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Utilization of Anion-Exchange Chromatography and Monoclonal Antibodies to Characterize Multiple Pilus Types on a Uropathogenic *Escherichia coli* O6 Isolate

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Multiple pilus types from a uropathogenic strain of *Escherichia coli* O6, strain 6260, were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), high-pressure liquid chromatography, binding assays, and erythrocyte adsorption. In addition, monoclonal antibodies were raised against purified pili of *E. coli* 6260 and used for immunological characterization. SDS-PAGE analysis of the purified pili showed at least three different subunits with molecular weights of 15,700, 17,800, and 19,300. SDS-PAGE analysis of four protein peaks from anion-exchange chromatography of intact pili showed polypeptides with molecular weights of 19,300 (fraction 1), 15,700 (fraction 2), and 17,800 and 15,700 (both fractions 3 and 4). Erythrocyte adsorption of the whole-pilus preparation removed the 17,800-molecular-weight subunit (17.8K subunit) and reduced the 15.7K subunit. Pili from an isogenic hemagglutination-negative variant of *E. coli* 6260, showing only the 15.7K and 19.3K subunits by SDS-PAGE, lacked the 17.8K subunit of fractions 3 and 4 present in the parent high-pressure liquid chromatography profile. Our data suggest that two of the pilus subunits, the 15.7K and 17.8K subunits, mediate mannose-resistant agglutination of human erythrocytes. Pili in fractions 1 and 2 from the parent strain bound specifically to mannose residues, while pili in fraction 4 bound to P-coated horse erythrocytes; no receptor specificity was identified for pili in fraction 3. Immunological analysis by the immunoblot technique showed that monoclonal antibody 11-2 reacted with the 19.3K subunit, monoclonal antibodies 34-3 and 73-3 reacted with the 15.7K subunit, and monoclonal antibodies 81-1, 82-1, and 91-1 reacted with polymers of subunits retained in the stacking gel. Intact pili precipitated by any of the six monoclonal antibodies showed two polypeptides by SDS-PAGE: 15.7K and 19.3K polypeptides for monoclonal antibody 11-2, and 15.7K and 17.8K polypeptides for monoclonal antibodies 34-3, 73-3, 81-1, 82-1, and 91-1. The cross-reactivity of the monoclonal antibodies with purified pili from other *E. coli* strains was determined by enzyme-linked immunosorbent assay. Monoclonal antibody 11-2 showed no significant cross-reactivity with heterogeneous pili. In contrast, the other monoclonal antibodies showed equivalent or greater reactivity with P pili from heterologous strains as compared with reactivity with *E. coli* 6260 pili. All six monoclonal antibodies inhibited agglutination of human erythrocytes by *E. coli* 6260; only monoclonal antibody 11-2 showed notable differences in reciprocal titers of hemagglutination inhibition (10 in the presence of mannose versus 1,000 in the absence of mannose). Our results suggest that *E. coli* 6260 pili are composed of at least four subunit types: 15.7 and 19.3K subunits involved in mannose-sensitive agglutination, and a second 15.7K subunit, in addition to a 17.8K subunit, involved in mannose-resistant agglutination.

Escherichia coli is the most common etiologic agent of urinary tract infection. The ability of a strain to attach to mucosal surfaces is usually a prerequisite for successful colonization of the urinary tract (12, 31); however, bacterial adherence capabilities are less important in urinary tracts of patients with reflux (23). Bacterial attachment to epithelial cells, as well as hemagglutination (HA), appears to be mediated by pili or accessory polypeptides which copurify with pili (13, 17, 24, 32). *E. coli* adhesins have been divided into two classes based on the ability of mannose to inhibit adherence reactions: mannose-sensitive (MS), or type 1, pili and mannose-resistant (MR) pili (6). A predominant MR adhesin of uropathogenic *E. coli*, termed P pilus, recognizes a glycolipid receptor found on human erythrocytes and epithelial cells (22, 35). Other types of MR pili bind to neuraminic acid-containing structures on human erythrocytes (S pili) or recognize a membrane glycoprotein common to M blood group antigens (27, 34).

The presence of multiple pilus types on uropathogenic *E. coli* strains is suggested by clinical isolates which show both MS HA and MR HA (11, 13), by the demonstration of multiple polypeptides in pilus preparations from individual strains by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16), by crossed immunoelectrophoresis (20), and by the detection of dual pilus types on single cells by using immunofluorescence (25). The purpose of our study was to separate and characterize the multiple pilus types of a uropathogenic *E. coli* strain. We have taken a different approach to pilus separation and isolation by using DEAE anion-exchange chromatography, a procedure which separates intact pili based on charge differences rather than differences in the susceptibility of pili to chemical depolymerization and subsequent isolation of pilus subunits (19). In some cases, chemical dissociation and reassembly of subunits alter biological activity (10, 36). Our technique, which separates distinct types of intact pili, circumvents the need to reassociate pilus subunits before determining the biological activity of a particular pilus type. In addition, we used a panel of pilus subunit-specific and quarternary struc-

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ture-specific monoclonal antibodies (MAb) to characterize the multiple pilus types.

MATERIALS AND METHODS

Strains, culture conditions, and piliation. *E. coli* strains from this laboratory, including 6260, 257, 6230, 1635, and 6824, are clinical isolates from adult patients with urinary tract infections. *E. coli* 6260, the strain used to prepare purified pili for immunization and subsequent MAb production, demonstrates both MR (P) and MS agglutinins. The representative HA-negative variant of *E. coli* 6260 was isolated by screening 1,384 colonies for the ability to agglutinate human erythrocytes in an MR manner. A total of 20 stable HA-negative variants which maintained this characteristic after four subcultures were identified. Strains 257 and 6230 demonstrated only MS agglutinins, and strains 1635 and 6824 demonstrated only MR agglutinins. Strain CSH50 (7), kindly provided by B. Eisenstein, expresses type 1 pili. Strain HU849 (15), kindly provided by R. Hull, contains genes for the expression of P pili cloned from a clinical isolate. Bacteria were routinely grown on tryptic soy agar (Becton Dickinson and Co., Cockeysville, Md.) at 37°C. The presence of pili on a strain was determined as follows: bacterial samples were applied to steel grids coated with 1% nitrocellulose in amyl acetate (Ernest F. Fullam Inc., Schenectady, N.Y.), shadow cast with chromium, and examined with an electron microscope (model 100 CX; JEOL Co., Tokyo, Japan).

