Antibodies Raised against the Outer Membrane Protein Interrupt Adherence of Enteroaggregative *Escherichia coli*

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Enteroaggregative *Escherichia coli* (EAggEC) is a distinct category of diarrheal pathogen implicated as the cause of persistent diarrhea. The pathogen exhibits a characteristic “stacked-brick” pattern of aggregation when incubated with HEp-2 cells. The outer membrane protein (OMP) profile of a prototype EAggEC strain (F03) reflected the presence of one major 30-kDa protein. The OMP is expressed in the presence of the 60-MDa plasmid that the strain harbors. Antibodies were raised against the OMP by injecting the protein into a rabbit. The manifestation of an adherence phenotype on HEp-2 cells was observed for F03 and other strains that express OMP in the presence and absence of anti-OMP serum. Clumps of bacteria forming an aggregative pattern were observed in the HEp-2 cell assay in the absence of OMP antibodies, whereas a few bacteria attached to the cells in the presence of OMP antibodies. Mannose-resistant hemagglutination of human erythrocytes observed in the presence of EAggEC strains was inhibited in the presence of anti-OMP serum. Sequence analysis of a peptide generated by protease digestion of OMP exhibited 90% homology to a peptide of flagellin protein encoded by the *hag* gene of *Serratia marcescens*. Immunolabeling of the outer membrane by colloidal gold confirmed the protein to be an OMP. Our results suggest that the OMP of EAggEC have common antigenic properties. Antibodies raised against the protein can prevent adherence in vitro and could potentially interrupt the natural disease.

Epidemiological studies have indicated that the newly discovered enteroaggregative *Escherichia coli* (EAggEC) is an important etiological agent in persistent diarrhea (3, 8, 22). This form of diarrhea, in which the episode continues for more than 14 days, accounts for a disproportionately large share of diarrhea-related morbidity and mortality among children (2, 5, 29, 33). EAggEC-induced persistent diarrhea is being increasingly recognized as a major public health problem (12, 20, 31). EAggEC strains exhibit a characteristic “stacked-brick” pattern of aggregative adherence (AA) when incubated with HEp-2 cells. On the basis of characteristic adherence pattern, enteroaggregative strains are grouped as a major category of diarrheagenic *E. coli* (11) distinct from the other well-established categories, i.e., enterotoxigenic, enteropathogenic, enterohemorrhagic, and enteroinvasive *E. coli* (19).

The EAggEC isolates harbor a 60-MDa plasmid that is required for the expression of the aggregative phenotype (9, 23) and elaboration of heat-stable enterotoxin (28). Several groups have described surface structures of EAggEC which might confer AA. Fimbriar adhesins were expressed by two EAggEC strains that conferred hemagglutination (HA) of erythrocytes of rodents and humans (26). Yamamoto et al. (34), while examining adherence of EAggEC to intestinal explants, demonstrated an adherence factor that conferred HA of human erythrocytes. Nataro et al. (21) reported bundle-forming fimbriae as a plasmid-associated AA factor in the prototype strain 17-2. This factor, designated AA fimbria I (AAF-1), conferred HA of human erythrocytes. A 12-kb fragment of the 60-MDa plasmid responsible for the AA determinant in strain 17-2 was cloned and designated the *aaf* region (21, 24). *TnphoA* insertion in one region of *aaf* simultaneously abolished AA, fim-

briae, and HA of erythrocytes. Antibodies raised against the protein (14 kDa) encoded by *aaf* and suspected to be a fimbrial subunit inhibited HA.

With an increase in the traveling population of the world and reports on colonization by EAggEC in travelers with and without diarrhea (6), the need for developing an efficacious vaccine against colonization is apparent. Fimbriae and toxins have been considered the major putative virulence determinants in classical diarrheagenic strains, and these genes have been targeted for vaccine development against *E. coli*. In EAggEC strains, however, the characteristic AA phenomenon seems to contribute to the virulence properties of the organism. Prevention of adherence would be an effective method of control which has not been previously addressed. In this paper, we provide evidence that antibodies raised against a 30-kDa outer membrane protein (OMP) inhibit both the AA phenomenon in a HEp-2 cell assay and HA of human erythrocytes. The protein appears to be immunogenic, with common antigenic sites shared by the OMP isolated from other EAggEC strains, and may be used to develop a vaccine efficacious against the EAggEC strains.

