15, 16, 28). As with the fimbriated bacteria, agglutination with isolated CFA/I was much more pronounced at 4°C than at room temperature.

In SDS-PAGE, CFA/I showed one subunit with a molecular weight of 15 kDa, as had been demonstrated before (17). Staining with the silver reagent (1) did not reveal additional bands. The fimbriae had an isoelectric point of 5.0 and was eluted from a MonoQ column with 90 mM LiCl in 20 mM Tris HCl at pH 8. The fimbriae could be dissociated by heating in deionized water. After dissociation, the samples were cooled to room temperature and subjected to SDS-PAGE without prior heating in dissociation buffer. As shown in Fig. 1, heating in water at 85°C for 20 min resulted in a mixture of single subunits and oligomers of the subunit. After heating in water at 100°C for 20 min, only single subunits were detected, as was true when the gels were stained with the silver reagent (1). SDS-PAGE of fimbriae without prior heating showed that no dissociation occurred during the electrophoretic run. The subunits obtained by heating in water remained stable, as shown by high-pressure liquid chromatography on MonoQ and by gel permeation chromatography on TSK SW2000. No protein larger than 15,000 Da could be observed in the eluate. Fractions of the material applied to the column from the peak materials gave the same band of 15 kDa in SDS-PAGE when run without prior heating in SDS. The free subunits had an isoelectric point of 5.3 and were eluted from MonoQ with 70 mM LiCl in 20 mM Tris HCl at pH 8.

TABLE 1. Characterization of MAbs against CFA/I

MAb	Immunoglobulin G subclass	Reciprocal titer by:		
		ELISA with:		Inhibition of hemagglutination[c]
		Fimbriae	Subunits	
CFA/I-1[a]	2a	5,120	40,960	218,700
CFA/I-2[a]	1	5,120	81,920	218,700
CFA/I-3[b]	1	10,240	5,120	24,300
CFA/I-4[b]	2b	10,240	2,560	8,100
CFA/I-5[b]	1	5,120	1,280	2,700
CFA/I-6[b]	2a	10,240	1,280	8,100

[a] Obtained by immunization with purified subunits.
[b] Obtained by immunization with purified fimbriae.
[c] Purified fimbriae and human RBC were used at pH 6 at 4°C.

Anti-CFA/I MAbs. For the production of MAbs against CFA/I, complete fimbriae and isolated subunits were used. As shown in Table 1, the antibodies obtained with the isolated subunits as immunogen (MAbs CFA/I-1 and CFA/I-2) reacted preferentially with the subunits in the ELISA, and the antibodies obtained with complete fimbriae as immunogen (MAbs CFA/I-3 to CFA/I-6) reacted with complete fimbriae and subunits. Results of the inhibition of CFA/I-induced hemagglutination are also included in Table 1. If the ELISA titer of a given antibody obtained with complete fimbriae is compared with its titer by CFA/I-induced hemagglutination (a reaction in which the complete fimbriae are used) it is apparent that MAbs CFA/I-1 and CFA/I-2 are better inhibitors by a factor of about 40.

To show whether the antibodies which strongly inhibit hemagglutination do so by binding at the receptor recognition site of CFA/I, we performed an ELISA in the presence of the receptor ganglioside GM2. This distinctly inhibited the reactivity of the MAbs with CFA/I (Fig. 2). The epitope specificities of the MAbs were not analyzed in detail. Competitive ELISAs with two MAbs in each test (10) resulted in addition indices of 30 for MAbs CFA/I-1 and CFA/I-2 and of 60 to 110 for all other combinations. Since control runs in which the same antibody was used twice gave an addition index of 13 and competition for the same epitope was reported to result in addition indices of 0 to 20 (10), the MAbs are probably not directed against the same epitope. To compare MAbs CFA/I-1 and CFA/I-2 further, both were biotinylated and used in an ELISA-type reaction with avidin-peroxidase and CFA/I as antigen. When homologous antibodies were used as inhibitors, the reciprocal inhibition titers were 32,000. MAb CFA/I-1 inhibited biotinylated MAb CFA/I-2 at a reciprocal titer of 16,000, and the same value was obtained with MAb CFA/I-1 as an inhibitor of biotinylated MAb CFA/I-2. This indicated that MAbs CFA/I-1 and CFA/I-2 probably react with identical or adjacent epitopes.

