Monoclonal Antibodies against Different Epitopes on Colonization Factor Antigen I of Enterotoxin-Producing Escherichia coli

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Hybridoma-secreting monoclonal antibodies (MAbs) against colonization factor antigen I (CFA/I) were produced by the fusion of spleen cells from immunized BALB/c mice with F/O myeloma cells. The 25 MAbs with the highest antibody titer against CFA/I, as determined by an enzyme-linked immunosorbent assay, were studied for specificity and the capacity to agglutinate CFA/I-carrying bacteria. Most of the MAbs agglutinated a majority of 16 CFA/I-positive strains tested but not any of a number of CFA-deficient mutants or strains carrying other colonization factors (CFA/II and CFA/IV). One MAb that agglutinated all the CFA/I-positive strains and one MAb that did not agglutinate any of these strains were compared for their reactivities with different preparations of CFA/I. Whereas both MAbs bound to or could be inhibited by isolated CFA/I fimbriae as well as the subunit protein, only the agglutinating MAb bound to CFA/I, as expressed on whole bacteria. The nonagglutinating MAb, on the other hand, bound considerably better than the agglutinating MAb to a peptide corresponding to the 46 N-terminal amino acid residues of the CFA/I subunit protein. These results suggest that the two MAbs are directed against different epitopes on CFA/I.

The capacity to colonize the small intestine seems to be an important prerequisite for enterotoxin-producing Escherichia coli (ETEC) to cause diarrhea. On ETEC strains isolated from humans, several distinct types of putative adherence fimbriae that are immunologically nonrelated have been identified. The best recognized of these fimbriae are the colonization factor antigens (CFAs) I and II (CFA/I and CFA/II) and CFA/IV (formerly designated PCF8775) (7, 9, 11, 26, 27, 29), but additional ones have been described previously (4, 15). The expression of CFAs is controlled by plasmids which, in most cases, also encode for the production of a heat-stable enterotoxin (23) and, in some instances, also for a heat-labile enterotoxin of E. coli (28).

CFA/I is expressed as threadlike appendages that are located on the surface of ETEC. In the electron microscope these fimbriae are seen as rather rigid structures of 0.5 to 1 μm in length with a diameter of 7 nm (20, 21). The fimbriae consist of more than 100 identical subunits of CFA/I that are made up of 147 amino acid residues; each subunit has a molecular weight of 15,058 (13).

The ability of ETEC to cause hemagglutination of human blood group A erythrocytes in the presence of n-dextran has been used to screen for the presence of CFA/I. In a recent report (1), we have shown that slide agglutination with polyclonal antiserum against purified CFA/I is a very sensitive and specific method for the identification of CFA/I on ETEC isolates.

Studies in humans have shown that CFA/I is important for the capacity of ETEC to colonize the intestine and to produce diarrhea (11). Antibodies against CFA/I are also important in preventing colonization and disease by CFA/I-positive strains in experimental animals (2, 6), and immunization with purified CFA/I has been reported to induce protective immunity in humans (10). Against this background it is most likely that CFA/I fimbriae, the subunit protein, or perhaps synthetic peptides harboring major antigenic determinants on CFA/I should be included in a future ETEC vaccine.

The aim of this study was to raise monoclonal antibodies (MAbs) against CFA/I for use in the identification of CFA/I-positive bacteria. Another aim was to analyze whether the MAbs produced were directed against the same or different epitopes on the CFA/I subunit protein. The results suggest that most but not all of the MAbs are directed against epitopes that are located on the surface of native fimbriae.

MATERIALS AND METHODS

Bacteria. The CFA/I-carrying ETEC strain H10407 (O78: K80:H11; CFA/I), which was kindly obtained from Dolores Evans (Houston, Tex.) and 15 previously CFA/I-positive strains isolated from patients with diarrhea during an epidemiological study in Dhaka, Bangladesh (12), were used. In addition, eight CFA/II-positive strains isolated in Dhaka (12) and two CFA/IV-positive strains kindly provided by B. Rowe (Colindale, London, England) were used for specificity analysis.

Antigens. Purified CFA/I was prepared from a flagellum-deficient mutant of strain H10407 (kindly provided by Dolores Evans) as described previously (8). The CFA/I preparation was checked for purity by double diffusion in gel (22) by using a polyclonal serum against strain H10407 or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16). CFA/II was similarly purified from a CFA/II-positive strain, E1392-75 (O6:H16), expressing E. coli surface antigens CS1 and CS3 and from a CFA/II-positive strain expressing CS2 and CS3 (3, 24).

