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Monoclonal Antibodies Against Colonization Factor Antigen I Pili from Enterotoxigenic *Escherichia coli*

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Hybridomas secreting monoclonal antibodies directed against intact colonization factor antigen I pili have been produced by the fusion of spleen cells from immunized BALB/c mice with NS1/SP2 myeloma cells. The four monoclones with the highest antibody titer, as detected by enzyme-linked immunosorbant assay (ELISA), were chosen for antibody amplification by production of mouse ascitic fluid. These four were examined for antibody specificity by ELISA and immunoblot assays, using six different pilus types. Three of the four monoclonal isolates were specific for only colonization factor antigen I pili in both assays, whereas the remaining isolate showed a distinct cross-reactivity with K99 pili in the ELISA assay but not in immunoblot analysis. These results indicate that this monoclone may be recognizing a common structural element between the two adhesive pilus types.

Colonization factor antigen I (CFA/I) consists of thread-like appendages located on the surface of enterotoxigenic *Escherichia coli*. These structures, or pili, are found on a 60 × 10^6-dalton plasmid which also encodes the heat-stable enterotoxin (4). Electron microscopy indicates that the CFA/I pilus is a rigid structure about 0.5 to 1.0 μm in length with a diameter of 7 nm (7). A single CFA/I pilus consists of about 100 identical protein subunits, each with a molecular weight of 15,058 (9).

CFA/I pili are necessary for the adherence and subsequent colonization of the intestinal mucosa of the human digestive tract (3, 4). Once colonized, the bacteria secrete toxins responsible for inducing the excretion of salts and water from the intestinal cells, resulting in diarrhea (3, 4). The antigenic characteristics of CFA/I pili are of interest in relation to the possible development of synthetic vaccines and in gaining an understanding of the mode of their attachment. This can be elucidated with the aid of hybridomas producing monoclonal antibodies possessing specificity for the antigenic determinant(s), be it sequence or conformation, of the intact pilus protein.

In this study, the identification of monoclonal antibodies specific to intact CFA/I pili is described. Data derived from the use of enzyme-linked immunosorbent assay (ELISA) and immunoblot analyses for the detection of antigen-antibody complexes with CFA/I pili as well as several other pilus systems are presented.

MATERIALS AND METHODS

Bacteria. The bacterial strain used for production of CFA/I pili was *E. coli* H-10407 (O78:H11), originally from a subject in Bangledesh (obtained from Margaret Finlayson, Provincial Laboratories, University of Alberta). This strain was grown on CFA agar (2), and random colonies were selected for mannose-resistant hemagglutination of human erythrocytes to check for the presence of CFA/I pili before additional pilus purification.

E. coli VIDO 1 (O101:K^-) $K99^+$ bacteria (obtained from J. F. C. A. Pantekoek, Alberta Agriculture, Edmonton, Alberta, Canada) grown on Minca medium (8) were used to examine the effect of CFA/I monoclonal and polyclonal antibodies on mannose-resistant hemagglutination of sheep erythrocytes.

Purification of whole CFA/I pili. H-10407 bacteria possessing CFA/I pili, as indicated by hemagglutination assays, were grown on CFA agar plates (2) at 37°C for 24 h. The bacteria were removed from the plate by gentle scraping and suspended in a solution containing 0.015 M sodium citrate and 0.15 M NaCl (pH 7.0) (SSC). A portion (0.2 ml) of this suspension was transferred to large petri dishes (14.4-cm diameter) containing CFA agar and incubated overnight at 37°C. Bacteria were removed from the large plates by scraping and resuspending in more SSC, after which the suspension was homogenized with a Sorvall Omnimixer at top speed for 5 min at 4°C to detach the pili. The cells were removed by centrifugation at 8,000 × *g* for 10 min, and the supernatant was subjected to an ammonium sulfate precipitation (25% [wt/vol]) at 4°C overnight. The precipitated pili were removed by centrifugation at 8,000 × *g* for 15 min. This crude pilus preparation was layered on a CsCl step gradient and subjected to ultracentrifugation at 20,000 rpm for 16 h,

using an SW27.1 rotor. The pilus band ($\rho = 1.307$) was removed from the gradient and dialyzed against deionized water to remove all CsCl. The resulting pilus preparation was checked for purity by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, as described previously by Armstrong et al. (1), and by electron microscopy.