Purification of pili and SDS-PAGE. Electrophoretic separations were done by the method of Laemmli (21). Samples of pili purified by the method of Dodd and Eisenstein (5) were diluted 1:2 in sample buffer (0.125 M Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.005% bromophenol blue [pH 6.8]) and heated to 100°C for 5 min, and 50-μl volumes were loaded onto a 5% stacking, 10% resolving gel. Polypeptide bands were stained with 0.25% Coomassie blue R250.

Erythrocyte adsorption of *E. coli* 6260 pili. Purified pili (50 μg suspended in 100 μl of phosphate-buffered saline [PBS]) were incubated with human type O erythrocytes (50% suspension in PBS; Sigma Chemical Co., St. Louis, Mo.) for 1 h at 25°C. Erythrocytes were pelleted by centrifugation; adsorbed supernatants were mixed with sample buffer (1:1) and applied to a gel for SDS-PAGE analysis.

Separation of intact pili by anion-exchange chromatography. High-pressure liquid chromatography (HPLC) profiles of pilus preparations were determined by using the 8800 series gradient liquid chromatographic system with an 860 absorbance detector (Du Pont Co., Wilmington, Del.). The pilus preparations were centrifuged (2,000 × *g*) and the pilus supernatant was filtered; 50-μl samples (200 to 1,000 μg of protein per ml) were applied to a protein PAK DEAE-5PW column (Waters Associates, Inc., Milford, Mass.) and eluted under the following conditions: a 20-min gradient (exponent, −3) of 0.02 M Tris hydrochloride buffer from pH 8.0 to pH 8.0, containing 0.5 M NaCl; and a flow rate of 1 ml/min, monitored at A_{285}. Data were recorded on a chromatography integrator (model SP4270; Spectra-Physics, San Jose, Calif.). Fractions were collected, dialyzed against deionized water, lyophilized, suspended in sample buffer, and applied to a gel for SDS-PAGE analysis.

Binding assays with purified pili. ^{125}I-labeled (4) *E. coli* 6260 pili were fractionated by HPLC as described, and the resultant four fractions were reapplied to the column for further purification. The repurified fractions were used in the following assays. Samples of the ^{125}I-labeled pilus fractions (0.06 to 3 μg) were incubated with immobilized D-mannose (25 μl of a 5% aqueous slurry; Pierce Chemical Co., Rockford, Ill.) or P-receptor-coated horse erythrocytes (10 μl of a 0.5% suspension) for 1 h at 25°C (18); sediments were washed extensively in PBS and counted in a gamma counter.

Production of MAb. The immunization protocol followed was similar to the procedure of Eisenstein et al. (8). Briefly, female BALB/c mice were immunized intraperitoneally with 0.25 ml of purified pili (5, 10, or 15 μg) emulsified in 0.25 ml of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). After 2 weeks, an intraperitoneal injection was given with Freund incomplete adjuvant. One month later, the mice received tail vein injections of 0.1 ml of purified pili diluted in PBS. Three days later, the spleens were harvested.

The protocol followed for MAb production was previously described (26). Briefly, 10^8 nonsecreting cells from the SP2/O-Ag14 BALB/c myeloma cell line (29) were combined with 6.8×10^8 spleen cells obtained from immunized mice and fused with polyethylene glycol. The hybridomas were screened for antibody production by enzyme-linked immunosorbent assay (ELISA). Antibody-producing cells were cloned by a limiting dilution technique and retested by ELISA. Selected clones were expanded in tissue culture for the collection of supernatants. The supernatants were tested for immunoglobulin isotypes by using a mouse immunoglobulin subtype identification kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

ELISA. ELISAs were performed as previously described (5, 9). Briefly, microtiter wells were coated with 150 μl of purified pili (5 μg/ml) suspended in 1 M $NaHCO_3$–0.5% polyethylene glycol (pH 9.6) overnight at 25°C and then rinsed in PBS containing 0.05% Tween 80 (PBS-T). A 50-μl vol of monoclonal supernatant was added to each well, incubated for 2 h at 25°C, and rinsed in PBS-T. A 150-μl volume of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (dilution, 1:1,500; Cooper Biomedical, Inc., West Chester, Pa.) was added to each well, and the wells were incubated for 3 h, washed with PBS-T, and incubated with 150 μl of substrate buffer (0.08% 5-aminosalicylic acid, 0.005% H_2O_2, [pH 6.0]) for 10 min at 25°C. Plates were read at 405 nm with an MR580 plate reader (Dynatech Laboratories, Inc., Alexandria, Va.).

Immunoelectron microscopy. For immunoferritin labeling (30), bacteria-coated grids were placed sequentially in drops of: PBS, twice for 30 s each; monoclonal supernatants, 60 min; PBS, twice for 30 s each; 1:10 dilutions of ferritin-labeled goat anti-mouse immunoglobulin (Cooper Biomedical), 15 min; and deionized water, twice for 30 s each. Grids were blotted dry after each step.