MATERIALS AND METHODS

Bacterial strains and serotyping. EAggEC strains used in this study were isolated from infants and children suffering from persistent diarrhea in India. Fecal material was directly cultured on MacConkey and deoxycholate agar plates and selenite enrichment broth within 1 h of collection for overnight incubation at 37°C (32). The identification of the bacteria followed standard methods recommended by the World Health Organization (32). O:H serotypes of EAggEC isolates were determined by the method described by Orskov et al. (27).

Adhesion assay. The mannose-resistant adherence pattern of EAggEC isolates to HEp-2 cells was assayed by the method described by Cravioto et al. (8). HEp-2 cells were grown in Dulbecco's modified Eagle's medium with Earle's salt supplemented with fetal bovine serum (5%). HEp-2 cells were infected with EAggEC isolates (10 µl of an overnight grown culture) and incubated for 3 h at 37°C. The cells were washed six times with phosphate buffer, fixed with methanol, stained with Giemsa, and observed under the microscope. To exhibit inhibition

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of adherence, 10 μ l of OMP antisera (diluted or undiluted) and 10 μ l of bacteria grown overnight were incubated for 15 min at room temperature and then added to a monolayer of HEP-2 cells. The degree of inhibition was estimated by counting the number of bacteria per cluster and the number of HEP-2 cells infected. The percent inhibition of AA was calculated from the average of five independent readings in different fields and from experiments conducted on two different days. The control experiment was conducted without any OMP antiserum but in the presence of serum collected prior to immunization (preserum) or with *E. coli* antilipopolysaccharide (anti-LPS) (10 μ l) obtained from Sigma Chemical Co., St. Louis, Mo.

HA. HA was performed with human type O erythrocytes as described by Yamamoto et al. (34). Briefly, *E. coli* was grown overnight in static L-broth cultures, pelleted in a microcentrifuge at 12,000 rpm for 5 min, washed twice in sterile phosphate-buffered saline (PBS), and resuspended in PBS to a concentration of 10^8 CFU/ml. The bacterial suspension (15 μ l) was mixed with an equal volume of a 3% (vol/vol) washed human type O erythrocyte suspension containing 1% D-mannose (Sigma) in PBS. The suspensions were briefly mixed on an antibody microslide and allowed to incubate at room temperature for 30 min. HA was evaluated under an inverted microscope with $\times 400$ magnification. Any HA discerned to be greater than that of a negative bacterium-free control was considered positive. Inhibition of HA was performed by incubating serial twofold dilutions of OMP antiserum in PBS with an equal volume of a washed bacterial suspension at room temperature for 20 min. To the suspension, 3% erythrocyte-1% mannose (15 μ l) was added, and HA was assayed as described above. Two separate experiments were conducted in duplicate, and HA was observed by two scientists independently in the presence and absence of anti-OMP serum.

OMP isolation. OMPs were isolated by the method described by Deneer and Potter (10) with minor modifications. Bacteria were grown overnight at 37°C in Luria broth. Cells were recovered by centrifugation (6,000 $\times$ g for 10 min at 4°C) and suspended in 3 ml of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; 10 mM, pH 7.4) solution. They were disrupted by sonication (Branson Sonifier; 60 s, 30% output). Cell debris was removed by centrifugation at 6,000 $\times$ g for 10 min at 4°C. The supernatant was added to 0.75 ml of 2% N-lauroylsarcosine (Sarkosyl) and incubated for 15 min at room temperature. The OMP was isolated after centrifugation of the mixture at 100,000 $\times$ g for 1 h. The pelleted protein was resuspended in 10 mM HEPES and stored at -20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1) was carried out with a 4% stacking and an 8% separating gel after OMP preparations were solubilized at 100°C for 5 min in sample solution (0.05 M Tris-HCl [pH 8.0], 2.5% SDS, 5% 2-mercaptoethanol, 25% glycerol, 0.003% bromophenol blue). The proteins were visualized after the gels were stained with Coomassie brilliant blue R250 and destained with 30% methanol-10% acetic acid.