Hemagglutination assays. H-10407 bacteria were tested for the presence of CFA/I pili by hemagglutination of human type A and AB erythrocytes. Colonies selected from CFA agar plates were suspended in 10 μl of 1% mannose (wt/vol) on glass slides. To this we added 10 μl of a 10% (vol/vol) erythrocyte suspension in 0.9% (wt/vol) saline. For inhibition of hemagglutination by CFA/I monoclonal antibodies and K99 pilus-specific antibodies (obtained from J. F. C. A. Pantekoek), the assay was performed as described above, except that 10 μl of the undiluted antibody source was added to the bacteria-mannose suspension before the addition of erythrocytes. Inhibition by the antibody was judged at 5 and 10 min after the addition of erythrocytes. K99-specific hemagglutination studies involved the agglutination of sheep erythrocytes with VIDO 1 $K99^+$ bacteria. The ability of CFA/I monoclonal antibodies, CFA/I polyclonal antibodies, and K99 polyclonal antibodies to inhibit this reaction was tested exactly as described above for the hemagglutination inhibition studies with CFA/I^+ bacteria.

Antisera production. Female BALB/c mice were injected intravenously via the tail vein with 10 μg of purified whole CFA/I pili in 0.2 ml of saline on days 0 and 7. The mice were bled by the tail vein, and antisera collected were tested for titer and specificity against whole CFA/I pili in an ELISA.

CFA/I pilus-specific polyclonal antisera were raised by injecting New Zealand white rabbits according to the method of Worobec et al. (14).

Fusion of BALB/c spleen cells and NS1/SP2 myeloma cells. Female BALB/c mice, immunized as described above, were injected intravenously with 10 μg of CFA/I pili in saline. Four days later, selected animals were sacrificed by cervical dislocation, the spleens were removed, and spleen cells were fused with cells from the NS1/SP2 myeloma cell line (an azoguanine-resistant, nonsecreting line derived from BALB/c mice) by the procedure of Kohler and Milstein (11). The resulting hybridomas were grown in hypoxanthine-aminopterin-thymidine (HAT) medium at 37°C and 95% humidity with 7% carbon dioxide. Eight days after the fusion, supernatants from 250 clones were screened by ELISA for anti-CFA/I pili antibody production. The positive clones were transferred to 24-well Linbro plates for additional growth. The clones were then subcloned by plating 100 μl of a 1 cell per ml suspension of each into 96-well Linbro plates. After allowing growth for 6 days, we tested the resulting monoclones for anti-CFA/I pili antibody activity by the ELISA. Ten monoclones were selected for additional growth; from these, four were chosen for antibody production in ascitic fluid. All 10 monoclonal cell lines were frozen in the presence of dimethyl sulfoxide and fetal calf serum, as described previously by Kohler and Milstein (11).

Production of ascitic fluid. Pristane (2,6,10,14-tetramethyl pentadecane)-primed BALB/c mice were irradiated with 500 rads. Twenty-four hours after irradiation, the mice were injected intraperitoneally with 3×10^6 to 6×10^6 hybridoma cells in 1 ml of saline. Ascitic fluid was collected 7 to 10 days later, using an 18-gauge needle. The fluid was tested for titer and specificity against CFA/I pili by the ELISA.

ELISA for hybridoma screening, titer determination, and monoclonal specificity. The ELISA used for hybridoma screening was a modification of the procedure of Voller et al. (13). Microtiter plates (96 wells) were coated with 100 μl of a solution (4 μg/ml) of CFA/I pili in 0.5 M bicarbonate coating buffer (pH 9.6) per well for 6 to 8 h at 4°C. After washing, the plates were incubated overnight at 4°C with 100 μl of supernatants from the growing hybridomas. Anti-CFA/I pili antibody activity was detected colorimetrically by using goat anti-mouse immunoglobulin G (IgG) conjugated to alkaline phosphatase (1:500; Boehringer Mannheim Corp.) and the alkaline phosphatase substrate *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets). Absorbance at 405 nm was determined with a Titertek multiscan ELISA plate reader. For titer determinations and specificity of supernatants and ascitic fluid from the CFA/I monoclones, the ELISA procedure was performed as described above except that the antigens were allowed to coat the microtiter wells for 16 h at 4°C, and after washing, the antigen-saturated plates were incubated for 2 h at room temperature with dilutions of supernatants or ascitic fluid in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20.