Immunoblots of pili. After SDS-PAGE, the polypeptides were electroeluted onto untreated nitrocellulose paper (NCP) by using an electroblot apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) at 6 to 8 V/cm for 16 h in 20 mM Tris–150 mM glycine–20% methanol (33). The NCP was soaked in TSGAN buffer (50 mM Tris, 0.15 M NaCl, 0.25% gelatin, 0.15% sodium azide, 0.1% Nonidet P-40 [pH 7.5]) (3) containing 5% bovine serum albumin, washed in TSGAN buffer, and incubated overnight at 25°C in MAb. The NCP was washed in TSGAN buffer, incubated for 2 h with iodinated (4) protein A (Sigma) diluted in 100 ml of TSGAN buffer to 30,000 cpm/ml (specific activity, 15,000 cpm/μg). The NCP was washed extensively with TSGAN buffer, air dried, and placed next to preflashed X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C with an intensifying screen (Du Pont).

RIP. The first radioimmunoprecipitation (RIP) method

used was a modification of techniques previously described (5, 14). Briefly, a 10% (wt/vol) suspension of protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was incubated with a 1:100 dilution of goat anti-mouse immunoglobulin (Cooper Biomedical) at 4°C for 18 h. The complex was washed with PBS-T, incubated in 5% bovine serum albumin–PBS-T at 25°C for 15 min, and then washed in PBS-T. The protein A-Sepharose–goat anti-mouse immunoglobulin complex was incubated with MAb at 4°C for 18 h. The washing and bovine serum albumin blocking steps were repeated. The protein A-Sepharose–goat anti-mouse immunoglobulin–mouse MAb complex was then incubated with ^{125}I-labeled (4) *E. coli* 6260 pili at 25°C for 2 h. The sediment was washed, suspended in sample buffer, and separated by SDS-PAGE, and the polypeptides were electroeluted onto NCP as described above.

The second RIP technique was performed by the method of Rhen et al. (28). Briefly, ^{125}I-labeled *E. coli* 6260 pili were dialyzed against 10 mM Tris hydrochloride buffer (pH 7.5) for 18 h. The pili protein concentration was adjusted to 200 μg/ml with buffer, deoxycholate (Sigma) was added to prevent pilus aggregation, and the mixture was incubated at 25°C. After 2 h, 250 μl of MAb was added, and the mixture was incubated for 3 h at 37°C with intermittent shaking and then incubated at 4°C for 18 h; the precipitates were collected by centrifugation (15 min at 15,000 × *g*), washed in buffer, suspended in sample buffer, and separated by SDS-PAGE. Polypeptides were electroeluted onto NCP and analyzed after autoradiography.

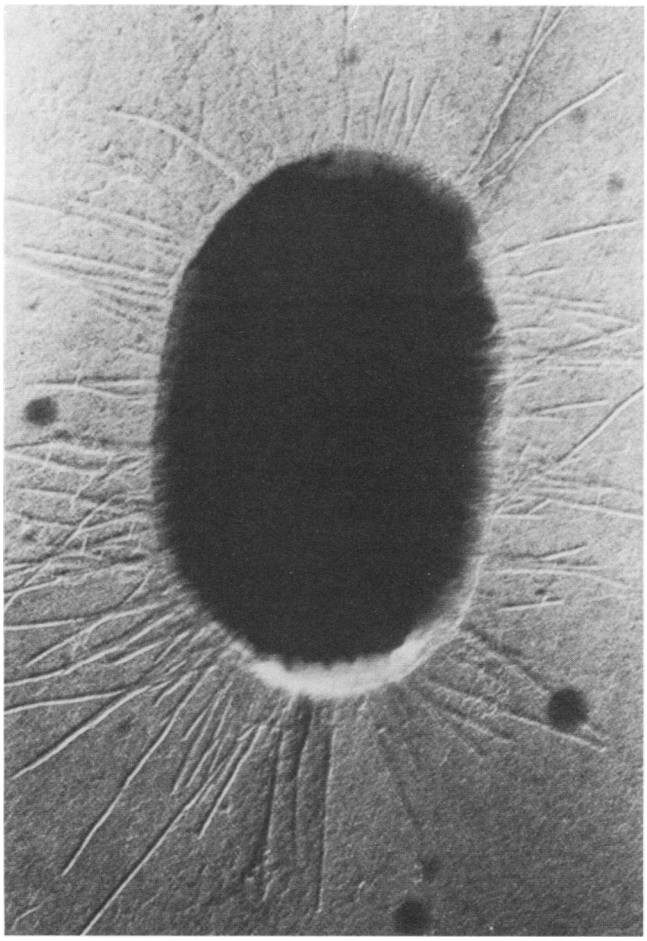

FIG. 1. Electron micrograph of piliated *E. coli* 6260. Magnification, ×72,000.

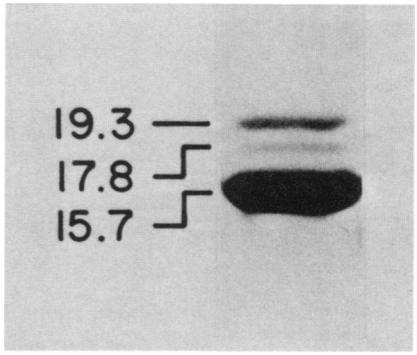

FIG. 2. SDS-PAGE analysis of purified pili from *E. coli* 6260. Molecular weights (in thousands) are indicated to the left of the lane.

HI assay. The lowest concentration of bacteria (4×10^8 cells per ml) that produced a strong agglutination reaction was used for the HA inhibition (HI) assays. Initially, 10-fold dilutions of MAb (50 μl) were combined with equal volumes of bacterial suspension; MAb were used in concentrations that did not agglutinate the bacteria. A 50-μl volume of human type O erythrocytes (0.5%), either in the presence or absence of 2.5% α-methyl-D-mannoside, was added to the mixture. The HI assay mixture was incubated initially at 25°C and read at 2 h and then incubated at 4°C and read again at 16 h. The HI titers are expressed as the reciprocal of the highest dilution of MAb which inhibited erythrocyte agglutination; each test well was compared with a positive control well.

