Characterization of Enteroadherent-Aggregative *Escherichia coli*, a Putative Agent of Diarrheal Disease


*Escherichia coli* that exhibit the aggregative pattern of adherence to HEp-2 cells (enteroadherent-aggregative *E. coli* [EA-AggEC]) have been epidemiologically incriminated as a cause of diarrhea. We undertook a preliminary microbiological and pathogenetic characterization of 42 isolates of this putative pathogen. The strains were negative by tests with DNA probes for enteropathogenic, enterotoxigenic, enteroinvasive, and enterohemorrhagic *E. coli* and, by serotype, did not fit these categories. Thirty-nine of 42 strains had a 55-65-megadalton plasmid; many shared DNA homology. With one representative strain, plasmid transfer was accompanied by transfer of smooth lipopolysaccharide, fimbriae expression, and the aggregative property. EA-AggEC caused characteristic lesions in rabbit and rat ileal loops. The intestinal lesions and (Shiga-like) limb paralysis and death in rabbits inoculated with live organisms suggest toxin involvement; assays for Shiga-like toxins were negative. These preliminary results support the contention that EA-AggEC may represent a distinct category of diarrheagenic *E. coli*.

Four major categories of *Escherichia coli* that cause diarrhea can readily be discerned, including enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and enterohemorrhagic (EHEC) [I]. Organisms in these categories differ in their pathogenesis, clinical features, epidemiology, and O:H serotypes [I] and can be identified by using specific DNA probes [2–7]. The property of adherence of *E. coli* strains to HEp-2 or HeLa cells has also been used to identify *E. coli* associated with diarrhea. Cravioto et al. [8] first noted that EPEC of most classic O:H serotypes associated with infant diarrhea attach to HEp-2 cells, although this property was rare among enterotoxigenic and enteroinvasive strains and among isolates from normal flora. Nataro et al. [9] and Scaletsky et al. [10] then described at least two distinct phenotypes of adherence, so-called localized and diffuse patterns. Localized adherence, characteristic of most classic EPEC, was shown to be encoded by EPEC adherence factor (EAF) plasmids. Nataro et al. [9] and Scaletsky et al. [10] did not correlate the diffuse adherence pattern with pathogenicity.

Very different findings were reported by Mathewson et al. [11, 12], who also used the HEp-2 assay to examine *E. coli* from travelers and infants in Mexico. They found that strains that adhered to HEp-2 cells by the localized pattern were isolated significantly more often from individuals with diarrhea than from non-ill controls; however, their strains were rarely of EPEC O serogroups. In addition, they reported that *E. coli* that exhibited the diffuse adherence pattern were also recovered significantly more often from patients with diarrhea than from controls. In contrast, Cravioto et al. [13], also working in Mexico, reported conflicting data. In their studies, *E. coli* strains giving localized adherence were found significantly more often in association with diarrhea, but such strains were largely of classic
EPEC O serogroups. Furthermore, Cravioto et al. [13] did not encounter *E. coli* giving the diffuse adherence pattern more frequently in children with diarrhea than in controls.

Two recent reports may resolve these confusing observations. Nataro et al. [14] and Levine et al. [15] described a putative new category of diarrheagenic *E. coli* that is identifiable by a characteristic pattern of adherence in the HEp-2 cell assay. This adherence pattern, referred to as *aggregative*, is distinct from the localized adherence pattern characteristic of EPEC, as well as from the true diffuse pattern [14]. *E. coli* that exhibit the aggregative pattern, hereafter referred to as enteroadherent-aggregative *E. coli* (EA-AggEC), have been epidemiologically incriminated as a cause of diarrhea [14, 15]. Thus, we considered it important to undertake a preliminary microbiological and pathogenetic characterization of these isolates. Herein we preliminarily characterize the EA-AggEC with respect to O:H serotypes, plasmid profiles, plasmid-encoded properties (including fimbriae), toxin production, and pathological lesions of the intestine in an animal model.