Class determination of CFA/I monoclonal antibodies. Supernatants of six CFA/I monoclones and ascitic fluid from the remaining four monoclones were used in the ELISA as previously discussed for immunoglobulin isotyping. Goat anti-mouse IgG1, IgG2A, IgG2B, IgG3, and IgM labeled with alkaline phosphatase were used as the second antibodies. Limiting dilutions of each monoclonal antibody were used with fixed dilution of the second antibody to distinguish between nonspecific binding and actual activity.

Transfer of proteins from SDS polyacrylamide gels to nitrocellulose paper (immunoblot). SDS-polyacrylamide gel electrophoresis was carried out as described previously by Armstrong et al. (1) except that thinner gels (0.75 mm) were used. Samples were boiled for 10 to 20 s in SDS before application to the gel. After electrophoresis, the proteins in the gel were transferred to nitrocellulose paper with an Electroblot Apparatus (E-C Apparatus) according to the procedure of Towbin et al. (12). The transfers were carried out for 3.5 h. Duplicate samples were stained directly with Coomassie blue according to Fairbanks et al. (5).

Immunological detection of proteins on nitrocellulose. Detection of proteins on nitrocellulose was as described previously by Towbin et al. (12). The monoclonal antibodies used were obtained from crude ascitic fluid. Antibody-antigen complexes on the nitrocellulose were labeled with ^{125}I-labeled protein A (New England Nuclear Corp.) and detected by X-ray film (Kodak X-AR) exposed to the dried nitrocellulose paper.

Electron microscopy. Intact CFA/I pili were incubated for 2 h at room temperature with the IgG component of mouse ascites induced by the WPC-5 cell line. (IgG was prepared with a protein A-Sepharose affinity column obtained from Pharmacia). One microliter of this preparation was placed on carbon-coated grids, negatively stained with 1% sodium phosphotungstate

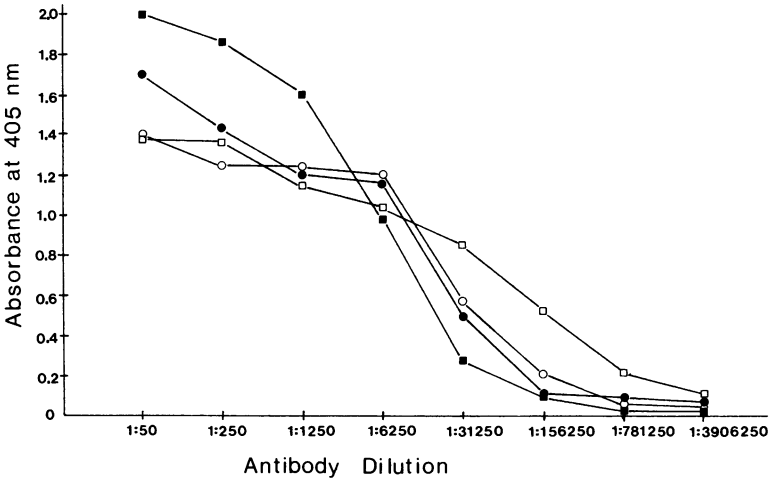

FIG. 1. Titer of CFA/I monoclonal antibodies as determined by ELISA. See text for details. Symbols: ○, WPC-1; ●, WPC-3; □, WPC-5; ■, WPC-6.

(pH 7.0), and photographed in a Phillips EM300 electron microscope. Antibody-free CFA/I pili were used as a control.

RESULTS

Monoclonal antibody production. After initial screening of 250 CFA/I hybridomas, 5 with the highest ELISA results were selected for subcloning. From the five 96-well plates obtained from subcloning, 10 monoclones were chosen for further growth and examination. These 10 clones were used for immunoglobulin isotyping, and all were found to belong to the IgG2A immunoglobulin class. The four monoclones with the highest titers, as determined by the ELISA, were used for the production of murine ascitic fluid, and it is this fluid which was used for subsequent examination. These four monoclones were termed WPC-1, WPC-3, WPC-5, and WPC-6.

Monoclonal antibody titer and specificity as determined by ELISA. Ascitic fluids from WPC-1, -3, -5, and -6 were used in an ELISA to estimate the titer of each monoclonal antibody. Figure 1 indicates the titer of each, each having a similar pattern with WPC-5 having the highest titer and WPC-3 the lowest.