RESULTS

SDS-PAGE of *E. coli* 6260 pili. An electron micrograph of *E. coli* 6260 showed numerous pili extending from the surface of the cell (Fig. 1). Purified pili were separated by SDS-PAGE, and at least three types of subunits (molecular weight range, 16,000 to 19,000) were consistently observed (Fig. 2). Additionally, a fourth, a 22,000-molecular-weight polypeptide (22.5K polypeptide), was observed when the gel was overloaded with sample or when the pilus preparation was treated with acid before application to the gel (not shown).

Investigation of the erythrocyte-binding activity of *E. coli* 6260 subunits. Pilus preparations from isogenic HA-negative variants were compared with pili from the HA-positive parent (Fig. 3). The subunit profile of a representative variant, which did not agglutinate human erythrocytes, lacked the 17.8K polypeptide (Fig. 3, lane 1) present in the parent strain (lane 2). This same polypeptide was adsorbed from the parent pilus preparations by human erythrocytes (lane 3). In addition, the 15.7K polypeptide was partially reduced by the erythrocyte adsorption. A low-molecular-weight erythrocyte polypeptide (Fig. 3, lane 3) was observed in the erythrocyte control (lane 4).

Separation of multiple intact pilus types by HPLC. Purified pili from *E. coli* 6260 were applied to a DEAE anion-exchange column; the elution profile of the pili consistently showed four peaks of protein with reproducible retention times (Fig. 4A). Examination by electron microscopy showed that each of the four corresponding fractions contained nonaggregated pili of similar physical appearance (not shown). When pili from *E. coli* 6260 were compared with pili

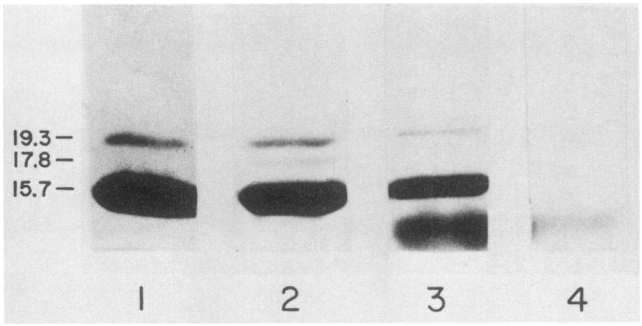

FIG. 3. SDS-PAGE analysis of *E. coli* 6260 purified pili to determine which subunits have erythrocyte-binding activity. Lane 1, Pilus preparation from HA-negative isogenic variant; lane 2, pilus preparation from HA-positive parent; lane 3, pilus preparation from HA-positive parent after adsorption with erythrocytes; lane 4, adsorption control (supernatant from erythrocyte suspension).

from HA-negative variants by HPLC (Fig. 4B), the most striking difference between the parent and the variant was the absence of the third protein peak in the variant profile. There was also a notable quantitative difference in the second peak when the unadsorbed parent and variant pilus preparations were compared. HPLC fractions were collected from the parent and variant pili, dialyzed against deionized water, freeze-dried, and analyzed by SDS-PAGE (Fig. 5). Fractions 1 and 2 from the parent pilus preparation (Fig. 5A, lanes 1 and 2) were composed of single types of polypeptides, while two polypeptides were demonstrated in each of the fractions 3 and 4 (Fig. 5A, lanes 3 and 4). Fractions 1 and 2 from the variant pilus preparation (Fig. 5B, lanes 1 and 2) resembled those of the parent pili. However, the 17.8K and 15.7K polypeptides collected in fraction 3 of the HA-positive parent were absent from the fraction 3 area of the HA-negative variant (Fig. 5B, lane 3). In addition, only the 15.7K polypeptide was demonstrated in fraction 4 of the variant pilus preparation (Fig. 5B, lane 4).

Binding specificities of HPLC fractions containing intact pili. The four labeled pilus fractions were tested for their

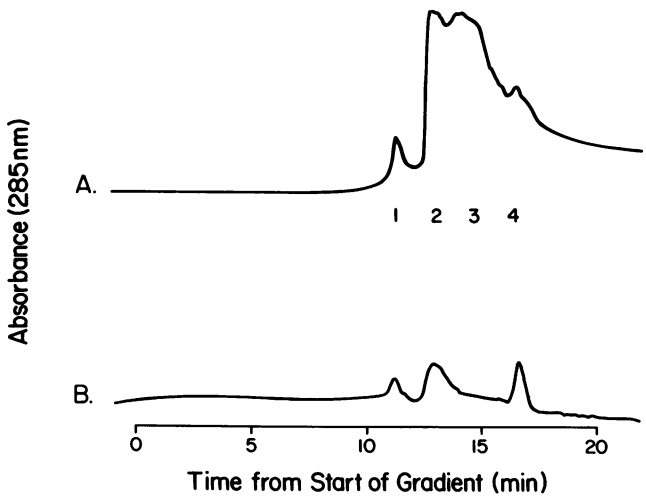

FIG. 4. HPLC protein profiles of pili preparations from the *E. coli* 6260 HA-positive parent (A) and the HA-negative isogenic variant (B). Peaks 1, 2, 3, and 4 correspond to retention times of 12.6, 13.7, 14.8, and 16.5 min, respectively.