### Materials and Methods

**Bacterial strains.** Forty EA-AggEC isolates recovered from patients in a prospective study of the etiology of diarrhea in young children in Santiago, Chile, were investigated. Two additional EA-AggEC isolates studied included 042 (O44:H18), from a Peruvian infant with diarrhea, and strain 221 (reported to be O78:H33/35; provided by Dr. J. Mathewson, University of Texas Medical School, University of Texas Health Science Center at Houston, Houston), from an American student who developed travelers’ diarrhea in Mexico [11]; strain 221 was originally reported to exhibit a pattern of localized adherence in their HEp-2 assay [12]. HB101 is a rough *E. coli* K12 × *E. coli* B hybrid strain that was used as the recipient in plasmid-transformation experiments. None of the above strains were positive with DNA probes that detect heat-labile enterotoxin [5, 6], human or porcine heat-stable enterotoxin [5, 6], EIEC [4], EAF-positive EPEC [2], or EHEC [3].

**Adhesion assay.** Patterns of adherence were assessed in the 3-h HEp-2 assay recommended by Cravioto et al. [8], in the presence of 1% methyl-α-D-mannoside, as previously described in detail [9, 14, 16]. Examples of the localized, true diffuse, and aggregative patterns detectable in this assay are shown in figure 1.

**Serotyping.** The EA-AggEC strains were serotyped at the Canadian Center for Disease Control (Ottawa) by using standard methods [17]. To determine O serogroup, we tested heated (100°C) bacterial suspensions in tube agglutinations to endpoint titer with specific O antisera. H antigens were similarly determined in tube agglutinations by using formalin-treated bacteria with H antisera.

**Reaction of *E. coli* strains with rough-specific phages.** For many strains, the aggregative phenomenon interfered with the determination of O serogroup, and these strains were reported as rough. To ascertain if the strains were truly rough (i.e., lacked smooth lipopolysaccharide [LPS]), we screened them for susceptibility to the rough-specific phages BR60, BR2, Ffm [18], and C21 [19]. Trypticase-soy agar was streaked with a bacterial suspension, allowed to dry, and spotted with one drop of each phage suspension. A strain was considered rough if lysis with even one phage was recorded.

**Purification of LPS.** Hot water–phenol extractions for LPS [20] were performed on several strains not sensitive to the phages; the extracts were analyzed by SDS-PAGE and silver stain [21].

**Plasmid analysis.** Extracted plasmid DNA [22] was electrophoresed in 0.7% agarose gel with molecular-weight sizing markers and was stained with ethidium bromide.

**Hydrophobicity studies.** Surface hydrophobicity after growth (37°C) on colonization-factor antigen (CFA) agar [23] was measured by precipitation with decreasing concentrations of ammonium sulfate [24, 25]. EPEC, ETEC, EHEC, and EIEC strains (20 each) were concomitantly tested. The test was repeated for some hydrophobic EA-AggEC strains; thus, we could compare bacteria grown at 18°C and 37°C.

HA. The EA-AggEC strains were examined for HA of guinea pig, bovine, and human erythrocytes, with and without D-mannose (0.1%), at 24°C and 4°C [26, 27].

**Electron microscopy.** Bacteria grown on CFA agar for 24 h at 37°C or 18°C were negatively stained as previously described [28]. Immunolabeling and electron microscopy of EA-AggEC fimbriae were also performed as described [28]; rabbit antiserum to purified strain 17-2 fimbriae that was adsorbed with bacteria grown at 18°C was the primary antibody (dilution, 1:100), with gold-labeled goat antise-
rum to rabbit immunoglobulin (Janssen Pharmaceutical, Piscataway, NJ) as the secondary antibody (dilution, 1:50).

Prefixed intestinal tissue (vide infra) was postfixed with 1% osmium tetroxide, dehydrated through a series of graded ethanol solutions, and embedded in Epon [29]. Ultrathin sections were stained with uranyl acetate and lead citrate, and the grids were examined in a Siemens 1A electron microscope (Siemens, Cherry Hill, NJ).

**Purification of fimbriae.** Fimbriae from several EA-AggEC strains that appeared highly fimbriated by electron microscopy were purified from bacteria grown on agar at 37 C, as described [28, 30].

