Epitope Analysis of the F4 (K88) Fimbrial Antigen Complex of Enterotoxigenic Escherichia coli by Using Monoclonal Antibodies

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So far, three subtypes of the F4 (K88) fimbrial antigen of porcine enterotoxigenic Escherichia coli, F4ab, F4ac, and F4ad, have been distinguished by using polyclonal antisera in agglutination and precipitation tests. The a factor represents one or more common epitopes, whereas each of the b, c, and d factors represents one or more subtype-specific epitopes. We further characterized the F4 antigen complex by using a panel of 40 F4-specific monoclonal antibodies (MAbs). The specificity of all MAbs was proven by enzyme-linked immunosorbent assays, agglutination and radioimmunoprecipitation tests, and immunoelectron microscopy. The MAbs either reacted with all F4 subtypes, reacted with two subtypes, or were subtype specific. Epitope analysis by competition enzyme-linked immunosorbent assays revealed at least 11 epitope clusters on the F4 antigen complex, designated a1 to a7, b1, b2, c, and d. The following antigenic formulas were found for the F4 subtypes: F4ab, a1a2a3a4a5a6a7b2; F4ac, a1a2a3a(a4)a5a6a7c; and F4ad, a1a2a3a4a7d. All MAbs were directed against conformational epitopes located on the 27,500-dalton major fimbrial subunits. Consequences for the replacement of polyclonal antisera by MAbs in diagnostic tests are discussed.

Fimbriae are long, nonflagellar, filamentous appendages on the bacterial cell surface. Fimbriae of Escherichia coli are composed of identical repeating protein subunits (major fimbrial subunits). In addition, they usually carry various minor protein components. At least four antigenically different fimbrial antigens, F4 (K88), F5 (K99), F6 (K987P), and F41, are important virulence factors of porcine enterotoxigenic E. coli (ETEC) (6, 24). They enable ETEC strains to colonize the small intestine by specific adhesion to the mucosa. The production and secretion of enterotoxin(s) may then cause disease characterized by diarrhea, dehydration, and death.

In the Netherlands, F4-positive (F4+) ETEC strains constitute the majority of ETEC strains isolated from diseased piglets, both neonatal and weaned. In contrast to F5, F6, and F41, several antigenic subtypes of F4 have been described. By using polyclonal antisera and monofactorial antisera obtained by absorbing polyclonal antisera with bacteria of heterologous subtypes, three subtypes are established in agglutination and precipitation tests: F4ab, F4ac, and F4ad (10, 25). The a factor represents one or more common antigenic determinants, whereas the b, c, and d factors are subtype specific. Almost all Dutch F4+ isolates are of the F4ac type. In vitro, F4+ strains do not express fimbriae when grown at 18°C (6).

The genetic determinants of F4ab and F4ac have been cloned (20, 27), and the F4ab genes in particular have been studied extensively (21, 22, 26). The genetic map of cloned F4ab DNA is given in Fig. 1. Genes C to H encode six polypeptides preceded by signal peptides (FaeC to FaeH). The G protein is the major fimbrial subunit; the D protein is an outer membrane protein that is necessary for transport of fimbrial components through the outer membrane and serves as an anchor protein. The other polypeptides probably are involved in the biosynthesis of the fimbriae; three of them, C, F, and H, are also minor components of the fimbrial structure (26; D. Bakker and F. K. de Graaf, unpublished observations). The major fimbrial subunit appears to be responsible for the adhesive properties of the fimbriae (13, 14).

The primary structures of the major fimbrial subunits of F4ab, F4ac, and F4ad have been determined from their nucleotide sequences (3, 7, 8, 15) or by amino acid sequencing (16); minor variations within one subtype have been described (3). Antigenic determinants on the major fimbrial subunits have been predicted (5, 17, 18). However, antisera raised against synthetic peptides of some of these predicted antigenic determinants did not react with native F4 antigens, except for antisera against the segment of the F4ab and F4ad variants consisting of amino acids 213 to 219 (18); whether this segment is also an immunogenic epitope of native fimbriae is unknown.

We have made panels of monoclonal antibodies (MAbs) against the F4, F5, F6, and F41 antigens to (i) study the structure and function of these fimbriae, (ii) replace polyclonal antisera in diagnostic tests by MAbs, and (iii) evaluate the protective value of MAbs against ETEC infections when MAbs are used for the passive oral immunization of neonates. This paper describes the antigenic characterization of the F4 antigen complex by using a panel of 40 F4-specific MAbs in competition enzyme-linked immunosorbent assays (ELISAs). All MAbs were directed against the F4 major fimbrial subunits.