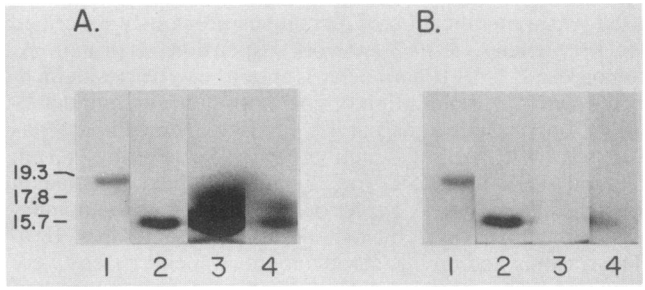

FIG. 5. SDS-PAGE analysis of HPLC fractions of purified pilus preparations from the *E. coli* 6260 HA-positive parent (A) and the *E. coli* 6260 HA-negative variant (B). Lanes 1, 2, 3, and 4 represent HPLC peaks 1, 2, 3, and 4, respectively.

ability to bind to mannose residues and to P (globoside)-coated horse erythrocytes (Fig. 6). The binding of HPLC fractions 1 and 2 to immobilized mannose particles was significantly higher than that of fractions 3 and 4 (Fig. 6A). The binding reactions of fractions 1 and 2 were blocked by 2.5% α-methyl-D-mannoside, while the reactions of fractions 3 and 4 were unchanged by the added carbohydrate. Fraction 4 demonstrated notable binding to P-coated horse erythrocytes, in contrast to fraction 3 (Fig. 6B). The interaction was specifically inhibited by free globoside. Fractions 1 and 2 showed no P-receptor specificity.

Immunoelectron microscopy. Six MAb were selected for

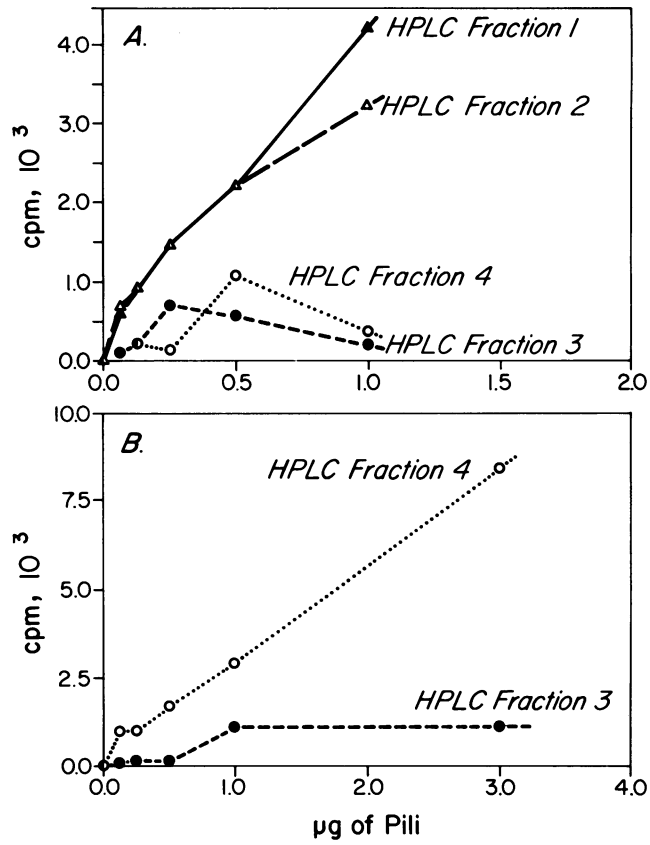

FIG. 6. Binding of HPLC fractions containing ^{125}I-labeled *E. coli* 6260 pili to immobilized D-mannose (A) or P-receptor-coated horse erythrocytes (B).

study: 11-2, 34-3, and 81-1 (all immunoglobulin M [IgM]); 73-3 (IgG1); 82-1 (IgG3); and 91-1 (IgG2b). Each of the MAb were shown to react with the pili of *E. coli* 6260 by using the immunoferritin technique (Fig. 7).

Binding of MAb to dissociated pili. The immunoblot technique was used to analyze the interaction between pilus subunits and each of the six MAb; pilus subunits were separated by SDS-PAGE, electroeluted onto NCP, and incubated with MAb (Fig. 8). MAb 34-3 (Fig. 8, lane 1) and 73-3 reacted with the 15.7K subunit; MAb 11-2 reacted with the 19.3K subunit (lane 2); MAb 81-1 (lane 3), 82-1, and 91-1 reacted only with polymers of the subunit retained in the stacking gel.

MAb reactivity with intact pili of *E. coli* 6260. Two RIP techniques were used to determine the specificity of each of the MAb for intact pili of *E. coli* 6260 (Fig. 9). Autoradiographic results obtained by the first technique, specific removal of antigen-antibody complexes by protein A-Sepharose CL-4B coated with goat anti-mouse immunoglobulins, are shown in Fig. 9A. The pili specifically reacting with MAb 11-2 (Fig. 9A, lane 1) showed two polypeptides with molecular weights of 15,700 and 19,300. Pili interacting with MAb 34-3 (lane 2) as well as those interacting with MAb 73-3, 81-1, 82-1, and 91-1 (data not shown) demonstrated the 15.7K and 17.8K subunits. In addition, the second precipitation method, involving centrifugation of precipitated pili complexed with MAb, gave similar results (Fig. 9B), except that negligible precipitates were collected at 16 h when MAb 73-3 or 82-1 was incubated with pilus preparations (Table 1).