The MAbs were subjected to Western blot (immunoblot) analysis. Figure 3 shows that MAbs CFA/I-1 and CFA/I-2

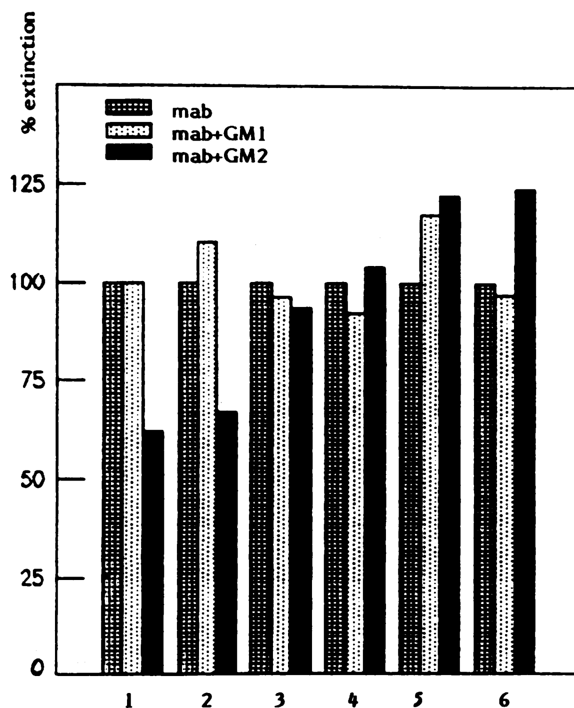

FIG. 2. ELISA of MAbs against CFA/I with CFA/I as antigen in the presence of GM1 or GM2. The wells were coated with suspensions (100 μg/ml) of CFA/I. After incubation of the wells with GM1 or GM2 (50 μg/ml each), the MAbs were added. Antibody designations and their dilutions (reciprocal titers): 1, CFA/I-1 (102,400); 2, CFA/I-2 (102,400); 3, CFA/I-3 (8,000); 4, CFA/I-4 (4,000); 5, CFA/I-5 (1,000); 6, CFA/I-6 (4,000).

reacted strongly with the monomeric subunit, whereas the other antibodies showed only weak reactivity with the same concentration of the subunit.

Coagglutionation of subunit-modified *S. aureus* and RBC. We wanted to learn whether the isolated and stable CFA/I subunit has receptor-binding properties. Therefore, *S. aureus* cells (protein A expressing) were modified with one of the weakly inhibitory MAbs (MAb CFA/I-6). These cells were then incubated with the purified subunit of CFA/I and, after being thoroughly washed, added to a suspension of human RBC. Microscopic inspection of the resulting suspension indicated that the RBC were agglutinated with the subunit-coated *S. aureus* cells. As a control, *S. aureus* cells to which the CFA/I subunit was attached with a strongly inhibitory MAb (MAb CFA/I-1) were used. In this case, no agglutination was observed.

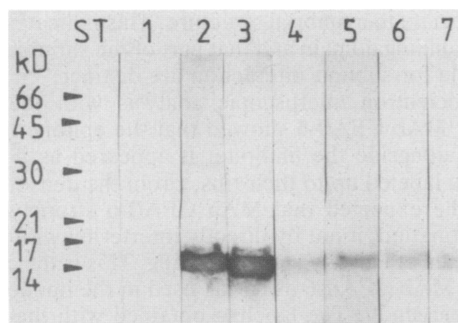

FIG. 3. Immunoblot analysis of MAbs on CFA/I fimbriae which had been dissociated by heating for 20 min at 100°C. Lanes: ST, molecular weight references; 1, blank; 2, MAb CFA/I-1; 3, MAb CFA/I-2; 4, MAb CFA/I-3; 5, MAb CFA/I-4; 6, MAb CFA/I-5; 7, MAb CFA/I-6.

To further demonstrate the receptor-binding capacity of the subunits, we attempted to inhibit the hemagglutination induced by fimbriated bacteria or isolated fimbriae. In this test, RBC (1.5% in 30 μl of PBS) were pretreated with the purified subunits (100 μg/ml, 30 μl) prior to the addition of bacteria or fimbriae. No hemagglutination could be observed under these conditions.

Immunoelectron microscopy. The fimbriated bacteria were incubated with a strongly inhibitory and a weakly inhibitory MAb and then labeled with a second (anti-mouse immunoglobulin) antibody which was attached to gold particles. As shown in Fig. 4, the weaker inhibitory antibody MAb CFA/I-6 bound densely and regularly alongside the fimbriae, and the strongly inhibitory antibody MAb CFA/I-2 labeled far fewer sites on the fimbriae. If these correspond to a tip-located receptor-binding domain, this labeling pattern should also be achieved with gold particles which were modified with the receptor ganglioside GM2. Figure 5 shows that the receptor seems to bind to the fimbriae in the same fashion as MAb CFA/I-2. No labeling with gold particles modified with GM1, which is not a receptor for CFA/I, was found (not shown).