MATERIALS AND METHODS

Bacterial strains and media. Fimbriae were purified from the E. coli strains G7 (O8K87:F4ab), 1072 (O8K87:F4ad), 1087 (O149K91:F4ac), and 1091 (O157K1:F4ac). In addition, all MAbs were tested against 7 other F4ab+ strains, 6 F4ad+ strains, and 73 F4ac+ strains. The main characteristics of these strains are listed in Table 1. All F4ac+ strains were isolated at the Central Veterinary Institute from diseased piglets. Each isolate originated from a different herd. Most of the F4ad+ strains were obtained from P. A. M.
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parts of the genes
dicate signal peptides. The numbers
refer to the molecular masses of the gene products in kilodaltons.
The major fimbrial subunit is indicated ( ).

Guinée and W. H. Jansen (National Institute of Public
Health and Environmental Protection, Bilthoven, The
Netherlands), and most of the F4ab+ strains (29) were kindly
donated by C. J. Thorns (Central Veterinary Laboratory,
Weybridge, Surrey, United Kingdom).

Five strains harboring different recombinant plasmids
(Table 2) were used in ELISAs to localize the protein against
which MAbS were directed. Other details of the construction
of these plasmids have been described before (20, 21) or will
be described elsewhere.

Strains were grown in Minca medium, Minca-IsoVitaleX
broth (11), or Trypticase soy broth or on 5% sheep blood
agar and Minca-IsoVitaleX agar. Ultrasonic extracts from
bacteria grown at 18°C (US18 extract) or 37°C (US37 extract)
were prepared as described by Guinee and Jansen (10).

Purification of fimbriae. Bacterial cells of 30- to 40-liter
fermentor cultures of F4+ strains were collected by cen-
trifugation and suspended to an A600 of approximately 150 in
0.05 M sodium phosphate buffer containing 2 M urea.
Fimbriae were detached from the bacteria by heating for 20
min at 58°C, and the cells were pelleted by centrifugation.
Fimbriae were purified from the supernatant as described by
de Graaf and Roorda (2) or as described by Jacobs and de
Graaf (12).

The purity of the purified fimbrial preparation was as-
essed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% slab gels.

Antisera. Rabbit antisera against purified F4 antigens
(RaF4ab, RaF4ac, and RaF4ad) were prepared as described
before for the F41 antigen (30). The specificity of the sera
was assessed by slide agglutination tests (SATs), immuno-
electrophoresis, and double immunodiffusion methods,
as described by Guinee and Jansen (10), and by ELISAs.

<table>
<thead>
<tr>
<th>F4 subtype</th>
<th>OK type</th>
<th>No. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>ab</td>
<td>O141:K85abc</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>O7:K8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>O100:K100</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O100:K?</td>
<td>1</td>
</tr>
<tr>
<td>ac</td>
<td>O149:K91</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>O8:K87</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>O138:K81</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>NT</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>O9:K204</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O20:K?</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O141:K85ac</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O157:K-</td>
<td>1</td>
</tr>
<tr>
<td>ad</td>
<td>O8:K8</td>
<td>3</td>
</tr>
<tr>
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<td>O8:K?</td>
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<tr>
<td></td>
<td>O32:K87</td>
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</tr>
<tr>
<td></td>
<td>O147:K89</td>
<td>1</td>
</tr>
</tbody>
</table>

* NT, Not typed.

Subtype-specific antisera were obtained by absorbing sera
against one subtype with whole bacterial cells of the heter-
ologous subtypes. Agglutinating subtype-specific rabbit
antisera, kindly donated by P. A. M. Guinee and W. H. Jansen,
were used as reference antisera.

MAbs. The immunization schedule of BALB/c mice, the
preparation of mouse hybridoma cell lines, the detection
of anti-F4 antibody-producing cell lines by ELISAs, the clon-
ing procedure, and the production of MAbs against the F4
variants were similar to those described previously for
MAbs against the F41 antigen (30). MAb of each stable cell
line was purified from ascitic fluid by ammonium sulfate
depreciation (35 to 40% saturation) followed by dialysis
against 0.01 M phosphate-buffered saline, pH 7.2. The purified
MAb preparations (8 mg of protein per ml in phosphate-
buffered saline) were stored in aliquots of 1 ml at
−70°C. The immunoglobulin isotype of each MAb was
determined in immunodiffusion tests with mouse isotype-
specific antisera.