Production of antibodies. For production of antibodies, the procedure described by Coligan et al. (7) was followed. A rabbit was immunized by injecting 200 μ g of the OMP preparation, isolated from F03, subcutaneously twice at 30-day intervals, first in complete and then in incomplete Freund's adjuvant. Blood was collected from the central auricular vein of each ear and then from the cephalic vein of the left leg 15 days later. The blood clot was removed from the sample, and the serum was prepared by centrifuging the blood at 2,000 rpm and 4°C for 20 min. The serum was stored at -20°C.

Immunoblotting. The proteins in the polyacrylamide gels were transferred to nitrocellulose filters (Trans-Blot Transfer medium; Bio-Rad Laboratories, Richmond, Calif.) by transblotting, according to the protocol described by Coligan et al. (7). The nonspecific antibody sites on the proteins captured on the nitrocellulose were blocked by incubating the filter with blocking solution (5% nonfat dry milk powder in PBS) for 1 h at room temperature. The electroblotted proteins were probed with the primary antibody. The OMP antiserum (50 μ l) was diluted in 5 ml of blocking solution and incubated for 1 h at room temperature with constant agitation. The nonspecifically bound OMP antibodies were removed by washing four times with 200 ml of PBS each time. The nitrocellulose filter was incubated with a solution of horseradish peroxidase-anti-immunoglobulin conjugate (Sigma Chemical) diluted 100-fold in blocking buffer for 1 h at room temperature. The bound antibody was detected by the color reaction developed by the addition of diaminobenzidine substrate solution (Sigma Fast tablets).

ELISA. The serum sample was tested for the presence of OMP antibodies by enzyme-linked immunosorbent assay (ELISA), according to Coligan et al. (7). Briefly, Immulon II microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with OMP antigen at 1-, 5-, and 10- μ g/ml concentrations and incubated overnight at room temperature. The plates were washed with PBS and blocked with blocking buffer (bovine serum albumin diluent blocking solution [Kirkegaard and Perry Laboratories, Gaithersburg, Md.]) for 30 min at room temperature. After an additional wash step, the sera, collected before (preserum) and after (postserum) OMP inoculation, were added in 10-fold serial dilutions beginning at a 1:10 concentration. Immunoglobulin G (IgG) titers were determined by binding alkaline phosphatase-linked anti-rabbit IgG (Sigma) and monitoring the color development with the alkaline phosphatase substrate paranitrophenylphosphate (Sigma Fast tablets). The plates were read and analyzed with a BioTek EL311 autoreader at 405 nm.

Electron microscopy. Electron microscopy was performed with a JEOL EXII microscope with 200-mesh copper grids coated with thin films of 2% Formvar. The bacterial colonies were overlaid with buffer (10 mM Tris hydrochloride [pH

TABLE 1. OMP profiles of EAggEC strains

Strain	Serotype ^a		HEP-2 assay results	OMP ^b (kDa)	Anti-OMP reaction ^c
	O	H			
F01	17	6	AA	43	+
F02	51	11	AA	38	+
F03	4	7	AA	30	+
F04	51	—	AA	ND	—
F05	75	NM	AA	ND	—
F06	77	18	AA	43	+
F07	162	NM	AA	ND	—
H08	111	32	AA	ND	—
H09	142	33, 35	AA	ND	—
H010	7	NM	AA	ND	—
H011	6	—	AA	ND	—
H012	—	32	AA	43	+
H013	68, 73	NM	AA	43	+
H014	—	NM	AA	ND	—
H015	—	—	AA	43	+
H016	78	—	AA	43	+
H017	78	—	AA	30	+
H018	85	—	AA	ND	—
H019	7	NM	AA	ND	—
H020	—	NM	AA	38	+
HBT	—	—	AA	30	+
PC	—	—	—	25	—
HB101	—	—	—	—	—

^a —, negative reaction. H09 and H13 showed multiple reactions. NM, nonmotile.

^b ND, not detected.

^c +, positive reaction; —, no reaction.