CFA/IV antigen was purified by the same methodology described above from strain C91682, which expresses CS4 and CS6; however, this purification only resulted in the isolation of purified CS4, whereas the nonfimbrial CS6 antigen was lacking in the fimbrial preparation.

Preparation of MAbs. Inbred female BALB/c mice (age, 8 to 14 weeks) were immunized with purified CFA/I by giving an initial intraperitoneal injection with 4 μg of purified CFA/I in Freund complete adjuvant followed by two intravenous

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injections with 4 μg of CFA/I without adjuvant in each injection 7 and 9 weeks later. Four days after the last immunization, the spleens were excised and spleen cells were prepared for the production of MAbs (5). After 10 to 14 days (6- to 14-day cultures) the hybridomas were tested for the production of antibodies against CFA/I in an enzyme-linked immunosorbent assay (ELISA), and the hybrids producing the highest titers were cloned and expanded by cultivation in 10 ml of fetal bovine serum-Iscove medium. Two of the hybridomas were selected for further growth, including preparation of ascites fluid as described previously (19). All cell lines were frozen in the presence of dimethyl sulfoxide and 10% fetal bovine serum in liquid nitrogen.

ELISA. MAbs against CFA/I were identified by a CFA/I ELISA method. ELISA microtiter plates (Nunc, A/S Roskilde, Denmark) were coated with a 5 μg of purified CFA/I per ml at 37°C overnight (100 μl per well). After the plates were blocked with 3% bovine serum albumin (BSA)-phosphate-buffered saline (PBS) solution at 37°C for 30 min, supernatants from hybridomas (culture fluids) diluted 1:2 in PBS containing 0.1% BSA (100 μl per well) were incubated in the plates at room temperature for 2 h. Bound antibody was then demonstrated by the addition of anti-mouse immunoglobulin-horseradish peroxidase conjugate and orthophenylenediamine-H2O2 enzyme substrate, as described previously (25). Culture fluids giving an A50 of ≥0.2 above the background at 20 min were considered to be positive for CFA/I.

The capacity of purified CFA/I, peptide fragments of the CFA/I subunit protein, and CFA-carrying E. coli to inhibit binding of the anti-CFA/I MAbs to solid-phase-bound CFA/I was assayed by an ELISA inhibition method. All of the different MAbs were diluted in 0.1% BSA-PBS to a concentration corresponding to 100 times the ELISA titer against purified CFA/I. The diluted MAbs were then mixed with equal volumes of twofold dilutions of purified CFA/I (1 mg/ml), peptide fractions (0.5 to 3 mg/ml), or whole bacteria (1010 bacteria per ml). After the mixtures were added to the CFA/I-coated plates and incubated at room temperature for 2 h, the plates were developed as described above for the CFA ELISA. The concentration of inhibitor that resulted in 50% inhibition of binding of the respective MAbs to solid-phase-bound CFA/I was determined.

The capacity of different anti-CFA/I MAbs to inhibit binding of heterologous anti-CFA/I MAbs to solid-phase-bound CFA/I was also studied by ELISA. Several dilutions of anti-CFA/I MAbs were incubated in a CFA/I-coated ELISA microtiter plate at room temperature for 2 h. After washing 3 times in PBS, the homologous or heterologous anti-CFA/I MAbs coupled to alkaline phosphatase (AP) (17) was added; the concentration of the different anti-CFA/I-AP conjugates to be used was determined by checkerboard titrations. After incubation of the AP-coupled MAbs at room temperature for 2 h, the plates were developed with enzyme substrate (nitrophenylphosphate, 1 mg/ml, in ethanolamine buffer [pH 9.6]).

Slide agglutination tests. Bacteria (10 μl; about 1010 organisms per ml) were mixed with 10 μl of either nondiluted or 3- to 10-fold-diluted ascites fluid of the different anti-CFA/I MAbs on a glass slide. Macroscopically visible agglutinates observed within 2 min were regarded as a positive reaction.

Hemagglutination inhibition was performed by mixing 10 μl of a suspension of CFA/I-positive bacteria with 10 μl of diluted MAbs on a glass slide for 1 min and then adding 10 μl of a 3% (vol/vol) human blood group A erythrocyte suspen-
sion containing 1% D-mannose; the highest dilution of MAbs that inhibited visible hemagglutination was determined.

Determination of isotype and concentration of MAbs. The isotypes of the MAbs were determined by means of double diffusion in gel (22) by using antisera specific for different isotypes of mouse immunoglobulins (immunoglobulin G1 [IgG1], IgG2a, IgG2b, IgG3, IgA, and IgM; Meloy Laboratories Inc., Springfield, Va.). The immunoglobulin concentration was determined by single radial immunodiffusion (18) by assaying appropriate dilutions of the MAb preparation in isotype-specific anti-mouse immunoglobulin and by using sera with known concentration of the respective immunoglobulin isotypes as standards (Meloy).