**Electrophoresis of fimbrial proteins.** The molecular weight of fimbrial subunits was determined by SDS-PAGE, in 15% acrylamide gels, against standard molecular-weight markers (prestained high; Bethesda Research Laboratories, Gaithersburg, Md) [30]. Gels were stained with coomassie brilliant blue R250.

**Preparation of antisera.** At three-day intervals, increasing doses (from $10^7$ to $10^9$) of live bacteria from EA-AggEC strains 17-2, 042, or 221 were inoculated intravenously into New Zealand white rabbits; the animals were bled 14 d after the last inoculation. Antisera to fimbriae were prepared by inoculating rabbits sc with 50 µg of purified fimbriae in Freund’s complete adjuvant, followed by two monthly boosters of 40 µg in Freund’s incomplete adjuvant. The antisera were adsorbed with nonfimbriated whole bacteria grown at 18 C. Endpoint titers were determined by colony blot immunoassay using bacteria grown at 37 C and 18 C.

**Western blot analysis.** Partially purified fimbriae were fractionated by SDS-PAGE in 15% gels and were electroblotted onto nitrocellulose filters [31]. Filters were blocked with 1% sodium caseinate, 0.05% Tween 20 (J. T. Baker Chemical, Phillipsburg, NJ), and 0.05% NaN₃ and incubated sequentially with antiserum to fimbriae (dilution, 1:5000) and alkaline phosphatase–conjugated protein A (dilu-
Enteroadherent-Aggregative E. coli

Blots were developed with indoxyl phosphate and 5-bromo-4-chloro-3-indolyl phosphate (U.S. Biochemistry, Cleveland).

**Dot immunoblot assay.** Colonies of all of the EA-AggEC strains, as well as of ETEC, EPEC, EIEC, and EHEC strains (10 each), after overnight growth on CFA agar at 37 C, were transferred to nitrocellulose filters and air dried. The filters were blocked as above, incubated for 2 h with antiserum to purified fimbriae of strain 17-2 (dilutions, 1:400, 1:1000, and 1:5000), washed, incubated for 2 h with alkaline phosphatase-conjugated protein A (dilution, 1:500), washed again, and developed as above.

Transformation of *E. coli* HB101 with plasmid DNA. Purified plasmid DNA from EA-AggEC strain 042 was cotransformed into *E. coli* HB101 [32] by using pMR5 as the marker plasmid; after cotransformation, pMR5 was cured by passage at 42 C [14]. Purified plasmid DNA from strain 221 was transformed into *E. coli* HB101 [33]. The transformants containing plasmids from strains 221 or 042 were examined for fimbriae, LPS, and adhesion to HEp-2 cells.

Plasmid homology. The 60-megadalton (MDa) plasmid of EA-AggEC strain 042 was initially cut with HindIII and then with Sau3A and was electrophoresed in agarose gel [3, 9]; the fragments were electroluted from the gel and labeled in vitro with [α-32P]dATP (New England Nuclear, Boston) [2, 9] for use as probes in colony blot hybridizations with other strains of EA-AggEC, ETEC, EPEC, EIEC, and EHEC [3].

Pathogenicity in animal models. After 24 h without food, New Zealand white rabbits (1.5–2.0 kg) and Fisher 344 rats (50–65 g) were anesthetized, and intestinal loops (4–5 cm long, 102 cm between loops) were isolated and ligated [34]. An inoculum of 10⁷ bacteria, in trypticase-soy broth (TSB), of EA-AggEC strain 17-2, 73-1, 042, or 221 was injected intraluminally into a loop in a randomized sequence; control loops received nonpathogenic *E. coli* HB101 or EPEC strain E2348/69 [16]. After 16 h the loops were surgically removed, and the intestinal tissue was prefixed in 4% formaldehyde–1% glutaraldehyde in cacodylate buffer (pH 7.3). After 48 h of fixation, tissue samples were prepared for light and electron microscopy. Histopathology slides were treated with hematoxylin and eosin and with gram stain.