Each of the four CFA/I monoclonal antibodies presented as ascitic fluid was examined for cross-reaction with other types of pili, using the ELISA. Microtiter wells were coated with 4 μg/ml of two conjugative, plasmid-encoded pilus types, EDP208 and ColB2, two adhesive pilus types, K99 from *E. coli* and gonococcal pili from *Neisseria gonorrhoea* (a gift from G. Schoolnik, Stanford University, Palo Alto, California), and pili from *Pseudomonas aeruginosa* K (kindly donated by T. Watts in our laboratory), along with CFA/I pili. Various dilutions of the four monoclonal antibodies were used, and antigen-antibody complexes were detected with goat anti-mouse IgG labeled with alkaline phosphatase. Figures 2B, C, and D show the ELISA results obtained with WPC-3, WPC-5, and WPC-6, respectively. These three showed no cross-reaction with any of the other types of pili. Figure 2A, on the other hand, indicates the distinct cross-reaction of WPC-1 with K99 pili and a trace reaction against ColB2 conjugative pili. This reaction could indicate the recognition of a common sequence or conformation but must be confirmed by other methods.

Monoclonal activity as detected by immunoblot analysis. An alternate method for examining the specificity of WPC-1, WPC-3, WPC-5, and WPC-6 was the immunoblot procedure. Five micrograms each of the six pilus types used in the aforementioned ELISA assay (EDP208, ColB2, K99, gonococcal pili, PAK, and CFA/I) were run in SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose paper. The paper was incubated with CFA/I monoclonal antibodies from one of the four ascitic fluids and then allowed to react with ^{125}I-labeled protein A from *Staphylococcus aureus*. The resulting antigen-antibody-protein A complexes were detected by autoradiography. Duplicate SDS-polyacrylamide gels were stained with Coomassie blue for direct visualization of the proteins. The positions of the six pilus types in each gel, as detected by staining, can be seen in Fig. 3A. The absence of these protein bands after transfer (Fig. 3B) shows that the transfer of proteins to nitrocellulose was efficient. Figures 3C, D, E, and F represent the autoradiographs obtained from the nitrocellulose papers treated

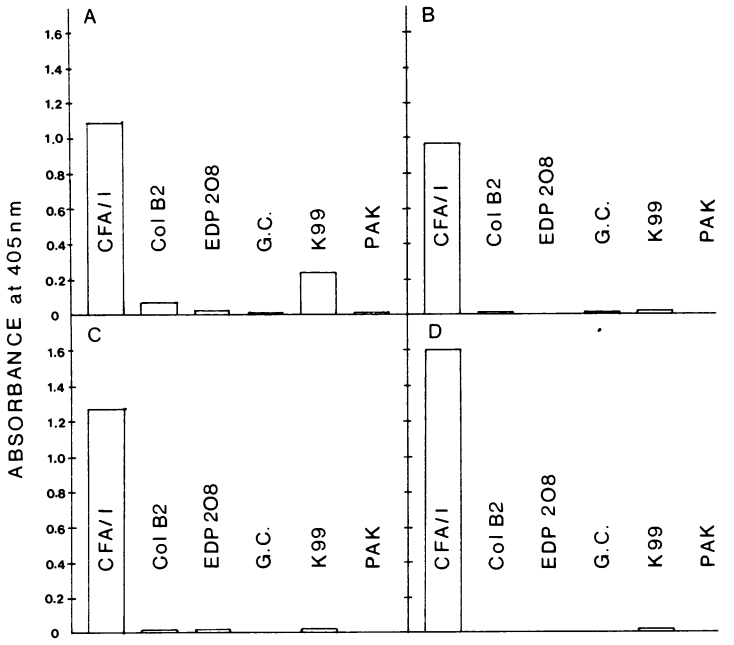

FIG. 2. Reaction of CFA/I monoclonal antibodies with various types of pili, as detected by ELISA. The ELISA assay was performed by coating microtiter wells with 4 μg of the indicated pili per ml, followed by incubation with a 1:1,250 dilution of ascitic fluid from (A) monoclone WPC-1, (B) monoclone WPC-3, (C) monoclone WPC-5, or (D) monoclone WPC-6. Antigen-antibody complexes were quantitated at 405 nm, using the goat anti-mouse IgG-alkaline phosphatase enzyme reaction. G.C., Gonococcal pili; PAK, *P. aeruginosa* K pili.

with monoclonal antibodies from WPC-1, WPC-3, WPC-5, and WPC-6, respectively. These results substantiate those obtained in the ELISA whereby WPC-3, WPC-5, and WPC-6 (Fig. 3D, E, and F, respectively) produced antibodies which recognized only CFA/I pili. WPC-1, contrary to the ELISA results, produced antibodies having a distinct specificity for CFA/I pili but no obvious cross-reactivity against the other pilus types, as detected by this assay.