7.5], 10 mM magnesium chloride), and the cell suspension was allowed to sediment for 15 min on a grid. The grids were incubated with the antiserum to OMP (1:1,000 dilution) for 5 min and washed with buffer; they were then incubated for 5 min with goat anti-rabbit IgG coupled to gold particles (10 nm) (Sigma). The grids were washed with deionized water, negatively stained for 10 s with 1% sodium silicotungstate (pH 7.0), and then washed with redistilled water (25) and observed under the microscope.

Peptide sequencing of OMP. The peptide from the OMP was identified and sequenced according to the method described by Gooderham (14). The protein was separated by SDS-PAGE as described above and then stained with Coomassie brilliant blue R250. The OMP band was then cut out of the gel and placed in a 10-ml snap-cap test tube containing 5 ml of equilibration buffer (0.125 M Tris hydrochloride [pH 6.8], 0.1% SDS, 1 mM EDTA). The tube was gently agitated. After 1 h, another 5 ml of fresh buffer was added and the equilibration was continued for another hour. The gel slice was placed in a sample well of an extra thick (1.5-mm) SDS-polyacrylamide gel with a 5-cm stacking gel. The gel slice was covered with 10 μ l of overlay buffer (0.125 M Tris hydrochloride [pH 6.8], 0.1% SDS, 1 mM EDTA, 20% glycerol, 1% bromophenol blue). This was followed by the addition of 10 μ l of protease buffer (same as overlay buffer except the glycerol concentration was 10%) containing 1 μ g of *Staphylococcus* V8 protease (Promega Corp., Madison, Wis.). The samples were then run at a constant current of 20 mA until the marker dye reached the top of the separating gel, when the current was increased to 40 mA for the remainder of the run. The peptides were blotted onto a polyvinylidene difluoride membrane filter (Bio-Rad Laboratories) in 0.5 $\times$ Towbin buffer (Tris, 0.0125 M; glycine, 0.096 M) with 10% methanol at 4°C and 100 V for 30 min. After transfer, the membrane was rinsed with water and stained with 0.5% Ponceau S. One of the major bands (~20 kDa) following digestion with protease was cut out from the membrane for the peptide sequencing that was performed at Hershey Medical Center at Penn State University. The sequence homology of the peptide was searched with the molecular biology program Intelligenetics Suite installed on the Sun SPARCstations at the Biotechnology Institute at Penn State. The package includes the major nucleotide and protein sequence databases (GenBank, EMBL, PIR, Swissprot, etc.) and provides a wide range of tools for sequence analysis.

RESULTS

Serotypes of EAggEC strains. Twenty EAggEC isolates obtained from patients suffering from persistent diarrhea in India and that exhibited AA properties were serotyped. The O serotypes varied considerably among the isolates (Table 1). The

FIG. 1. Electrophoretic analysis of OMP in SDS-PAGE. Staining with Coomassie blue. Lane 1, marker; lane 2, plasmid-cured strain (PC); lane 3, prototypic strain F03; lane 4, HB101 transformed with a 60-MDa plasmid isolated from F03 (HBT); lane 5, HB101.

H serotype was found to be nonmotile in several strains.

SDS-PAGE of OMP preparations. The OMP profile of a prototype EAggEC strain, F03, exhibited the presence of one major protein of 30,000 molecular weight. To determine if the OMP was expressed in the presence of the 60-MDa plasmid, two isolates that we had previously constructed were used (9). One of them was the plasmid-cured prototype strain F03, called PC, and the other one was the nonpathogenic HB101 transformed with the 60-MDa plasmid from F03, called HBT (9). OMP were isolated from PC, HB101, and HBT. The OMP profiles from these strains were compared with the F03 profile on SDS-PAGE (Fig. 1). One major protein (30 kDa) was observed in F03 and HBT strains, whereas PC showed the presence of a smaller protein (25 kDa). No protein was detected in the control HB101, implying that HB101, unlike F03, did not express any Sarkosyl (0.4%)-insoluble proteins. The OMP preparation from F03, upon high-performance liquid chromatography (HPLC), also exhibited a single peak, confirming the presence of only one major protein in the preparation (data not shown). Since F03 and HBT both expressed the 30-kDa protein whereas PC expressed a different protein, it was evident that the 60-MDa plasmid is required for the expression of the 30-kDa OMP.