SATs. Each MAb was used at a dilution of 1:20 in
phosphate-buffered saline in agglutination tests with all F4+
strains grown at 37°C and randomly selected F4+ strains
grown at 18°C. If no agglutination was observed, SATs were
also performed with lower dilutions (1:5 and 1:10).

PEPSCAN for mapping of epitopes on F4. The PEPSCAN
method has been described in detail elsewhere (9, 19).
Briefly, the total amino acid sequences of the major subunits
of all subtypes of F4 (7, 8, 15) were synthesized in overlap-
ing peptides of seven amino acids on polyethylene rods,
i.e., peptide 1 of F4ab consisted of amino acids 1 to 7 of the
primary sequence of its major subunit, peptide 2 consisted of
amino acids 2 to 8, peptide 3 consisted of amino acids 3 to 9,
and so on. In this way, 257 peptides of F4ab, 255 peptides
of F4ac, and 257 peptides of F4ad were made to cover the total
sequences of the three variants. The peptides still coupled to
the polyethylene rods were then tested against selected
MAbs of different epitope specificity in an indirect ELISA.
A known epitope of the VP1 protein of foot-and-mouth
disease virus and its corresponding MAb (19) were included
as appropriate controls on peptide synthesis and the subse-
quent ELISA procedure.

ELISAs. The ELISAs used in this study were similar to
those used in a study of the characterization of the F41
antigen (30). For details of the buffers and substrate used,
the length of incubation steps, and the conjugation of MAbs
and polyclonal antisera to hors eradish peroxidase (HRPO),
we refer to that study. Optimal dilutions of HRPO-conju-
gated MAbs were determined by checkerboard titration
against purified antigens of all subtypes. Briefly, the follow-
ing ELISAs were used.

(i) Indirect ELISA for screening hybridoma culture super-
натants and titer determination of purified MAbs. The wells
of microtiter plates were coated with 0.5 μg of purified
F4ab, F4ac, or F4ad per well. After the plates were washed,
1:2 dilutions of hybridoma culture supernatants or serial twofold dilutions of purified MAb preparations were added. The plates were incubated and washed, and HRPO-labeled rabbit anti-mouse immunoglobulins (Dakopatts, Copenhagen, Denmark) were added. After the plates were washed, substrate solution was added, and the plates were read after 2 to 3 h of incubation.

Titers of purified MAb preparations were expressed as the logarithm of the reciprocal of the highest dilution giving an $A_{490}$ of 50% of the maximum obtainable absorbance value.

(ii) Indirect ELISA for epitope mapping by the PEPSYN method. The polyethylene rods, each carrying a different peptide of seven amino acids, were placed in the wells of microtiter plates with diluted MAb or polyclonal antisera. After incubation, the rods were washed and placed in microtiter plates containing HRPO-labeled goat anti-mouse or anti-rabbit immunoglobulins. After incubation and washing, the rods were placed in microtiter trays with substrate solution, and each plate was read.

(iii) Direct-competition ELISA for epitope analysis. Non-conjugated MAbs (competition MAbs) were added in serial twofold dilutions (range, 1:2 to 1:10,240) in volumes of 50 μl to the wells of plates coated with purified F4ab, F4ac, or F4ad antigen. After incubation for 30 min, 50 μl of the optimal dilution of each of the HRPO-conjugated MAbs was added per well; in this way, the dilutions of each nonconjugated MAb were allowed to compete with individual MAb conjugates for its epitope on all F4 subtypes. Incubation was continued for 1 h, and the substrate solution was added after the plates were washed.

The titers of competition MAbs against a conjugated MAb were expressed as the logarithm of the reciprocal of the highest dilution giving an $A_{490}$ of 50% of the absorbance of wells to which only the conjugate was added.

Epitopes were defined on the basis of the assumption that two MAbs showing no competition at all recognize two different epitopes. Two MAbs showing reciprocal competition with high titers were assumed to recognize the same epitope or two nearby epitopes. In general, no conclusions were drawn from nonreciprocal competition, because it may be caused by several possible factors (30), such as differences in affinity. An epitope cluster was defined as a single epitope or a group of adjacent or overlapping epitopes that cannot be distinguished as separate epitopes by epitope analysis with competition ELISAs because of steric hindrance. Epitope clusters present on two or more subtypes were designated as clusters, whereas subtype-specific clusters were designated as b, c, or d clusters.