Inhibition of hemagglutination. Equal volumes of 1% mannose, 10% human erythrocyte suspension, undiluted CFA/I monoclonal antibodies, and a 1/5 dilution of K99 antisera were used in the hemagglutination assay of CFA/I-bearing H-10407 bacteria. Antibodies from WPC-1, WPC-3, WPC-5, and WPC-6 were capable of inhibiting hemagglutination by CFA/I$^+$ bacteria, thus providing an alternate test of the specificity of these antibodies for CFA/I pili.

K99-specific antiserum (polyclonal) was also capable of inhibiting mannose-resistant agglutination of human erythrocytes by *E. coli* H-10407 (data not shown). However, attempts to inhibit K99-specific agglutination of sheep erythrocytes (6) with the CFA/I-specific monoclonal antibodies (WPC-1, WPC-3, WPC-5, and WPC-6) were unsuccessful. Thus, although some immunological cross-reactivity between CFA/I and K99 pili is suggested by the ELISA analysis of CFA/I monoclonal antibodies (Fig. 2A) and by the fact that K99 polyclonal antisera block CFA/I-specific hemagglutination, the extent of the homology is difficult to assess and must await additional elucidation by comparison of CFA/I and K99 amino acid sequences.

Electron microscopy studies of antibody-pilus binding. Protein A-Sepharose-purified IgG (see above) from WPC-5 murine ascitic fluid was diluted 1:100 and incubated with 5 μg of whole CFA/I pili. The attachment of these monoclonal antibodies to pili was examined by electron microscopy (Fig. 4B). Intact CFA/I pili, free of antibodies, were used as the control (Fig. 4A). The antibodies appear to be binding along the surface of the entire pilus, but no distinct pattern of binding seems obvious.

DISCUSSION

Klemm has determined the amino acid sequence of CFA/I pili (9) and has predicted the location of six antigenic fragments (10). In our laboratory, CFA/I pili have been cleaved with trypsin and CNBr with a view to identifying antigenically active peptides, using the CFA/I monoclonal antibodies discussed in this communication.