Gel electrophoresis and Western blot analyses. Sodium dodecyl sulfate–15% polyacrylamide gels were prepared as described by Laemmli (16) by using 1.5-mm-thick gels. Samples of purified CFA/I and outer membrane preparations of the CFA/I-positive strains 258909-3 (O128:H7) and H10407 and of CFA/I-negative mutants of these strains (258909-3M and H10407P, respectively) were boiled for 5 min and dissolved in sample buffer (sodium dodecyl sulfate, 10% glycerol, 0.06 M Tris hydrochloride [pH 6.8]) and then applied to the gel in 20-μl volumes corresponding to 20 μg of CFA/I per ml. After electrophoresis was carried out as described previously (25), the separated samples were transferred to a nitrocellulose sheet for 4 h at 180 mA. The nitrocellulose sheet was then incubated in a 1% BSA–PBS solution for 1 h, followed by washing 3 times in PBS and incubation with anti-CFA MAb diluted in a 0.1% BSA–PBS–TWEEN solution for 16 h at room temperature. The nitrocellulose sheets were developed with enzyme conjugate and substrate as described previously (17, 25).

Nitrocellulose replica method. In some experiments CFA/I-positive and CFA-negative E. coli were cultured onto CFA agar, and replicas of the cultures on nitrocellulose filter papers were performed as described previously (17). After the paper was blocked with 1% BSA–PBS, ascites fluid of anti-CFA/I MAbs diluted in 0.1% BSA–PBS was added for 1 h, and the paper was developed with anti-mouse immunoglobulin-horseradish peroxidase conjugate and enzyme substrate as described previously (17).

Proteolytic and chemical cleavage of CFA/I fimbriae. Digestion of purified CFA/I fimbriae with trypsin and subsequent isolation of soluble peptides with different amino acid lengths, as well as cleavage with cyanogen bromide, was performed as described previously (14).

RESULTS

Frequency and isotype of anti-CFA/I MAbs. Culture fluids from all stable hybrids from the different cell fusion experiments were studied for production of anti-CFA/I antibody by ELISA. Altogether, 25 stable hybridomas were expanded and characterized with regard to their MAb product. All but one of the hybridomas produced anti-CFA/I antibodies of the IgG1 isotype; the remaining one produced IgM antibodies. The antibody concentration in the culture fluids was 4 to 64 μg/ml, and in ascites fluids it was 1 to 4 mg/ml.

Specificity of anti-CFA/I MAbs. Ten of the CFA/I-positive MAbs were tested for their reactivities with an outer membrane preparation of the CFA/I-positive strain H10407, as well as with purified CFA/I, by Western blot analyses. The results of these studies indicated that all of the 10 MAbs recognized a single 15-kilodalton band in the outer membrane preparations of H10407 at the same position at which the MAbs developed a band with purified CFA/I (data not
Mabs against colonization factor antigen I of E. coli

When CFA/I-carrying bacteria and the corresponding CFA-negative mutants adsorbed onto separated nitrocellulose replicas were reacted with these anti-CFA/I Mabs and the filter papers were developed by ELISA (17), a positive reaction was only obtained for replicas that were treated with CFA/I-fimbriated bacteria (Fig. 1).

To evaluate further the specificity of the different Mabs for CFA/I, they were tested for inhibition of binding to solid-phase-bound CFA/I by various purified CFAs and CFA-carrying bacteria. Although all of the 17 Mabs tested were adjusted to the same antibody concentration, i.e., 10 times the ELISA titer against CFA/I, slightly varied concentrations of purified CFA/I (1 to 6 μg/ml) were needed to obtain 50% inhibition of their binding to solid-phase antigen. This difference in inhibitory capacity of CFA/I suggests slightly different avidities of the Mabs for the specific antigen. In no instance were any of the anti-CFA/I Mabs inhibited by 1 mg of purified CFA/II (CS1 and CS3 or CS2 and CS3) per ml or by the purified CS4 component of CFA/IV. When CFA/I-positive bacteria were tested, it was found that all but one of the Mabs tested were inhibited by such organisms but that none of the Mabs was inhibited by bacteria expressing CFA/II or CFA/IV or by CFA/I-deficient mutants (strains H10407P and 258909-3M).