The direct-competition ELISA with polyclonal RaF4 conjugates were used to detect whether single MAbs or combinations of MAbs were able to block the signal obtained by the polyclonal RaF4-HRPO conjugates alone.

(iv) Double-antibody sandwich ELISA for screening epitopes on the F4 antigen of field strains and recombinant DNA strains. Microtiter plates were coated with RaF4ac immunoglobulins. After the plates were washed, supernatants of heated (20 min at 58°C) cultures or US37 and US18 extracts were added. After the plates were incubated and washed, conjugates of all MAbs, RaF4ab, RaF4ac, and RaF4ad were added in their optimal dilutions. After the plates were incubated and washed and the substrate solution was added, the plates were read.

RIP assays for specificity control of MAbs. Radioimmunoprecipitation (RIP) assays with crude antigens of strains G7, 1077 (O8:K?:F4ad), and 1087 were performed similarly to those described previously for MAbs against the F41 antigen (30). Briefly, bacteria were grown in broth containing $^3$H-labeled amino acids (TRK 550; Radiochemical Centre, Amersham, United Kingdom). The cultures were heated for 20 min at 58°C, and the cells were pelleted by centrifugation. The supernatants to which sodium deoxycholate (1%), Triton X-100 (1%), and SDS (0.1%) were added were used as crude antigen preparations in RIP assays. Each MAb (1 μl) was added to 200 μl of crude antigen, and after incubation, 50 μl of rabbit anti-mouse immunoglobulins was added. The resulting precipitates were collected, washed, and used in SDS-PAGE as described before.

IEM. Immunoelectron microscopy (IEM) was performed as described before (30) either with colloidal gold-labeled MAbs or by an indirect assay with gold-labeled rabbit anti-mouse immunoglobulins. If present, at least two MAbs against each epitope cluster were tested. Appropriate controls with MAbs against F5, F6, and F41 antigens were incorporated.

RESULTS

Production and characterization of MAbs. Fusions were performed with spleens of 17 mice. The cloning and selection procedures resulted in 40 stable hybridoma cell lines; 14 lines were obtained from mice immunized with F4ab (CVI F4ab-1 to CVI F4ab-14), 10 from mice immunized with F4ac (CVI F4ac-1 to CVI F4ac-10), and 16 from mice immunized with F4ad (CVI F4ad-1 to CVI F4ad-16). The main characteristics of the purified MAbs (8 mg of protein per ml) are listed in Table 3.

In SATs, all MAbs agglutinated F4$^+$ strains of one or more subtypes grown at 37°C and none of the randomly selected F4$^-$ strains grown at 18°C.

Four of the F4ab MAbs (F4ab-1, -4, -5, and -7), five of the F4ac MAbs (F4ac-1, -2, -3, -7, and -10), and eleven F4ad MAbs (F4ad-4, -5, -7 to -9, and -11 to -16) reacted similarly in SATs, RIP assays, and indirect ELISAs to all F4 variants and hence were directed against one or more a epitopes. Each of these MAbs showed similar titers against the three different F4 subtypes in the indirect ELISA. Each of the HRPO conjugates of these MAbs had similar optimal dilutions when tested against purified antigens of the three subtypes, except for the F4ab-5 conjugate, which had optimal dilutions of 1:4,000 with F4ab and F4ac and 1:250 with F4ad. MAb F4ab-3 recognized all F4ab$^+$ and F4ad$^+$ strains but agglutinated only a part of the F4ac$^+$ strains; its ELISA titer against F4ac also differed depending on the strain used for antigen production.

Ten MAbs (CVI F4ab-2, -8, -9, -10, -11, and -14; CVI F4ac-5 and -8; and F4ad-6 and -10) were subtype specific in agglutination tests and hence were directed against one or more b, c, or d epitopes; some of them had low titers in the indirect ELISA with heterologous types. HRPO conjugates of these MAbs reacted only with antigen of the homologous subtype.

Four MAbs (F4ab-6, -11, and -12 and F4ac-4) reacted in a similar way with the F4ab and F4ac antigen and did not recognize the F4ad antigen.