DISCUSSION

CFA/I of enterotoxigenic bacteria has been studied extensively with respect to chemistry, immunology, epidemiology, and receptor specificity (5–8, 17, 24, 30, 34, 36). However, no information on the functional architecture of these fimbriae was hitherto available. The present study approaches this problem with biochemical as well as serological and immunoelectron microscopic techniqes with the aid of MAbs raised against complete fimbriae and their subunits.

The fimbriated bacteria agglutinated human RBC, but this was not the case with isolated fimbriae when the test was performed at pH 7.4. At pH 6, however, the fimbriae readily agglutinated RBC. The same observation has been previously reported with P and S fimbriae and was attributed to a change of the state of fimbriae in suspension (15, 16, 28). At pH 7.4, the suspension contains free and monovalent fimbriae, whereas at pH 6, the fimbriae form aggregates which are polyvalent and thus agglutinating. In support of this interpretation, suspensions of CFA/I fimbriae became increasingly turbid with lowering of the pH. These results indicated that CFA/I fimbriae are monovalent and that the adhesin is probably at their tips.

Since the P and S adhesins could be released from the respective fimbriae in an active form by heating the fimbriae in detergent (15, 16), we attempted to obtain a CFA/I adhesin in the same way. When the CFA/I fimbriae were heated, even in the absence of detergents, the fimbriae dissociated completely. Subsequent SDS-PAGE revealed only one type of subunit. This is in contrast to the findings with other fimbriae, which all revealed several subunits on SDS gels (13, 15, 16). This finding suggested that, in contrast to P, S, and type 1 fimbriae, the CFA/I fimbriae were made up of only one type of subunit. The CFA/I subunits as obtained by dissociation of their fimbriae remained in the monomeric form, as demonstrated by gel permeation chromatography and SDS-PAGE.