**In vitro studies for cytotoxins.** EA-AggEC strains, grown overnight at 37 C in TSB or in TSB pretreated with Chelex 100 (Bio-Rad Laboratories, Richmond, Calif; to deplete the iron content), were assayed for cytotoxins by adding culture supernatants and sonicated bacterial pellets [35] (dilutions, 1:10–1:400) to microtiter plates containing tissue culture cells. Cell lines tested included the following: MRC-5, HeP-2, 293, CaCo-2 (human colon carcinoma), MA-104, human fetal intestine (EOL-01-2700; East Clay Laboratories, Noveto, Calif), Vero, and HeLa cells. Cells were examined daily for four days for CPE.

**DNA probes for Verotoxins 1 and 2.** The EA-AggEC strains were hybridized with DNA probes that detect Verotoxins 1 and 2 (Shiga-like toxins 1 and 2) [7]; O157:H7 strains 933J and 933W served as positive controls for Verotoxins 1 and 2, respectively.

**Results**

**Adhesion assay.** Two independent examiners confirmed that the 40 Chilean strains, as well as strains 042 and 221, exhibited the aggregative pattern (figure 1, top left), whereas strain HB101 showed no adherence.

**O Serogroups.** The serotyping results of the 40 Chilean EA-AggEC strains are shown in table 1. Notably, 27 strains (68%) appeared as O nontypable or rough.

**H Typing.** Twenty-nine strains (73%) had an identifiable H antigen with H33 (nine strains), H2 (three strains) and H10 (three strains); the H antigen occurred on multiple occasions.

**Reaction with rough-specific phages.** Although many EA-AggEC strains were initially reported serologically as rough, we surmised that if they were intestinal pathogens they should possess LPS O antigens. To rapidly test this hypothesis, we screened the strains with rough-specific phages. Only five of the 27 strains reported serologically as rough or nontypable were attacked by the phages (table 1), a result implying that the other 22 elaborated smooth LPS.

**Extraction of LPS.** Silver-stained, electrophoresed, hot water–phenol extracts of four randomly selected strains resistant to the rough-specific phages showed the characteristic ladder pattern of LPS. In contrast, rough control strain HB101 was susceptible to the phages and yielded no smooth LPS.

**Hydrophobicity studies.** Sixteen (40%) of the 40 Chilean EA-AggEC strains were hydrophobic (i.e., precipitated by <0.06 M ammonium sulfate), compared with 16 (75%) of 20 ETEC strains. In contrast, only 4 (20%) of 20 EIEC, 1 (5%) of 20 EPEC, and 0 of 20 EHEC strains were hydrophobic. With
Table 1. Characterization of 40 strains of enteroadherent-aggregating *E. coli* from Chilean patients.