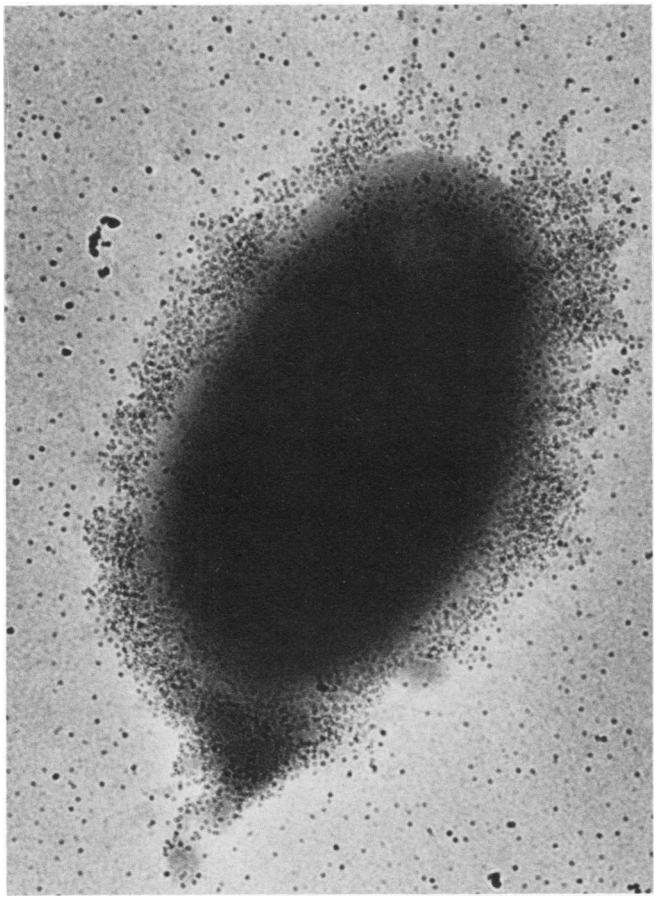

FIG. 7. Electron microscopy by the immunoferritin technique for visualization of the binding of MAb to the surface of *E. coli* 6260. Magnification, ×48,000.

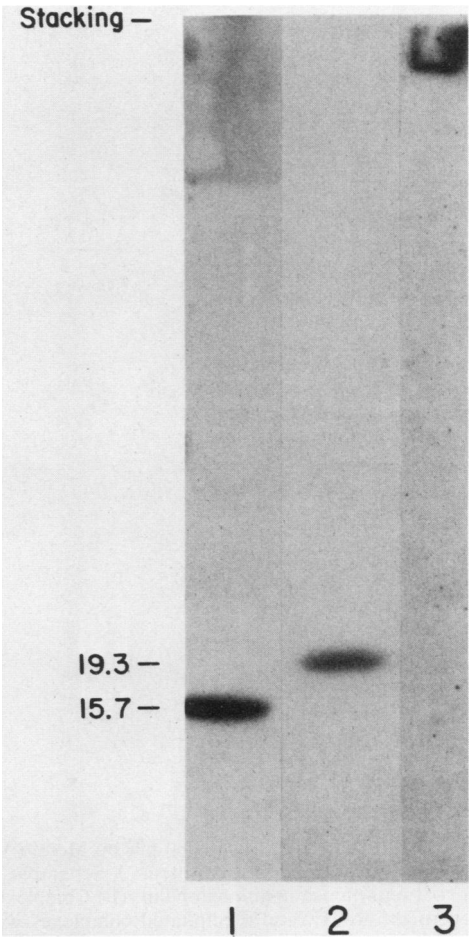

FIG. 8. Immunoblot analysis of *E. coli* 6260 pilus subunits incubated with MAb 34-3 (lane 1), 11-2 (lane 2), or 81-1 (lane 3).

Cross-reactivity of heterogenous pili. The ELISA technique was used to detect the reactivity of the MAb with purified pili from six other *E. coli* strains as well as pili from the homologous strain, *E. coli* 6260 (Table 2). MAb 11-2, specific for the 19.3K pilus subunit, reacted strongly only with pili from the homologous strain. MAb 34-3 and 73-3, both reacting specifically with the 15.7K subunit, showed equivalent or greater reactivity with heterologous P pili purified from two clinical isolates. In addition, MAb 73-3 cross-reacted with pili isolated from a strain showing MS agglutination. MAb 81-1 was similar to 73-3, reacting with both P pili and type 1 pili, although the cross-reaction with the MS pili was weaker. Similarly, MAb 82-1 and 91-1 both showed notable cross-reactivity with P pili from one *E. coli* strain, 1635, and weak reactivity with type 1 pili.

MAb inhibition of *E. coli* 6260-mediated HA. Results from the HI assays demonstrated that MAb directed against *E. coli* 6260 pili blocked the agglutination of human type O erythrocytes by intact *E. coli* 6260 (Table 3). Inhibition of MR HA was detected by HA assays in the presence of mannose; inhibition of MR HA or MS HA was detected by HA assays in the absence of mannose. The reciprocal titers observed for HI with MAb 11-2 were 100-fold different when MRHA only (10) was compared with MR HA and MS HA

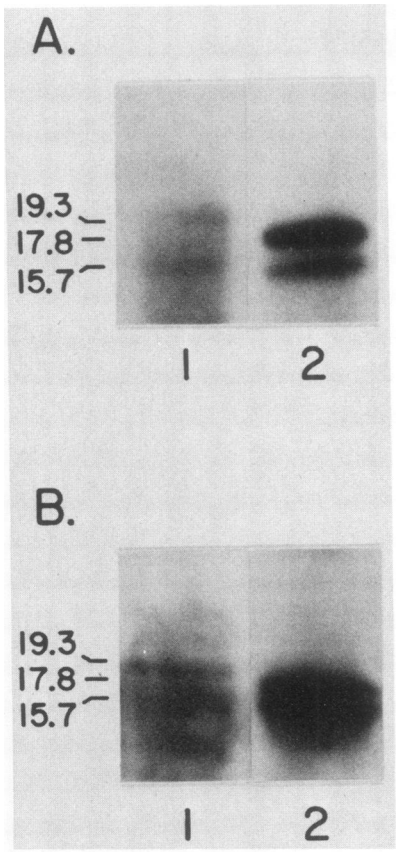

FIG. 9. RIP of ^{125}I-labeled *E. coli* 6260 pili by MAb. (A) Pilus-MAb complexes were adsorbed to protein A-Sepharose CL-4B coated with goat anti-mouse immunoglobulin. (B) Complexes were incubated overnight at 4°C, and precipitated complexes were collected by centrifugation. Each pilus preparation was incubated with MAb 11-2 (lane 1) or 34-3 (lane 2).