Reactivities of anti-OMP sera to outer membrane preparations. The antiserum raised against the OMP isolated from strain F03 was tested for the presence of antibodies in an ELISA. The IgG titer of anti-OMP postserum was found to be in the range of 2,560 to 5,120 (data not shown).

OMP were isolated from EAggEC strains and electrophoresed on SDS-PAGE. Ten of the isolates exhibited the presence of one major protein band (Fig. 2A; Table 1). The other five strains did not show any Sarkosyl-insoluble protein. The molecular weights of the OMPs varied among the different isolates. They were approximately 43,000 in strains F01, F06, H12, H13, H15, and H16; 38,000 in F02 and H20; and 30,000 in F03 and H17. OMP of these strains were further immunoblotted against the OMP antiserum. The results demonstrated (Fig. 2B) that anti-OMP serum recognized the proteins from all 10 isolates. The antisera also reacted positively with the OMP of HBT but did not recognize the protein isolated from PC or HB101, which served as negative controls. The molecular weights of OMP from different EAggEC strains ranged between 30,000 and 43,000 and were found to be antigenically related (Fig. 2B; Table 1) in 10 of 20 (50%) isolates tested.

Inhibition of AA phenomena by antisera raised against

FIG. 2. Electrophoretic analysis of OMP isolated from EAggEC strains. (A) Staining with Coomassie blue. (B) OMP expressed by EAggEC strains were blotted onto nitrocellulose paper, incubated with anti-OMP serum, washed, and finally bound to goat anti-rabbit IgG conjugated to horseradish peroxidase. The bound antibody was detected by the color reaction developed by the addition of diaminobenzidine substrate. Molecular mass in kilodaltons and the strain numbers are indicated.

OMP. Serial dilutions of antisera raised against the OMP were incubated with the prototype strain F03 before the mixture was added to monolayers of HEp-2 cells. Compared with control experiments conducted without added antiserum, dilutions of the OMP antiserum of 1:1, 1:10, 1:25, and 1:50 reduced the percentage of infected HEp-2 cells from 45.6% to 6.9, 10, 15.2, and 20%, respectively (Table 2). The number of bacteria per cluster was reduced as well, to 18 in wells containing a 1:1 to 1:25 dilution of antibody from an average of 50 in the control wells to which no antiserum was added. This result clearly showed inhibition of AA upon addition of OMP antiserum to the HEp-2 adherence assay. Incubation of F03 in the presence of preimmune serum did not exhibit any inhibition of AA properties of the bacteria. Antiserum against other surface determinants of *E. coli* such as anti-LPS was also employed to ascertain whether the inhibition of the AA was due to nonspecific steric hindrance of the bacterial association with tissue culture cells. Anti-LPS did not cause any inhibition of the AA properties exhibited by F03.

All strains that expressed OMP were tested for inhibition of AA in the presence of OMP antibodies. There was a marked reduction in the number of infected cells in the presence of

TABLE 2. Effect of OMP antiserum on the AA of EAggEC strain F03 (O4:H7) to HEp-2 cells

Antiserum dilution	% Inhibition of AA ($\pm$ SD) ^a	No. of cells per cluster
No antiserum added	45.6 ($\pm$ 2.0)	50.4
1:1	6.9 ($\pm$ 3.3)	18
1:10	10 ($\pm$ 3.4)	19.2
1:25	15.2 ($\pm$ 2.6)	18.5
1:50	20.0 ($\pm$ 3.1)	43.1

^a Inhibition of the AA phenotype by the indicated dilutions of OMP-specific antiserum, expressed as the mean percentage of HEp-2 cells containing one or more clusters of bacteria (based on values from five readings in different fields and from experiments conducted in duplicate).

TABLE 3. Effect of anti-OMP sera on the AA phenotype manifested by EAggEC strains

Strain	% Cells infected		% Inhibition of AA ^a
	– Antibody	+ Antibody ^b	
F01	39.1	10.8	78.1
F02	35.2	15.4	56.3
F03	45.6	6.9	85
F06	37.3	12.0	68.0
H12	56.9	32.3	44
H13	19.5	15.2	22.1
H15	35.0	9.1	74
H16	8.5	0.6	93
H17	50.6	17.5	65.5
H20	100	100	0
HBT	6.0	0.45	92.5

^a Based on values from five readings in different fields and from experiments conducted in duplicate.