<table>
<thead>
<tr>
<th>Strains</th>
<th>O</th>
<th>H</th>
<th>Sensitivity to rough phages†</th>
<th>Presence of plasmids‡</th>
<th>Bacterial aggregation in given concentration of ammonium sulfate (M) at§</th>
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<tr>
<td>5-2</td>
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<td>37 C: 2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>8-1</td>
<td>11</td>
<td>16</td>
<td>–</td>
<td>mul (–)</td>
<td>37 C: &gt;2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>14-1</td>
<td>?</td>
<td>33</td>
<td>–</td>
<td>mul</td>
<td>37 C: 2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>17-2</td>
<td>R</td>
<td>2</td>
<td>–</td>
<td>sing</td>
<td>37 C: 0.01 NT 18 C: 0.01</td>
</tr>
<tr>
<td>32-1</td>
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<td>37 C: 2.0 NT 18 C: NT</td>
</tr>
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<td>8</td>
<td>–</td>
<td>mul</td>
<td>37 C: &gt;2.0 NT 18 C: NT</td>
</tr>
<tr>
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<td>–</td>
<td>mul</td>
<td>37 C: 0.5 NT 18 C: 0.5</td>
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<td>NM</td>
<td>–</td>
<td>mul</td>
<td>37 C: 0.02 NT 18 C: 2.0</td>
</tr>
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<td>33</td>
<td>+</td>
<td>mul</td>
<td>37 C: 1.0 NT 18 C: NT</td>
</tr>
<tr>
<td>45-2</td>
<td>?</td>
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<td>–</td>
<td>mul</td>
<td>37 C: 0.06 NT 18 C: &gt;2.0</td>
</tr>
<tr>
<td>54-1</td>
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<td>28</td>
<td>–</td>
<td>mul</td>
<td>37 C: 2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>61-1</td>
<td>?</td>
<td>33</td>
<td>–</td>
<td>mul</td>
<td>37 C: &gt;2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>62-1</td>
<td>?</td>
<td>33</td>
<td>+</td>
<td>mul</td>
<td>37 C: &gt;2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>63-2</td>
<td>R</td>
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<td>–</td>
<td>mul (–)</td>
<td>37 C: 0.01 NT 18 C: 2.0</td>
</tr>
<tr>
<td>68-1</td>
<td>44</td>
<td>?</td>
<td>–</td>
<td>sing</td>
<td>37 C: 0.5 NT 18 C: 2.0</td>
</tr>
<tr>
<td>69-1</td>
<td>130</td>
<td>26</td>
<td>–</td>
<td>mul</td>
<td>37 C: 2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>70-1</td>
<td>?</td>
<td>5</td>
<td>+</td>
<td>mul</td>
<td>37 C: 0.01 NT 18 C: 2.0</td>
</tr>
<tr>
<td>73-1</td>
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<td>–</td>
<td>sing</td>
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<td>–</td>
<td>mul</td>
<td>37 C: 0.01 NT 18 C: 0.01</td>
</tr>
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<td>80-1</td>
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<td>NM</td>
<td>–</td>
<td>mul</td>
<td>37 C: &gt;2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>105-1</td>
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<td>–</td>
<td>mul</td>
<td>37 C: 0.5 NT 18 C: NT</td>
</tr>
<tr>
<td>108-2</td>
<td>R</td>
<td>10</td>
<td>–</td>
<td>mul</td>
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</tr>
<tr>
<td>115-1</td>
<td>R</td>
<td>2</td>
<td>–</td>
<td>mul</td>
<td>37 C: &gt;2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>117-2</td>
<td>26</td>
<td>32</td>
<td>–</td>
<td>sing</td>
<td>37 C: 2.0 NT 18 C: NT</td>
</tr>
<tr>
<td>121-2</td>
<td>?</td>
<td>?</td>
<td>–</td>
<td>sing</td>
<td>37 C: 0.06 NT 18 C: 0.06</td>
</tr>
<tr>
<td>122-2</td>
<td>R</td>
<td>10</td>
<td>–</td>
<td>mul (–)</td>
<td>37 C: 0.5 NT 18 C: NT</td>
</tr>
<tr>
<td>129-2</td>
<td>?</td>
<td>NM</td>
<td>–</td>
<td>mul</td>
<td>37 C: 0.5 NT 18 C: NT</td>
</tr>
<tr>
<td>135-1</td>
<td>R</td>
<td>?</td>
<td>–</td>
<td>mul</td>
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</tr>
<tr>
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<td>R</td>
<td>45</td>
<td>–</td>
<td>mul</td>
<td>37 C: 1.0 NT 18 C: NT</td>
</tr>
<tr>
<td>137-1</td>
<td>R</td>
<td>?</td>
<td>–</td>
<td>mul</td>
<td>37 C: 0.02 NT 18 C: NT</td>
</tr>
<tr>
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<td>78</td>
<td>10</td>
<td>–</td>
<td>mul</td>
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<tr>
<td>148-3</td>
<td>128</td>
<td>35</td>
<td>–</td>
<td>mul</td>
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<tr>
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<td>4</td>
<td>–</td>
<td>mul</td>
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<td>–</td>
<td>mul</td>
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<td>164-1</td>
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<td>mul</td>
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<td>169-3</td>
<td>?</td>
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<td>+</td>
<td>mul</td>
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<td>171-3</td>
<td>6</td>
<td>1</td>
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<td>mul</td>
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<td>174-3</td>
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</tr>
<tr>
<td>177-1</td>
<td>15</td>
<td>18</td>
<td>–</td>
<td>mul</td>
<td>37 C: 0.01 NT 18 C: NT</td>
</tr>
</tbody>
</table>

* R, rough; ?, nontypable; NM, nonmotile.
† Data are positive (+) or negative (–).
‡ Sing, a single plasmid of 55–65 MDa; mul, multiple plasmids, one of which was 60–70 kDa; mul (–), multiple plasmids <55–65 MDa.
§ NT, not tested.

seven EA-AggEC strains (042, 45-2, 63-2, 68-1, 70-1, 74-2, and 160-2), growth at 18 C, which suppresses fimbrial expression, resulted in a fourfold or greater decrease in hydrophobicity, compared with cells grown at 37 C.