Inhibition ELISA. The ability of different anti-CFA/I MAbs to inhibit one of the anti-CFA/I MAbs, 1:6, coupled to AP was tested by ELISA. This was done by incubating one dilution of the different Mabs, corresponding to 10 times the

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**TABLE 1. Agglutination of ETEC strains carrying different fimbrial antigens by 20 different anti-CFA/I Mabs**

<table>
<thead>
<tr>
<th>Strains agglutinated and %</th>
<th>No. of Mabs agglutinating the indicated % of test strains (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA/I-positive strains (n = 16)</td>
<td>100:13, 94:2, 88:3, 81:1, 0:1</td>
</tr>
<tr>
<td>CFA/II-positive strains (n = 8), 0:20</td>
<td></td>
</tr>
<tr>
<td>CFA/IV-positive strains (n = 2), 0:20</td>
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</table>
Comparison of agglutinating and nonagglutinating MAbS.

For further analyses, two IgG1 MAbS that both reacted in high, almost similar, titers with CFA/I in the ELISA (MAb 1:6, which agglutinated all the 20 CFA/I-positive strains, and MAb 65D, which did not agglutinate any of these strains) were compared in different assays for their reactivities with CFA/I. The capacities of E. coli expressing CFA/I to inhibit binding of diluted MAbS 1:6 and 65D to solid-phase-bound CFA/I were compared. Whereas purified CFA/I was very efficient to inhibit the binding of the two MAbS to the solid phase, only MAb 1:6 was inhibited by CFA/I-carrying bacteria (Fig. 3). The hemagglutination-inhibitory capacity of the two MAbS was also tested. It was found that whereas the culture medium of MAb 1:6 effectively inhibited hemagglutination of human blood group A erythrocytes by CFA/I-carrying bacteria at a dilution of 1/100, even concentrated medium of MAb 65D had no such inhibitory effect.

In a further attempt to evaluate whether MAbS 65D and 1:6 were directed against different epitopes on CFA/I, we performed cross-inhibition experiments with these MAbS by ELISA. After incubation of different dilutions of the respective MAbS in CFA/I-coated ELISA plates, MAb 1:6 or 65D covalently coupled to AP was added and assayed for binding to the solid phase. It was found that although the two MAb preparations contained equal concentrations of specific IgG1 antibodies, only MAb 1:6 effectively inhibited subsequent binding of either of the two enzyme-conjugated MAb preparations. Thus, ascites fluid of MAb 1:6 diluted 1/10,000 inhibited subsequent binding of both MAb 1:6-AP and MAb 65D-AP by 50%. Initial incubation with ascites fluid of MAb 65D gave only partial inhibition (50 to 60%) of subsequent binding of the homologous and heterologous MAb-AP conjugate; MAb 65D diluted 1/300 inhibited both conjugates by 50% (data not shown).

Binding of CFA/I MAbS to fragments of CFA/I subunit protein. To confirm that MAbS 1:6 and 65D were directed against different epitopes on the CFA/I subunit protein, we studied the capacity of soluble peptides corresponding to various parts of the CFA/I protein (for amino acid sequences of peptides; Fig. 4) to inhibit binding of the two MAbS to solid-phase-bound CFA/I. We found that the only one of the six fragments tested that effectively inhibited binding of either MAb was a peptide corresponding to the N-terminal 46 amino acid residues of the CFA/I protein; the inhibition of MAb 65D was considerably better than that of MAb 1:6 (Table 2). A number of shorter peptides covering various parts of the N-terminal 46 amino acid residues (Fig. 4) (15) at concentrations of up to 200 nmol/ml failed to inhibit the binding of either of the MAbS to solid-phase CFA/I (compared with purified CFA/I, which inhibited the binding by 50% at concentrations of 0.03 to 0.07 nmol/ml). Attempts to determine the direct binding of the anti-CFA/I MAbS to the different peptides failed since none of the MAbS bound significantly to ELISA plates coated with a wide range of concentrations (0.1 to 100 μg/ml) of the different peptides presented in Fig. 4.