Of the four monoclones used in this study,

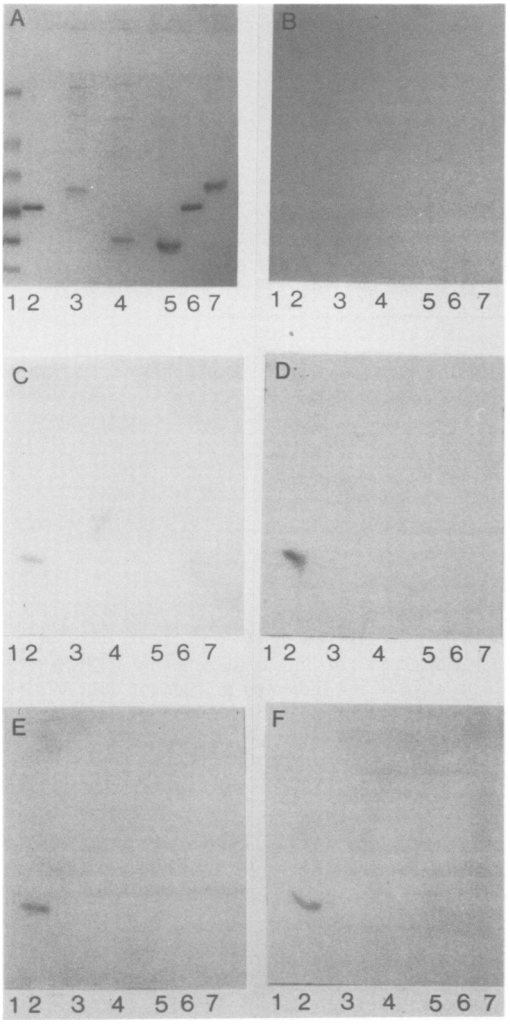

FIG. 3. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose paper. (A) Coomassie blue-stained pilus proteins after SDS-polyacrylamide gel electrophoresis. Lane 1, 5μg of prestained protein standards (Bethesda Research Laboratories); ovalbumin (43,000 daltons); α-chymotrypsinogen (25,700 daltons); β-lactoglobulin (18,400 daltons); lysozyme (14,300 daltons); cytochrome *c* (12,300 daltons); bovine trypsin inhibitor (6,200 daltons); and insulin (3,000 daltons); lane 2, 5 μg of CFA/I pili; lane 3, 5 μg of K99 pili; lane 4, 5 μg of F pili; lane 5, 5 μg of EDP208 pili; lane 6, 5 μg of *P. aeruginosa* K pili; lane 7, 5 μg of gonococcal pili (provided by G. K. Schoolnik, Stanford University). (B) Coomassie blue-stained duplicate of gel A after electrophoretic transfer of proteins to nitrocellulose. (C) Autoradiograph of proteins transferred from a duplicate of gel A to nitrocellulose paper and reacted with WPC-1 monoclonal antibodies (1:25) and ^{125}I-labeled protein A. (D) Autoradiograph of proteins transferred from a duplicate of gel A to nitrocellulose paper and reacted with WPC-3 monoclonal antibodies (1:50) and ^{125}I-labeled protein A. (E) Autoradiograph of proteins transferred from a duplicate of gel A to nitrocellulose paper and reacted with WPC-5 monoclonal antibodies (1:50) and ^{125}I-labeled protein A. (F) Autoradiograph of proteins transferred from a duplicate of gel A to nitrocellulose paper and reacted with WPC-6 monoclonal antibodies (1:25) and ^{125}I-labeled protein A.

three were highly specific for CFA/I pili and showed no reaction with any of the other pilus types. We plan to determine the fine specificity of these antibodies by using tryptic fragments of these pili. From the binding curves (Fig. 1), it appears that WPC-3 and WPC-6 are derived from the same mother clone. WPC-5, on the other hand, has a higher binding affinity and is probably derived from a distinct clone. WPC-1 displays a binding affinity similar to that of WPC-3 but also binds to K99 pili. Therefore, it must be a distinct monoclonal antibody from the others. The cross-reactivity displayed by WPC-1 is probably due to the recognition of a shared determinant between CFA/I and K99 pili. The very weak interaction with ColB2 pili is probably not significant.

The fact that all of these monoclones are of the IgG2A isotype is not surprising. Since we used hyperimmune mice for the fusions, it is possible that the only cells available for the fusion are the high-affinity IgG2A-producing B cells. This could also explain the similar affinity of all of the monoclones obtained in these experiments.

All four monoclonal antibodies will be used in mapping the antigenic determinants on CFA/I pili. Once determined, these antigenic fragments will be examined for their ability to induce antibody formation in a variety of animals. Such an undertaking has already been initiated, using a nonpathogenic, conjugative pilus type, EDP208 (14). Large quantities of the peptide were produced by solid-phase synthesis and covalently coupled to the carrier protein, bovine serum albumin. This peptide-bovine serum albumin conjugate was found to be immunogenic in rabbits, and antipeptide antibodies reacted strongly with native pili (14).

It is of interest that WPC-1 was found to react with another pilus protein involved in bacterial adhesion, K99. K99 is one of the factors responsible for bacterial colonization of calf and lamb intestines. Faris et al. (6) indicated that CFA/I and K99 pili attach to ganglioside GM_2-like glycoconjugates on erythrocytes, suggesting a common receptor. It is possible that WPC-1 is recognizing a common sequence in these two proteins.