HA. Type 1 somatic fimbriae were detected in 27 strains. Mannose-resistant HA of human and bovine erythrocytes was seen in nine strains, of human red blood cells alone in seven strains, and of bovine cells alone in three strains. None of the strains reacted
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with antisera to CFA I or CFA II (antibody to CS3) [28, 36].

Presence of fimbriae. Only strains lacking type 1 somatic fimbriae were examined by electron microscopy. By negative staining, rigid fimbriae that were 6-7 nm in diameter were visualized on several EA-AggEC strains after growth at 37 C (figure 2). Fimbriae of strain 17-2, the first strain examined, were composed of 16-kilodalton (kDa) subunits. Antiserum prepared against the purified native fimbriae of strain 17-2 was shown, by gold-immunolabelling technique, to react with the fimbriae on the strain 17-2 bacteria (figure 2, bottom). This antiserum was used to screen for the presence of this fimbria in the 42 EA-AggEC strains by using colony blot immunoassay. Six Chilean EA-AggEC strains (17-2 and five others) and Peruvian strain 042 were positive; there was no correlation with any HA pattern. By gold-immunolabeling electron microscopy, the adsorbed antiserum to strain 17-2 fimbriae was shown to label the rigid fimbriae from the seven strains. Partially purified fimbriae from six of these strains showed subunits of 16 kDa; tested by western blot, these strains reacted with adsorbed antiserum to purified strain 17-2 fimbriae.

Two additional fimbriae, 6-7 nm in diameter, with rigid morphology were identified in strains 38-2 and 159-1; these fimbriae are expressed at 37 C but not at 18 C and are composed of subunits of 12 and 18 kDa, respectively. These fimbriae do not react with antiserum to strain 17-2 fimbriae in immunoblots or with antisera to type I somatic fimbriae, CFA/I, CS1, or CS3.

Plasmid profiles and transformation of E. coli HB101. Thirty-seven of 40 Chilean EA-AggEC strains had a 55-65-MDa plasmid; the remaining three strains had smaller plasmids. In 5 of these 37 strains, the 55-65-MDa plasmid was the sole plasmid, whereas in the remaining 32 strains, smaller plasmids coexisted.

Strain 042, with a single 60-MDa plasmid and strain 221 with multiple plasmids, were selected as representative EA-AggEC strains and were used to transform E. coli HB101. Three of the four plasmids of strain 221, among them the 60-MDa plasmid, were simultaneously transformed into E. coli HB101; the small plasmids were cured from the transformant to leave the single ~60-MDa plasmid. The transformed HB101 containing the strain 042 plasmid and the HB101 with the plasmids from strain 221 exhibited the aggregative pattern in the HEp-2 cell assay and expressed fimbriae; these properties were lacking in the HB101 parent. The 042 transformant also acquired the capacity to produce LPS, as shown by extraction of LPS (figure 3).

Plasmid homology. A 1.0-kilobase fragment of the plasmid from strain 042 hybridized with 20 (49%) of the other 41 EA-AggEC strains tested but did not hybridize with any of the 40 ETEC, EPEC, EIEC, and EHEC strains. Subsequent experiments using

Figure 2. Top, an electron photomicrograph of EA-AggEC strain 17-2 grown at 37 C. Note the long, rigid fimbriae (6-7 nm in diameter) at the surface of the bacterium, as shown by negative staining. Bottom, the same fimbrial structures after incubation with adsorbed rabbit antiserum to strain 17-2 fimbriae and with gold-labeled goat antibody to rabbit IgG. The rigid fimbriae are specifically labeled with 10-nm gold particles.
purified plasmid DNA confirmed that the homologous sequences resided in plasmid DNA.