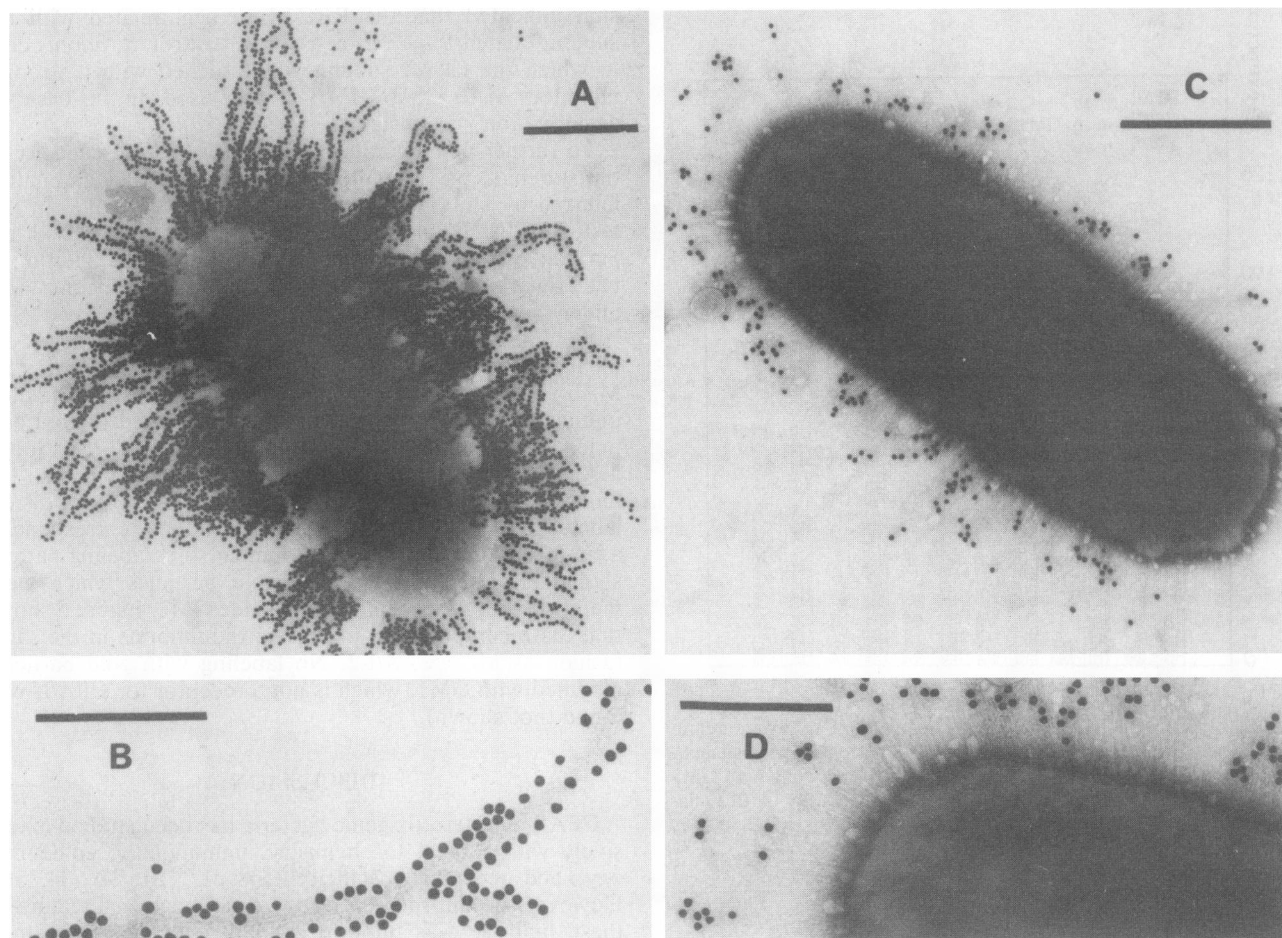

FIG. 4. Immunoelectron microscopy of *E. coli* H10407 (O78:H1:CFA/I) with antifimbrial MAbs. (A) Bacteria labeled with MAb CFA/I-6 (magnification, ×30,800; bar, 0.5 μm); (B) isolated fimbriae labeled with MAb CFA/I-6 (magnification, ×91,000; bar, 0.25 μm); (C) bacteria labeled with MAb CFA/I-2 (magnification, ×39,200; bar, 0.5 μm); (D) magnification of panel C (×79,400; bar, 0.25 nm).

The fact that the subunits did not reassociate could be taken as an indication of their (at least partial) denaturation. We have found in an independent study of nonfimbrial adhesins (14a) that reassociation seems to depend on the presence of a disulfide bridge in the subunit. It is noteworthy that the CFA/I subunit does not contain cysteine (17), which may result in a low probability of the monomeric subunit attaining the conformation necessary for reassociation.

MAbs prepared in BALB/c mice with purified subunits (MAbs CFA/I-1 and CFA/I-2) reacted in ELISA preferentially with the subunits and less well with complete fimbriae. They strongly inhibited CFA/I-induced hemagglutination. MAbs prepared against complete fimbriae (MAbs CFA/I-3 to CFA/I-6), which reacted in ELISA with the complete fimbriae and with the subunit to a comparable extent, inhibited CFA/I-induced hemagglutination only slightly. From the results of the inhibition studies, it appears that the subunit is a good candidate for the production of antiadhesive and protective antibodies. Competitive ELISA using pairs of the MAbs indicated that the antibodies did not recognize the same epitope in the subunits and that the two antibodies which were induced by and reacted with the isolated subunit (MAbs CFA/I-1 and CFA/I-2) reacted with epitopes which are either close to each other or identical.

The adhesive function of the subunit was tested in a coagglutination experiment in which the subunit was bound by MAbs to protein A on the surface of *S. aureus* cells. When MAb CFA/I-2 was used, the receptor-binding domain was accessible and the complex obtained agglutinated RBC. In contrast, when MAb CFA/I-6 was used, the recognition site of the subunit was blocked and the complex obtained did not agglutinate RBC. It is interesting to note that the subunit exhibited the receptor-binding property even though it did not reassociate to a fimbrial structure. This indicates that the receptor-binding domain and that part of the subunit which is responsible for subunit interaction are distinct.