MAbs F4ad-1, -2, and -3 agglutinated all F4ac$^+$ and F4ad$^+$ strains and none of the F4ab$^+$ strains. Their ELISA titers were high against the F4ad antigen, intermediate against the F4ac antigen, and low against the F4ab antigen. A similar reaction pattern was observed for MAb F4ac-6, which agglutinated only F4ab$^+$ and F4ac$^+$ strains and showed high ELISA titers against F4ac, intermediate titers against F4ab, and low titers against F4ad. HRPO conjugates of MAbs...
F4ad-1 to -3 and F4ac-6 could be used in high dilutions in the direct-competition ELISA against the homologous subtype and, in general, against one of the other subtypes in low dilutions; the conjugates did not recognize the subtype, against which unconcatenated homologs had the lowest titers.

All attempts to make suitable HRPO conjugates of MAb F4ac-7 and F4ac-9 failed. Their conjugates could only be used in very low dilutions against the F4ac antigen; their titers in the indirect ELISA suggest that they recognize all subtypes, with a preference for F4ac.

None of the MAbs showed titers in the indirect ELISA with F41 as antigen.

**Specificity control by RIP.** The reaction patterns of all MAbs against the three subtypes are shown in Table 3; they correlated with the findings of SATs. SDS-PAGE on 10% slab gels of precipitates obtained with the MAbs showed single radiolabeled protein bands with apparent molecular masses of approximately 27,500 daltons for all subtypes, similar to the molecular masses of 27,540 (F4ab), 27,239 (F4ac), and 27,562 (F4ad) reported for the F4 major subunits (4, 7, 8, 15). A typical example of the results of the RIP assay is given in Fig. 2. On some autoradiograms, a second, minor band of a protein with a molecular mass of 1,000 to 1,500 daltons more than those of the F4 bands was observed.

**Epitope analysis by direct-competition ELISA.** Each MAb in serial twofold dilutions was allowed to compete with conjugated MAb for its epitope on the three F4 subtypes. MAb were placed in epitope cluster specificity classes according to the criteria described in Materials and Methods. In addition, the reaction pattern of each MAb against all
other MAbs in the competition ELISA and, if necessary, the results of other tests were also taken into account in deciding the epitope specificity of each MAb.

In Fig. 3, the titers of all MAbs recognizing all subtypes are given against their conjugates. The titers shown in this figure are the averages of the titers found in competition ELISAs with the three subtypes. We concluded that these MAbs were directed against four epitope clusters designated a1 to a4. MAb F4ac-1 and especially Mab F4ac-3 did not block conjugates of some of the other MAbs classified in epitope cluster specificity class a1 or had low titers. The antigens used in the competition assay were F4ab from strain G7, F4ad from strain 1072, and F4ac from strain 1087. When a competition assay was performed with F4ac antigen from strain 1091 (the antigen used for immunizing mice from which MAbs F4ac-1 and -3 were obtained), F4ac-1 and -3 did block all conjugates directed against epitope cluster a1. For that reason, both MAbs were classified in epitope specificity class a1. Some variation probably exists in this cluster; however, we could not exclude the possibility that F4ac-1 and -3 are directed against another epitope. It was also difficult to classify MAb F4ab-5; it may be classified as a
FIG. 4. Titers of F4ab MAbs in the direct-competition ELISA with F4ab antigen. All MAbs against each of the epitope clusters a1 to a4 are represented by single squares. Partial shading of these squares represents the percentage of MAbs showing competition.

FIG. 5. Titers of F4ad MAbs in the direct-competition ELISA with F4ad antigen. All MAbs against each of the epitope clusters a1 to a4 are represented by single squares. Partial shading of these squares represents the percentage of MAbs showing competition.

FIG. 6. Titers of F4ac MAbs in the direct-competition ELISA with F4ac antigen. All MAbs against each of the epitope clusters a1 to a3 are represented by single squares. Partial shading of these squares represents the percentage of MAbs showing competition.

(Fig. 5). The remaining F4ac MAbs were classified in epitope cluster specificity classes a5, a6, and c (Fig. 6). F4ac-4 was probably directed against the same epitope cluster as F4ab-6, -11, and -12, although F4ac-4 blocked the conjugates of F4ab-6, -11, and -12 only in low dilutions when F4ab was used as antigen, and F4ab-6, -11, and -12 showed low titers against the F4ac-4 conjugate when F4ac was used as antigen (not shown). Because of its reactions in other tests, MAb F4ac-6 was placed in a separate class, a6, although some interference was noticed in the competition assay with MAbs of classes a5 and c. Competition assays with MAbs of classes a5, a6, and a7 on F4ac antigen (not shown) revealed that these classes were probably unrelated, indicating that the MAbs recognized three different clusters and not three variants of the same epitope cluster. F4ac-7 and F4ac-9 were not classified because their conjugates could be used only on F4ac antigen in low dilutions; for instance, the F4ac-7 conjugate was blocked by almost all other MAbs. These two MAbs may be directed against one or two epitope clusters other than the ones already established.