Pathogenicity in animal models. All four EA-AggEC strains tested in rabbit and rat intestinal loops induced striking histopathologic changes that were characterized by shortening of the villi, hemorrhagic necrosis of the villus tips, and a mild inflammatory response with edema and mononuclear infiltration of the submucosa (figure 4). The pathological lesions, although varying in severity, were qualitatively consistent. These preliminary histological data suggest that EA-AggEC may elaborate a toxin that damages enterocytes. No pathological lesions were seen in loops infected with HB101. In loops infected with E2348/69, the classic histopathology of EPEC was seen, including attachment of E. coli; villous effacement, as shown by light microscopy; and microvillar dissolution at the site of attachment of bacteria, as shown by electron microscopy [37].

Search for toxins. After inoculation with live 17-2, 042, and 221 bacteria to prepare antisera, five of eight rabbits developed severe limb paralysis and died. The natural course of this syndrome was followed up in three rabbits. All rabbits had fatal outcomes with no regression of the paralysis; two rabbits were killed 48 h after the onset of the apparent neurological deficit. Because of the similarity of this syndrome in rabbits to that seen following inoculation with Shiga toxin or with bacteria that elaborate Shiga toxin [38, 39], a search was made for such potent cytotoxins. Neither supernatants nor sonicated cell pellets from bacteria cultured in plain or iron-depleted TSB induced a CPE on the eight cell lines tested, including HeLa cells sensitive to Shiga toxin. Furthermore, none of the 42 EA-AggEC strains hybridized with the Verotoxin 1 or 2 gene probes.
Discussion

A prospective case-control study of diarrhea due to *E. coli* of the different categories in infants and young children in Santiago, Chile, using DNA probes and the HEp-2 assay, incriminated EA-AggEC as an important agent associated with diarrhea [14, 15]. EA-AggEC were isolated significantly more often from 154 patients (36%) than from 66 controls (23%; \( P < .03 \)), and they appeared to comprise a distinct category of diarrheagenic *E. coli*, because they were rarely recovered from patients infected with ETEC, EPEC, EIEC, or EHEC. In view of these incriminating epidemiological data, we initiated a preliminary characterization of some representative strains.

In a recent review, Levine [1] noted that the ETEC, EPEC, EIEC, and EHEC categories of diarrheagenic *E. coli* have certain features in common: distinct sets of O:H serotypes are encountered, critical virulence properties are encoded by plasmids, there is a characteristic interaction with intestinal mucosa, and they tend to elaborate enterotoxins or cytotoxins. Consequently, our characterization of EA-AggEC proceeded with these features in mind.

We could not correlate any specific O group with EA-AggEC, because the aggregative phenomenon often interfered with its determination by tube agglutination. Alternative O-grouping methods may have to be used [40]. Nevertheless, the recurrence of certain H types, particularly H33, suggests that common serotypes exist. With one exception (ETEC serotype O148:H28), the O:H serotypes identified were not characteristic of other categories of diarrheagenic *E. coli* (table 1).

Our observations suggest that plasmids appear to be important in encoding or expressing some putative virulence properties of EA-AggEC. In the two strains investigated so far by using bacterial genetic methods, transfer of the 55–65-MDa EA-AggEC plasmid also transferred the ability to express smooth LPS, fimbriae, and the aggregative phenotype. This preliminary investigation did not identify the plasmid-encoded products responsible for the aggregative phenomenon. Possibilities include hydrophobic outer membrane proteins (as in *Yersinia* [41]), hydrophobic fimbriae [42], or an unusual LPS. Because the plasmid of strain 042 is associated with the expression of smooth LPS, as well as the aggregative phenotype, LPS may, in part, be involved.

Fimbriae serologically related to the prototype fimbriae of strain 17-2 were found in seven EA-AggEC strains. Already two other antigenic types of fimbriae have been found. EA-AggEC may thus resemble ETEC, in which many distinct fimbrial colonization factors have been recognized [1]. The role of the fimbriae is conjectural, but we are investigating the hypothesis that they mediate adherence to intestinal epithelium (figure 5). Fimbriae may also be responsible for bacterial aggregation. Hydrophobicity determinations are not, however, conclusive—40% of the EA-AggEC strains were hydrophobic, and in some, this property disappeared under conditions that inhibited fimbrial expression.