(1,000); MAb 34-3 and 73-3 both showed 10-fold differences in titers. In contrast, the other three MAb showed identical reciprocal titers of HI in the presence and in the absence of mannose. In addition, the MAb were tested to determine whether the supernatants inhibited yeast agglutination or globoside-coated horse erythrocyte agglutination induced by *E. coli* 6260; none of the MAb blocked agglutination of yeast or globoside-coated horse cells by *E. coli* 6260 (data not shown).

DISCUSSION

In a recent study of 211 strains of uropathogenic *E. coli* (11), we reported that *E. coli* expressing MR agglutinins concurrently with MS agglutinins predominated in urinary tract infections. This observation suggested the presence of multiple pilus types on these strains. We selected *E. coli* 6260, a clinical isolate demonstrating both MR and MS agglutinins from the original study, to use in the biochemical and immunological characterization of multiple pilus types on a single clinical strain.

Our results indicated that a wide array of pili, four or more pilus types, were expressed by *E. coli* 6260. The pili were purified by the method of Dodd and Eisenstein (5), in which a 5 M urea buffer was used to disaggregate flagella contaminating the pilus preparation. Thus, the pilus types we have studied are urea resistant. This may not always be the case with *E. coli* pili, as Karch et al. (19) observed two pilus types

TABLE 1. Immunoblot and RIP results for *E. coli* 6260 pili incubated with MAb

MAb	Isotype	Molecular weight (10^3) of reactive pilus subunits in the following assay:	
		Immunoblot	RIP
11-2	IgM	19.3	19.3, 15.7
34-3	IgM	15.7	17.8, 15.7
73-3	IgG1	15.7	17.8, 15.7
81-1	IgM	—[a]	17.8, 15.7
82-1	IgG3	—	17.8, 15.7
91-1	IgG2b	—	17.8, 15.7

[a] —, MAb reacted with polymers of subunits retained at the top of the stacking gel.

that were susceptible to disruption by 6 M urea. It is possible that our strain 6260 has urea-sensitive pili undetected by our methods. However, SDS-PAGE analysis of pilus preparations showed multiple subunits, regardless of sample preparation; the non-acid-treated pilus mixture was composed of at least three subunit types, with an additional polypeptide seen after treatment of the pili with acid. Amino-terminal sequencing of the pilus mixture (data not shown) showed four different amino acids at 3 of the 11 positions which were sequenced; these data corroborate the SDS-PAGE analysis, suggesting the presence of at least four different polypeptides in the pili preparation. The anion-exchange chromatographic profiles showed that purified intact pili of *E. coli* 6260 were separated into four subpopulations based on their net charge differences. Two of these four subpopulations contained only one pilus type: fraction 1 contained a 19.3K subunit and fraction 2 contained a 15.7K subunit. Fractions 3 and 4 both contained 17.8K and 15.7K polypeptides, suggesting in each case that two of the pilus types have similar net charges or that a single pilus type is composed of two subunits. The 15.7K polypeptide was detected in three of the fractions, suggesting that the pilus preparation contains several polypeptides with similar migration rates on SDS-PAGE but which can be separated by net charge by using HPLC.

Initially, we studied the biological activity of the multiple pilus types by subunit analysis. Results of the study comparing pili purified from an HA-negative variant to pili from the HA-positive parent, *E. coli* 6260, suggested that the 17.8K subunit is a functional binding unit in the MR agglu-

TABLE 2. ELISA results, showing cross-reactivity of MAb with pilus preparations from other *E. coli* strains

MAb	% Absorbance for purified pili from *E. coli* strain[a]:						
	6260	CSH50	257	6230	HU849	1635	6824
11-2	100	0	0	9	3	15	2
34-3	100	25	35	29	13	100	185
73-3	100	16	11	144	0	325	192
81-1	100	38	0	16	0	115	225
82-1	100	15	0	24	0	54	8
91-1	100	19	0	29	2	157	2

[a] Absorbance values for homologous strain 6260 are represented as 100%; absorbance values for other strains are expressed as percentages relative to the homologous strain; samples were tested in triplicate. The homologous strain demonstrated MR agglutination of human erythrocytes and globoside-coated horse erythrocytes and MS agglutination of yeast cells; strains CSH50, 257, and 6230 demonstrated MS agglutinins only; strains HU849, 1635, and 6824 demonstrated agglutinins for globoside-coated horse erythrocytes only.

TABLE 3. MAb inhibition of *E. coli* 6260-mediated human erythrocyte agglutination

MAb	Reciprocal of highest dilution of MAb supernatant which demonstrates HI[a]	
	With mannose	Without mannose
11-2	1,000	10
34-3	1,000	100
73-3	1,000	100
81-1	100	100
82-1	100	100
91-1	100	100

[a] All tests were done in duplicate. The HI assay was performed in the absence of α-methyl-D-mannoside to detect both MR HA and MS HA and performed with the addition of 2.5% α-methyl-D-mannoside in the erythrocyte suspension to detect MR HA reactions only.

tination of human type O erythrocytes. SDS-PAGE analysis of HPLC fractions from variant pili showed that the 17.8K subunit was missing from protein peaks 3 and 4. Findings from the erythrocyte adsorption of a parent pilus preparation also supported the conclusion that the 17.8K subunit is important in erythrocyte binding; in addition to the removal of the 17.8K subunit, the 15.7K polypeptide was partially removed by the erythrocyte adsorption, suggesting a possible functional association between these two polypeptides. The immunoprecipitation results suggest antigenic relatedness between these two subunits because both the 15.7K and 17.8K polypeptides were associated with pili precipitated by MAb 34-3, 73-3, 81-1, 82-1, and 91-1.