Finally, the CFA/I monoclonal antibodies are being used in electron microscopic studies of antibody-pilus interactions. Preliminary results indicate that the antibodies from WPC-5 attach

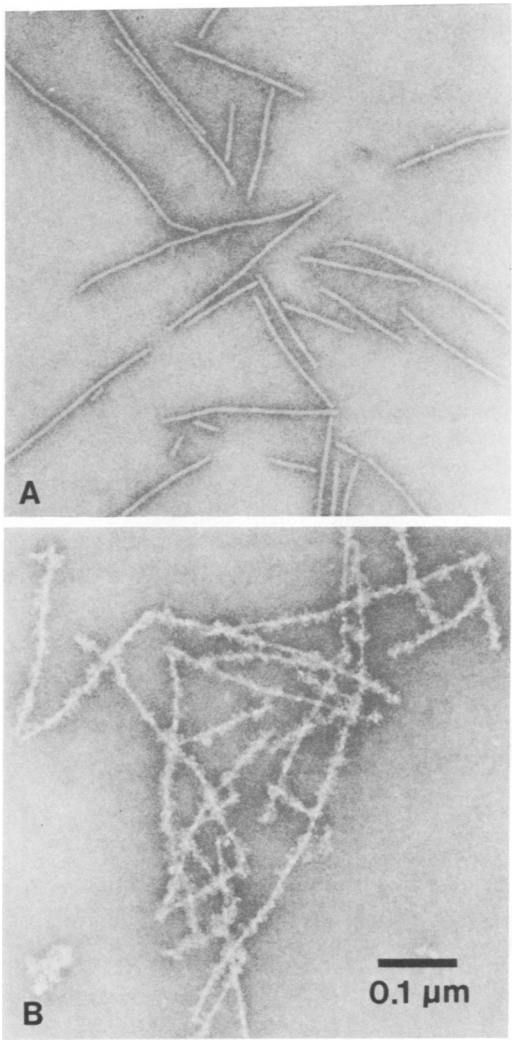

FIG. 4. Electron microscopy of CFA/I pili before and after addition of monoclonal antibodies. (A) Intact CFA/I pili with no antibodies added. (B) CFA/I pili treated with WPC-5 monoclonal antibodies before grid preparation.

to the entire surface of intact CFA/I pili and that no noticeable pattern is evident. Identification of the peptide domains involved in antigen-antibody interactions involving all four monoclones should provide clues as to those regions of the pilus polypeptide chain which are located on the surface of native pili.

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LITERATURE CITED

1. **Armstrong, G. D., L. S. Frost, H. J. Vogel, and W. Paranchych.** 1981. Nature of the carbohydrate and phosphate associated with ColB2 and EDP208 pilin. J. Bacteriol. **145**:1167–1176.
2. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
3. **Evans, D. G., D. J. Evans, Jr., W. S. Tjoa, and H. L. DuPont.** 1978. Detection and characterization of colonization factor of enterotoxigenic *Escherichia coli* isolated from adults with diarrhea. Infect. Immun. **19**:727–736.
4. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12**:656–667.
5. **Fairbanks, G., J. L. Stick, and D. F. J. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
6. **Faris, A., M. Lindahl, and T. Wadstrom.** 1980. GM_2-like glycoconjugate as possible erythrocyte receptor for the CFA/I and K99 haemagglutinins of enterotoxigenic *Escherichia coli*. FEMS Microbiol. Lett. **7**:265–269.
7. **Gaastra, W., and F. K. de Graaf.** 1982. Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. Microbiol. Rev. **46**:129–161.
8. **Guinée, P. A. M., J. Veldkamp, and W. H. Jansen.** 1977. Improved Minca medium for the detection of K99 antigen in calf enterotoxigenic strains of *Escherichia coli*. Infect. Immun. **15**:676–678.
9. **Klemm, P.** 1982. Primary structure of the CFA/I fimbrial protein from human enterotoxigenic *Escherichia coli* strains. Eur. J. Biochem. **124**:339–348.
10. **Klemm, P., and L. Mikkelsen.** 1982. Prediction of antigenic determinants and secondary structures of the K88 and CFA/1 fimbrial proteins from enteropathogenic *Escherichia coli*. Infect. Immun. **38**:41–45.
11. **Kohler, G., and C. Milstein.** 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur. J. Immunol. **6**:511–519.
12. **Towbin, H., J. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
13. **Voller, A., D. E. Bidwell, G. Huldt, and E. Engvall.** 1974. A microplate method of ELISA and its application to malaria. Bull. WHO **51**:209–221.
14. **Worobec, E. A., A. K. Taneja, R. S. Hodges, and W. Paranchych.** 1983. Localization of the major antigenic determinant of EDP208 pili at the N-terminus of the pilus protein. J. Bacteriol. **153**:955–961.