DISCUSSION

We produced and characterized a number of MAbS which bind to CFA/I-fimbriated bacteria as well as to isolated CFA/I fimbriae. As many as 65% of these MAbS agglutinated all 16 CFA/I-positive E. coli strains tested, whereas none of the MAbS agglutinated CFA/I-deficient mutants or CFA/I- and CFA/I-positive bacteria. Against this background several of these anti-CFA/I-specific MAbS may be used as immunodiagnostic reagents in the identification of CFA/I-positive ETEC, e.g., in epidemiological studies. We also identified a MAb, 65D, that did not recognize native CFA/I fimbriae when they were present on bacteria, but it was able to react with isolated and dissociated CFA/I subunits, sug-

**TABLE 2. Inhibitory activities of different fragments of the CFA/I subunit protein in CFA/I ELISA**

<table>
<thead>
<tr>
<th>CFA/I fragment</th>
<th>50% Inhibitory concn (μg/ml)</th>
<th>MAb 1:6</th>
<th>MAb 65D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–46</td>
<td>100</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>1–78</td>
<td>&gt;100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>79–143</td>
<td>&gt;100</td>
<td></td>
<td>&gt;100</td>
</tr>
<tr>
<td>45–57</td>
<td>&gt;100</td>
<td></td>
<td>&gt;100</td>
</tr>
<tr>
<td>90–96</td>
<td>&gt;100</td>
<td></td>
<td>&gt;100</td>
</tr>
<tr>
<td>125–147</td>
<td>&gt;100</td>
<td></td>
<td>&gt;100</td>
</tr>
<tr>
<td>Purified CFA/I</td>
<td>0.4</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

* Values are means of four different experiments.

**FIG. 3.** ELISA inhibition of binding of anti-CFA/I MAbS 1:6 (A) and 65D (B) to solid-phase-bound CFA/I by purified CFA/I (C) and by two CFA/I-positive strains, 304688 (A) and 255875 (B). The initial concentrations tested were 1 mg of purified CFA/I per ml and 10^10 organisms per ml.

**FIG. 4.** Amino acid sequences of the CFA/I subunit protein given in standard single-letter code. Predicted continuous antigenic determinants (14) are indicated by heavy bars. Peptides used in this study are indicated by lines.
gesting that it is directed against an epitope that is not normally accessible on native fimbriae. The advantage of using anti-CFA/I MAbs over polyclonal anti-CFA/I sera for diagnostic purposes is that the MAbs are homogeneous and can be produced in practically unlimited amounts; however, the MAbs often have lower avidities for the corresponding antigens than do polyclonal sera. Of practical importance for the use of specific MAbs in CFA/I ELISA detection methods, such as the nitrocellulose replica assay (17), is that they can be used specifically to detect CFA-carrying E. coli excreted in the stools of people without prior isolation of ETEC.

Worobec et al. (30) have previously reported the production of MAbs against CFA/I. The four MAbs they studied by electron microscopy appeared to bind with a similar pattern along the surface of the entire CFA/I pilus, suggesting that they were all directed against the same epitope that was accessible on whole fimbriae. On the other hand, only one of the four MAbs cross-reacted with K99 antigen, suggesting that it has a specificity for at least partially different epitopes. The fact that MAb 65D recognized denatured fimbriae as well as the 15-kilodalton subunit, but failed to agglutinate whole bacteria carrying CFA/I, suggests that it recognizes an epitope which is partially or completely masked on native fimbriae but that is exposed on denatured or depolymerized CFA/I protein. This is further supported by our finding that isolated CFA/I fimbriae, but not E. coli expressing comparatively high levels of CFA/I fimbriae, inhibited the binding of MAb 65D to solid-phase-bound CFA/I. The failure of MAB 65D, at variance with MAB 1:6, to inhibit hemagglutination of human erythrocytes by CFA/I-carrying bacteria strongly supports the fact that MAB 65D is not directed against an epitope that is expressed on the receptor-binding portion of native CFA/I.

Based on the elucidated amino acid sequence of the CFA/I subunit protein (13), six potential antigenic determinants have previously been predicted (14). Two of these potential determinants are located within the N-terminal 46 amino acid residues. The two different MAbs, 1:6 and 65D, which are agglutinating and nonagglutinating, respectively, were inhibited by a peptide that encompasses this region of the protein. This finding may suggest that the two MAbs are directed against two different, but proximate, epitopes within the first 46 amino acid residues. Alternatively, only the epitope to which MAB 65D binds specifically is restricted to this region. Thus, whereas the 46-amino-acid fragment was almost as effective as purified CFA/I in inhibiting the binding of MAb 65D to solid-phase-bound CFA/I, this fragment was, on a molar basis, at least 15-fold less effective than the CFA/I protein in inhibiting the binding of MAb 1:6. This might suggest that the MAB 1:6 epitope is only partially located within the N-terminal 46 amino acid residues or that it is dependent on the tertiary configuration of the CFA/I subunit protein.

It is somewhat suprising that a peptide corresponding to the N-terminal 78 amino acid residues only has a marginal inhibitory effect on MAb 65D (Table 2), and we must assume that this peptide folds up in a conformation in which the epitope in question is either shielded or in the wrong configuration.

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LITERATURE CITED