A salient feature of experimental EA-AggEC infection in isolated rabbit and rat intestinal loops is the striking histopathologic lesions, which are not described for intestinal mucosa infected with ETEC [43], EPEC [37], EIEC [44], or EHEC [45, 46]. The nature of the intestinal lesions and the “neurotoxic” syndrome in rabbits inoculated with live bacteria suggest the involvement of toxins.

One must now consider if a relationship exists between EA-AggEC and the “enteroadherent” *E. coli* referred to by Mathewson et al. [11]. These investigators used this term to refer to *E. coli* strains, almost entirely of non-EPEC serogroups, that they reported as adhering to HEp-2 cells in patterns of localized or diffuse adherence. Mathewson et al. [11] recognized the quandry raised by their findings with respect to earlier reports and wrote “This finding raises an interesting, but confusing, question as to the relationship of non-EPEC, enteroadherent *E. coli* strains...
to EPEC strains. . . . The description and preliminary characterization of EA-AggEC goes a long way toward resolving the question. For instance, strain 221 was cited by Mathewson et al. as an example of a non-EPEC serogroup strain that gave localized adherence and was found to cause diarrhea when fed to volunteers [12]. In contrast, in the HEp-2 assay as we perform it (i.e., as recommended by Cravioto et al. [8]), strain 221 clearly gives the aggregative pattern and not the localized adherence pattern characteristic of EAF-positive EPEC. Moreover, strain 221 is EAF negative by probe and has H33 flagellar antigen. The explanation for the conflicting observations may reside in differences in the HEp-2 assay as performed by ourselves and Cravioto et al. [8] versus the method used by Mathewson et al. [11]. The assay as recommended by Cravioto et al. [8] involves a single 3-h incubation of bacteria with HEp-2 cells before washing and staining. Mathewson et al. [11] describe a preliminary 1-h incubation of bacteria with HEp-2 cells, followed by washing of the monolayer before a 2-h incubation. Conceivably, with this modification of Cravioto’s recommended assay, the aggregative pattern cannot be discerned. Accordingly, we surmise that for this or other reasons, Mathewson et al. inadvertently could not detect the aggregative pattern in strain 221 and that this pattern appeared to them to be localized adherence; we presume this occurred with many other strains they refer to as “enteroadherent.” To resolve this important point, we have initiated a collaborative study with Mathewson and co-investigators.

Data from recent studies on molecular genetics demonstrate that E. coli strains that manifest localized, diffuse, or aggregative adherence represent three distinct and separate categories. Ninety-seven percent of E. coli that manifest localized adherence are positive with the EAF gene probe [13] and usually are of classic EPEC O serogroups [14, 15]; totally distinct are strains that manifest diffuse adherence. With 96% specificity and 75% sensitivity [15], these strains can now be identified by means of a DNA probe [47]. Finally, preliminary results with a candidate DNA probe for EA-AggEC are also promising and show high specificity and sufficient sensitivity to detect 49% of the EA-AggEC strains; these data also demonstrate genetic heterogeneity among EA-AggEC.

The results of this preliminary characterization support the epidemiological data that contend that EA-AggEC may represent a distinct category of diarrheagenic E. coli. These results should serve to stimulate further research on this putative diarrheal pathogen.

References

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23. Faris A, Wadstrom T, Freer JH. Hydrophobic adsorptive and hemagglutinating properties of Escherichia coli possessing colonization factor antigens (CFA-I or CFA-II), type 1 pili, or other pili. Current Microbiology 1981;5:67-72
28. Eiklid K, Olssen S. Animal toxicity of Shigella dysenteriae cytotoxin: evidence that the neuroparalytic, entero-toxic, and cytotoxic activities are due to one toxin. J Immunol 1983;130:380-4
29. Engley FB Jr. The neurotoxin of Shigella dysenteriae (Shiga) Bacteriological Reviews 1952;16:153-78