^b Dilution of anti-OMP serum used was 1:1.

antibodies at a 1:1 dilution for all strains tested except one (H20). The degree of inhibition of AA in the presence of anti-OMP serum varied considerably among the strains (Table 3). The manifestation of AA was considerably less for the transformed strain HBT, to an extent of only 6%, while in the presence of the antibodies the number of infected cells was drastically reduced (0.45%). It was apparent from the results that OMP antibodies inhibited adherence properties of most of the EAggEC strains.

HA. To determine if OMP plays a role in mannose-resistant HA of human erythrocytes, the EAggEC strains that expressed OMP were selected. While 9 of 10 strains conferred HA of erythrocytes, two isolates (F06 and H13) conferred greatly diminished HA and H16 did not confer HA (Table 4). When anti-OMP serum at a 1:1 dilution was added, HA of erythrocytes was completely inhibited in 8 of 10 strains. However, in two strains, H12 and H15, the presence of OMP antibodies did not completely inhibit HA but exhibited a weak inhibition. HBT did not confer HA, reflecting that additional host factors are probably required for HA manifestation. However, OMP from most (80%) of the EAggEC strains examined were found to be involved in the manifestation of mannose-resistant HA of human erythrocytes.

Amino acid sequence. The sequence of 10 amino acids from a peptide generated by digestion of OMP with *Staphylococcus* V8 enzyme was determined to be Leu-Thr-Val-Gln-Ala-Gln-Gln-Gly-Ser-Asn. A data bank sequence search generated sequence homology of the peptide to the peptide sequence of the flagellin protein encoded by the *hag* gene of *Serratia marcescens* (15) for which the sequence of the peptide between amino acid residues 95 and 105 is Leu-Thr-Val-Gln-Ala-Gln-Asn-Gly-Ser-Asn. The seventh amino acid of this peptide, Asn, is replaced by Gln in OMP of EAggEC. A partial homology was also observed for a peptide of the flagellin protein of *E. coli* (18). In this protein, the amino acid sequence is Leu-Thr-Val-Gln-Ala-Thr-Thr-Gly-Thr-Asn. The two Thr residues at the sixth and seventh positions of the peptide are replaced by Gln residues and the Thr residue at position 9 is replaced by Ser in OMP of EAggEC strain F03.

Immunoelectron microscopy. To confirm that the protein was isolated from the outer membrane and not from fimbriae or flagella, the prototype strain F03 was immunolabeled with colloidal gold and observed under the electron microscope. As seen in Fig. 3, the outer membrane of the bacteria was deco-

rated with gold, confirming that the protein is associated with the outer membrane.

DISCUSSION

The mechanism by which the EAggEC strains cause acute and persistent diarrhea is not yet clearly understood. The pathogenicity of the organism is attributed to the AA properties. Binding of the pathogen is a primary step in pathogenesis because it promotes internalization. Adherence also promotes delivery of bacterial exoproducts, including enterotoxins, to eukaryotic cell surfaces (35). Histopathological observations of rabbit ligated intestinal loop exhibited adherence of EAggEC strains to the mucosa, with hemorrhage and disruption of the villi (17, 30). The EAggEC isolates have recently been shown to elaborate a heat-stable enterotoxin that is encoded by the 60-MDa plasmid (28).