None of the MAbs blocked polyclonal RaF4 conjugates. Complete blocking of polyclonal RaF4 conjugates could be achieved by combining at least three MAbs of different epitope specificity.

Occurrence of epitopes on F4⁺ strains. In double-antibody sandwich ELISAs and SATs, all strains of each F4 subtype grown at 37°C reacted similarly with individual MAbs, as shown in Table 3, except for F4ac strains with MAb F4ab-3. This MAb agglutinated all F4ab⁺ and F4ad⁺ strains but only 26 of the 73 tested F4ac⁺ strains. Most of the F4ac⁺ strains belonged to three OK types (Table 1); all O138:K81 strains and 31 of the O149:K91 strains were not recognized by F4ab-3, whereas 6 O149:K91 strains and all 15 O8:K87 strains showed positive tests with this MAb.

Locating epitopes on F4 proteins by using recombinant DNA strains. The conjugates of all MAbs recognizing F4ab (Table 3) reacted in the double-antibody sandwich ELISA with US37 extracts of all strains listed in Table 2 except for the conjugates of F4ab-2, -3, -8, -9, -13, and -14, which did not react with strain M5(pDB88-103). This demonstrates that the expression of epitope clusters a4, b1, and b2 is dependent only on amino acids located on the F4ab major fimbrial subunit. The conjugates of the F4ad-specific MAbs, F4ad-5 and -8, reacted only with antigen from strain M5(pDB88-103), which indicates that their epitope is restricted to the F4ac major fimbrial subunit or perhaps the H protein.
Mapping of epitopes on the F4 major fimbrial subunit by the PEPS CAN method. MAbs F4ab-1, -2, and -3; F4ac-1, -2, -5, and -6; and F4ad-3, -4, -5, -6, and -10, representing epitope clusters a1, a2, a3, a4, a6, a7, b1, c, and d were tested in the indirect ELISA against the synthesized peptides. MAbs against epitope clusters a5 and b2 were not available at the time of testing. None of the tested F4 MAbs or polyclonal RaF4 antisera showed a significant reaction; the control MAb against the VP1 protein of foot-and-mouth disease virus invariably showed positive reactions against its synthesized epitope.

IEM. IEM was performed with one or two representatives of each epitope specificity class. Their reaction pattern against the three subtypes correlated completely with those in the tests listed in Table 3. They were all directed against fimbriae of approximately 2 to 3 nm in diameter, and the gold particles were regularly distributed along the whole fimbrial structure (Fig. 7) with small intervals, as was also seen by Thiry et al. with our F4ad-9 MAb (28). Fimbriae often had a strong tendency to aggregate or to collapse against the bacterial cell wall in IEM.

FIG. 7. IEM of strain 1087 with MAb F4ad-11. Magnification, ×150,000. Bar, 100 nm.

DISCUSSION

We produced a panel of 40 MAbs directed against the F4 antigen complex of E. coli. The specificity of the MAbs was proven by ELISAs, SATs, RIP assays, and IEM. MAbs either reacted with all F4 subtypes (F4ab, F4ac, and F4ad) or with two subtypes or were subtype specific. These findings agree with those of another study of F4 MAbs (4). On the basis of the results of competition ELISAs and the behavior of MAbs in other tests, at least 11 epitope clusters, called a1 to a7, b1, b2, c, and d, on the F4 antigen were defined. The common factor found with conventional serologic tests could be subdivided into at least seven epitope clusters shared by two or more subtypes. Although the number of F4ad+ and F4ab+ strains used in this study was low, we propose the following antigenic formulas for the three subtypes of F4: F4ab, a1a2a3a4a5a6a7b2; F4ac, a1a2a3(a4) a5a6ac7c; and F4ad, a1a2a3a4a7d.

The number of epitope clusters found on each F4 subtype is relatively large compared with the five epitope clusters found on F41 (30) and compared with the number of clusters found on F5 and F6, which have fewer epitope clusters (F. G. van Zijdeveld, unpublished observations). Because all strains of each F4 subtype had similar reaction patterns with our MAbs, the epitopes present on a particular subtype seemed to be conserved, except for the epitope recognized by MAB F4ab-3 (epitope cluster a4). In SATs or RIP assays, this MAb distinguished two types of F4ac strains (a4+ and a4-).