Immunoelectron microscopic analysis with the weakly inhibitory MAb CFA/I-6 showed that the epitope is spaced regularly alongside the fimbriae. It appeared as if the fimbriae were labeled up to their tips. From the dense labeling, it would be expected that MAb CFA/I-6 strongly inhibits hemagglutination, if not by directly interfering with receptor binding, then at least by steric blocking. This is the case with undiluted MAb CFA/I-6 as it was used in the imunoelectron microscopic study. The labeling obtained with the strongly inhibitory MAb CFA/I-2 was less distinct, although it was obvious that far fewer sites on the fimbriae were labeled without any apparent regularity. This pattern is in agreement with our assumptions that the CFA/I fimbriae expose the receptor-binding site at their tips and that those sites are

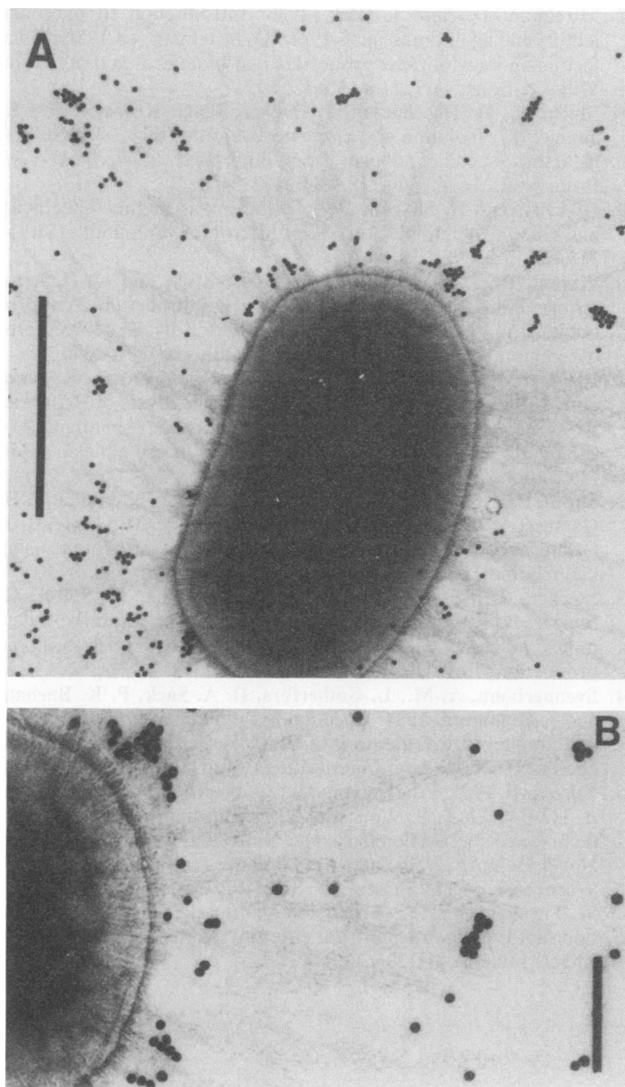

FIG. 5. Electron microscopy of fimbriated bacteria with receptor-gold. (A) *E. coli* H10407 (O78:H11:CFA/I) labeled with ganglioside GM2-gold particles (magnification, ×39,200; bar, 0.5 μm); (B) magnification of panel A (×78,400; bar 0.2 μm).

recognized by MAb CFA/I-2. The labeling obtained with gold particles modified with receptor ganglioside GM2 is much the same as that obtained with MAb CFA/I-2. A similar pattern of labeling found with S-specific fimbriae was interpreted as indicative of a tip-located S adhesin (28).

The biochemical, immunological, and electron microscopic data taken together indicate that the CFA/I fimbriae have an architecture which differs from that reported for the P, S, and type 1 fimbriae (12). In the last three, the adhesin is a distinct minor subunit which, together with other minor subunits, forms a tip-located functional complex (13, 16, 23, 28). When this complex is removed, e.g., by heating the fimbriae with detergents in vitro, the remaining fimbrial rod, which consists of the major subunit, is not adhesive. In contrast, CFA/I fimbriae consist of only one type of subunit, which, also after dissociation of the fimbriae, retains at least part of its receptor-binding property. In spite of consisting of only one type of (adhesive) subunit, CFA/I fimbriae are polar and monovalent like P, S, and type 1 fimbriae (15, 16, 28). We interpret this to mean that only the tip-located subunit is accessible to the receptor, whereas the potential receptor-binding sites of the internal subunits are covered by the neighboring subunits. Thus, breakage of the fimbriae would liberate a new adhesive tip. Such an exposure of new adhesive sites after breakage may be important when the adhering bacteria are subject to shearing forces such as can be expected in intestinal peristalsis. In an attempt to block the adhesion of *E. coli* cells which express CFA/I fimbriae with receptor, the reappearance of adhesive sites by fimbrial breakage should be taken into consideration.

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