All EAggEC strains checked harbored a common 60-MDa plasmid in addition to one or several other plasmids (9). We earlier confirmed that the AA phenotype and expression of fimbriae were associated with the 60-MDa plasmid. The prototypic strain F03 and HBT (HB101 transformed with the 60-MDa plasmid from F03) both manifested AA properties and expressed fimbriae. PC (plasmid-cured F03), on the other hand, did not exhibit any AA phenomena (9). In this report, we have shown that a large proportion of EAggEC isolates expressed one major OMP of molecular weight between 30,000 and 43,000. The OMP preparation was composed of proteins that were insoluble in 0.4% Sarkosyl. In the prototypic strain F03, a 30-kDa OMP is expressed. HBT also expressed a 30-kDa OMP that is not expressed by HB101 (Fig. 1). The OMP isolated from F03 and HBT were antigenically related (Fig. 2). When F03 was cured of both the 60-MDa and 4-kb plasmids it harbored, the resulting strain PC exhibited flagella and was found to be motile, unlike F03 (9). PC exhibited a 25-kDa protein that did not interact with the antibodies to OMP isolated from F03, reflecting that it is an unrelated protein (Fig. 1 and 2). Since PC is devoid of all plasmids, the 25-kDa protein may be the flagellar protein expressed by the host. The expression of the 25-kDa protein was found to be inhibited in the presence of the 60-MDa plasmid since this protein is not expressed by F03. Plasmid-encoded genes have been found to be essential for the expression of specific adherence structures such as CS1 and CS2 in enterotoxigenic *E. coli* (4) and the *eae* gene for enteropathogenic *E. coli* (16).

Fifty percent of EAggEC strains tested express one major OMP, and these OMP are antigenically related (Fig. 2). HPLC analysis of the crude OMP preparation from the prototypic strain exhibited one major peak, confirming the presence of a single protein in the preparation. Therefore, further purification of the protein was not considered essential. Antisera raised against the 30-kDa OMP from the prototypic strain F03 inhibited aggregation of EAggEC strains in a HEP-2 cell assay (Table 3). Anti-LPS sera or preimmune sera did not inhibit adherence, confirming the specificity of the anti-OMP serum. HBT, the transformed HB101 strain, did not exhibit aggregation to the extent observed in the wild-type strain in a HEP-2 cell assay. Therefore, other factors may be essential for the manifestation of AA. Similar observations have been reported (13) when antiserum raised against the subunit of bundle-forming pili (19,500 Da) from enteropathogenic *E. coli* strains reduced their capacity to infect cultured epithelial cells and exhibit a localized adherence phenotype (13). Until now there have been no reports concerning the integral role played by OMP in the adherence process characteristic of EAggEC.

A 14-kDa fimbrial protein from an EAggEC strain was

FIG. 3. Immunogold staining of EAggEC strain F03. The bacteria were incubated with anti-OMP serum and further immunolabeled by standard procedures, using colloidal gold, and observed under the electron microscope. (A) Magnification, $\times 100,000$; (B) magnification, $\times 250,000$.

TABLE 4. HA exhibited by EAggEC strains^a

Strain	HA without anti-OMP serum	HA with anti-OMP serum ^b
F01	+	—
F02	++	—
F03	++	—
F06	w+	—
H12	+++	w+
H13	w+	—
H15	++	—
H16	—	—
H17	+	—
H20	+	—
HBT	—	—
No bacteria	—	—

^a +, positive reaction; —, negative reaction; w+, weak positive reaction.^b Dilution of anti-OMP serum used was 1:1.

found to confer HA of human erythrocytes (21). The EAggEC strains also exhibited HA of human erythrocytes. An inhibitory response of HA was observed in the presence of antibodies to OMP (Table 4), suggesting that OMP may also be involved in the manifestation of HA of human erythrocytes.

The amino acid sequence was determined from a peptide generated by protease digestion of the protein. Sequence homology was observed between a 10-amino-acid sequence of a peptide generated from OMP with 9 amino acids of a peptide from the flagellin protein of *S. marcescens* (15) and a 7-amino-acid-sequence of a peptide from the same protein from *E. coli* (18). Electron microscopic observation of F03 immunolabeled with gold exhibited binding of the gold to the outer membrane and not with flagella or fimbriae.

Microbial cell surface components and secreted proteins are often immunogenic and are thus potentially rich sources of antigen for vaccine development. The role of the outer membrane in pathogenesis and adherence is not well understood for this group of bacteria. Stimulation of gut-associated immunity in the host through immunization with these antigens may be of considerable assistance in the development of a vaccine against this group of bacteria. Further knowledge on the genetics, structure, and immunogenicity of the OMP will assist in the understanding of the role of this protein in pathogenesis and in developing a vaccine efficacious against the EAggEC group of pathogens.

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