The reaction patterns of MAbs in RIP assays, SATs, and IEM agreed completely; the results of the indirect ELISA also corresponded well with those of SATs and RIP assays, although low titers against a particular subtype were often found for MAbs that were negative in SATs and RIP assays. Because none of the MAbs reacted with F41 antigen, these low titers seemed to be specific, indicating that small parts of (for instance) subtype-specific epitopes not detectable by less sensitive techniques such as RIP assays and SATs are present on the other subtypes. When MAbs showed titers of ≥3.4 in the indirect ELISA, they also yielded positive SATs and RIP assays. In general, MAbs directed against two F4 subtypes had the highest affinities to the F4 subtype used for
immunization, indicating that their epitopes on both subtypes were partially identical.

The ELISAs with recombinant DNA strains demonstrated that epitope clusters a4, b1, and b2 were located on the F4ab major fimbrial subunit(s) and that epitope cluster c was located on the F4ac major fimbrial subunit(s) or perhaps the H protein. Gaastra and Amstrup-Pedersen (5) also showed that amino acids 80 to 264 of the major fimbrial subunits are important for the expression of subtype-specific epitopes. On the basis of these findings combined with the identical distribution patterns of MAbs of all epitope clusters along the fimbriae in IEM, we concluded that all MAbs were directed against epitopes on the major fimbrial subunit. In contrast to the findings of others (4), none of our MAbs reacted with denatured antigen in immunoblotting procedures (results not shown); this was also shown by Thiry et al. (28) with our MAb F4ac-3. These findings indicate that the MAbs are probably not directed against linear (continuous) epitopes. Attempts to map epitopes on the F4 major subunits by using the PEPSSCAN method failed. This method is especially suited for locating amino acids contributing to linear epitopes. Thus, it is probable that all epitopes recognized by our MAbs are conformational epitopes dependent on the configuration of the subunit or the quaternary structure of the whole fimbrial filament. The lack of MAbs against linear epitopes in this study probably is not due to the selection procedures of hybridoma supernatants because, if present, MAbs against linear epitopes accessible on native fimbriae should have been noticed in the ELISA. Another study (18), in which immunization with synthetic peptides from the sequence of F4 major fimbrial subunits failed to induce antibodies reacting with native fimbriae, also indicated that F4 epitopes are conformational epitopes. Further studies with recombinant DNA strains (D. Bakker et al., Microb. Pathog., in press), in which a part of the nucleotide sequence of the major fimbrial subunit of F4ab was replaced by heterologous sequences encoding viral epitopes, revealed parts of the F4 subunit essential to the expression of some epitopes recognized by our MAbs.

The results of the RIP assay, as shown in Fig. 2, suggest that F4 fimbriae are composed of a repeating single 27,500-dalton subunit; however, minor components that should coprecipitate may not have been detected because their concentrations were too low. Sometimes a second band of a protein with a molecular mass of 1,000 to 1,500 daltons greater than that of the major fimbrial subunit was observed. The H protein is the most likely candidate for this protein.

Previous work on the F41 antigen of ETEC, which is closely related to F4 (1, 23), showed that MAbs against only one of the five epitope clusters of F41 inhibited the in vitro adhesion of F41 (F. G. van Zijderveld, submitted for publication). Further studies with the F4 MAbs are in progress to evaluate their antiadhesive capacities in vitro and their protective capacities in challenge experiments when given orally to neonatal piglets.

For the replacement of polyvalent antisera by MAbs in diagnostic tests such as SATs and ELISAs to directly detect the F4 antigen in clinical specimens, almost all MAbs or combinations of MAbs are suitable, because the epitopes should be highly conserved, despite the existing antigenic variation found by conventional serologic tests. In particular, MAbs against epitope clusters a1, a2, and a3, with high titers in the different tests, seem to be the MAbs of choice for this purpose. Subtype-specific MAbs can be used for further typing of F4 strains in SATs. We successfully use MAb F4ad-11 as a coating antibody and a combination of MAb F4ac-5 and F4ab-1 as a conjugate in ELISAs to directly detect F4+ ETEC in feces. The results of these ELISAs correlate well with those of conventional bacteriological examination followed by SATs.

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LITERATURE CITED