Anion-exchange chromatography was extremely helpful in the separation of intact pilus types of *E. coli* 6260. This was an important step which enabled us to characterize the binding functions of the different subpopulations of pili. Our technique, which separated intact pilus types, avoided potential problems encountered when pili are separated by chemical dissociation (19); reassembled subunits may vary from intact pili in their ability to mediate binding reactions. The receptor specificities of *E. coli* 6260 pili from three of the HPLC fractions were determined in binding assays. Pili contained in fraction 1, with 19.3K subunits, bound specifically to mannose residues, indicating the presence of type 1 pili. In addition, pili in fraction 2, with 15.7K subunits, also bound to mannose residues, suggesting the presence of a second type of mannose-sensitive pilus on *E. coli* 6260. Klemm et al. (20) also characterized multiple type 1-like pili, termed 1A, 1B, and 1C, although these pili were grouped together based on similar N-terminal sequences rather than on similar biological function. The receptor specificity for pili in fraction 3 is not known. Strikingly, fraction 4, with 15.7 and 17.8K subunits, which had migration rates by SDS-PAGE similar to those of fraction 3 showed binding to globoside-coated horse cells, suggesting the presence of P pili in this fraction. A 15.7K polypeptide was detected in three of the fractions, yet each fraction demonstrated distinct characteristics in the binding assays, suggesting that the pilus preparation contains polypeptides with similar migration rates on SDS-PAGE but which can be separated by anion-exchange chromatography.

The pilus-specific MAb serve as useful immunological markers to differentiate *E. coli* 6260 pilus types. Each of the monoclonals reacted with antigenic determinants present on SDS- and heat-treated preparations of *E. coli* 6260 pili. The immunoblot data demonstrated that three of these MAb have specificities for single subunit types. The group of MAb which bound only to the partially dissociated and undissociated pili remaining in the stacking gel showed that these antibodies reacted with a polymeric but not the monomeric form of the antigen. Since a 10% resolving gel was used for electrophoretic separations, these MAb were recognizing pilus polymers with molecular weights of greater than 205,000. These results are similar to those reported by Abraham et al. (1) and Eisenstein et al. (8) in which both IgG and IgM MAb reacted with polymers of the pilus subunits remaining in the stacking gel, with no reactivity for pilus polypeptides in the 10 to 12% resolving gels. These investigators suggested that the MAb are specific for epitopes extending across more than one subunit, which are formed by the quarternary structural conformation of fully assembled pili.

The MAb were used in RIP techniques with purified intact pili; two polypeptides were precipitated by each of the MAb. The most direct explanation for these results is that the MAb recognizes similar antigenic determinants on two different native pili; thus, two different subunit types were observed by autoradiography. Antigenic determinants on one of the pilus types may be altered on SDS-PAGE, resulting in nonrecognition by the MAb in the immunoblot assay. A contrasting explanation is that each MAb recognizes one pilus determinant on a single subunit of a subpopulation of pili, and that this pilus type is composed of two different subunits. This explanation is supported by the proposal of Normark, that Pap pili are composed of two types of polypeptides, a major structural subunit and a minor adhesin subunit (24). In addition, all pili immunoprecipitated by a particular MAb may not be visualized in the RIP results because the pilus types may not have been labeled with equal efficiency in the iodination procedure. However, the reactivities of the MAb with *E. coli* 6260 pili were also examined by an immunoblot technique in which unlabeled pili were used, thereby circumventing the possible problem of inefficient iodination.

Five of the six MAb cross-reacted with purified pili prepared from heterologous *E. coli* strains. Notably, MAb 34-3, 73-3, and 81-1 reacted more strongly with pili from strain 6824 than with pili from the homologous strain. These results suggest that *E. coli* 6824 has a larger number of pili expressing complementary antigenic determinants or that the appropriate antigenic determinants are found repeated more frequently on individual pili of *E. coli* 6824 or both. Five of the MAb cross-reacted with P pili isolated from clinical isolates, but none of the MAb reacted with P pili from the laboratory strain, HU849. Other investigators (2, 20) have observed antigenic heterogeneity within the MR pilus group. By the ELISA technique, MAb 73-3 and 81-1 appeared to react with both MR (P) pili and MS (type 1) pili. Researchers have indicated that type 1 and P pili are antigenically dissimilar, suggesting that homologous regions between the pili are immunorecessive (20). The two MAb showed lower activity with type 1 pili than with P pili. Perhaps buried antigenic determinants on the type 1 pili, common to P pili, were uncovered during the pili purification process.

All six of the pilus-specific MAb blocked the interaction between *E. coli* 6260 and human erythrocytes. One possible explanation for the antiagglutination properties of these MAb is that the antibodies bound to epitopes in close proximity to adhesin sites on the pilus structures, sterically hindering the interaction between adhesins and receptors on the erythrocytes. Abraham et al. (1) have observed that

CD3, an MAb directed against quarternary determinants on type 1 pili, blocks the adhesion of *E. coli* CSH50 to guinea pig erythrocytes, oral epithelial cells, and yeast cells. Preliminary data suggested that CD3 binds to sites on the pili other than the adhesin sites. Another possibility is that the observed inhibition was produced by cross-linking of the pilus filaments by the MAb, since Fab fragments were not used. A third possible explanation for the inhibition results is that the MAb are specific for determinants on the pili which mediate adherence to erythrocytes. Notably, inhibition data for MAb 11-2, associated with 15.7K and 19.3K subunits by the RIP results, suggest that this antibody specifically binds to MS adhesin sites. HI in the presence of mannose required significantly more concentrated MAb supernatant than did HI in the absence of mannose. The mannose residues could compete with the MAb for sites on the pili.

In summary, in our studies of *E. coli* 6260 pili, four types of intact pili were separated by HPLC, and four subunit types were determined by their reactions with pilus-binding MAb. Two of the subunits, the 15.7K and 17.8K subunits, were associated with P pili and MRHA. Results of the binding and HI assays suggest that the 19.3K and 15.7K subunits are involved in MS agglutination.

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