HEp-2 Cell Adherence Patterns, Serotyping, and DNA Analysis of Escherichia coli Isolates from Eight Patients with AIDS and Chronic Diarrhea

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Three morphologic patterns of interaction between bacteria and enterocytes have been observed in colonic biopsy specimens from AIDS patients with chronic diarrhea in the United States. The DNA encoding virulence factors and the HEp-2 cell adherence patterns of Escherichia coli strains isolated from the stools of eight symptomatic AIDS patients were compared with those of five control strains with known adherence patterns. One clinical isolate from a patient with attaching-and-effacing enteropathy displayed the localized adherence attaching-and-effacing pattern typical of enteropathogenic E. coli on HEp-2 cells, five isolates displayed the “stacked-brick” aggregative adherence pattern typical of enteroaggregative E. coli strains, and one isolate showed the pattern characteristic of diffusely adherent E. coli. One patient’s isolate displayed features of all three patterns. No clinical isolate hybridized with standard probes for enteropathogenic, enteroaggregative, diffusely adherent, enterotoxigenic, and enteroinvasive E. coli strains. Thus, isolates from symptomatic AIDS patients in the United States can display the same interactive patterns with HEp-2 cells as the agents of pediatric or traveler’s diarrhea, but lack their typical virulence factors.

At least half of all human immunodeficiency virus (HIV)-infected patients in developed countries and more than 90% of those in the developing world suffer from chronic diarrhea and wasting during the course of their HIV disease (1, 18). The diarrhea is associated with high rates of morbidity and mortality. Despite significant advances, including the identification of new intestinal pathogens, the etiology of diarrhea still remains obscure in a substantial portion of these patients (34).

In the non-HIV-infected population most susceptible to diarrhea, infants and young children, the most important worldwide diarrheagenic bacterial pathogen is Escherichia coli, although Salmonella and Shigella are common in the United States (26). Six categories of diarrheagenic E. coli are defined by the presence of specific genes that encode for their virulence traits: enteropathogenic E. coli (EPEC), enteroinvasive E. coli (EIEC), enterotoxigenic E. coli (ETEC), enterohemorrhagic E. coli (EHEC), enteraggregative E. coli (EAEC), and diffusely adherent E. coli (DAEC) (17, 21, 43). In developing countries, EPEC, ETEC, and EAEC are particularly common in young children (43, 48). EPEC, EAEC, and DAEC are detected by the HEp-2 assay, and molecular probes which reveal specific virulence factors are now available (9, 12, 23).

Until recently, outbreaks of diarrheagenic E. coli have seldom been identified in industrialized countries; now EHEC O157:H7 is recognized as a significant problem (42). However, since clinical microbiology laboratories do not routinely attempt to isolate and verify lactose-fermenting organisms from stool specimens, the true prevalence of E. coli diarrhea is unknown.

The immunodeficiency of HIV disease adds a new dimension to the problem of bacterial enteritis; e.g., EIEC has been shown to cause recurrent bacteremia (4). Enteric bacterial infections, including the classic attaching-and-effacing EPEC type, have recently been observed in colonic biopsy specimens from AIDS patients with chronic diarrhea in the United States (35). Bacterial enteropathy was demonstrated in colonic biopsy specimens from almost 20% of AIDS patients with idiopathic chronic diarrhea (20). EPEC has been isolated from infants with diarrhea born to HIV-seropositive mothers in Zaire (36). Enteroadherent E. coli strains (i.e., EPEC, EAEC, and DAEC) were suspected to be the cause of 60% of cases of diarrhea in AIDS patients studied in Zambia (27).

The aim of this study was to compare the DNA virulence factors and the light and electron microscopic patterns of HEp-2 cell adherence of E. coli isolates from eight symptomatic patients with AIDS to those of control isolates with known serotypes.

MATERIALS AND METHODS

Strains and patients. Pure E. coli isolates from the stools of eight AIDS patients with severe immune depression, weight loss, and chronic diarrhea (three or more loose or watery stools per day for at least 30 days [1, 20, 35]) were evaluated. The stools from seven of the patients (patients A to G) were selected for study because a recent colonic biopsy specimen showed bacteria associated with enterocyte pathology, as reported previously (35). The eighth patient (patient H) was selected because his stool was negative for pathogens that would otherwise explain his diarrhea, and endoscopy was not performed on patient H. Four of the patients’ colonic biopsy specimens had rare cytomegalovirus-infected endothelial cells that did not appear to be associated with epithelial damage. For only two patients was another disease identified in the small bowel: a low level of Enterocytozoon bieneusi microsporidiosis. Three of the patients (A, B, and H) who were known to have been treated with antibiotic therapy (e.g., ciprofloxacin) responded well (35). Four patients (patients C to F) were from George Washington University Medical Center, and four patients (patients A, B, G, and H) were from the St. Luke’s-Roosevelt Hospital Center. For seven patients CD4 T-cell counts were determined within a few months of the E. coli isolation, and while the counts for two patients (patients E and F) were above 100 cells/mm³ (patient E, 136 cells/mm³, patient F, 133 cells/mm³), the counts for the remaining patients were below 50 cells/mm³.
For *E. coli* isolation, stool specimens were first streaked onto MacConkey, Hektoen enteric, and MacConkey-sorbitol agar plates. Following overnight incubation five suspicious colonies from each plate were tested for motility and indole, ornithine, and citrate utilization. Subcultures of isolated strains were then tested in the API 20E system for final identification of members of the family Enterobacteriaceae or other gram-negative bacteria. The strains were usually sensitive to ciprofloxacin.

Bacterial cultures were grown static at 35°C overnight in Trypticase soy broth, and the predominantly growing *E. coli* strain was isolated and propagated. Nonadherent commensal *E. coli* II, serotype O9:H14, isolated from a normal stool from a healthy man, served as a negative control. Four *E. coli* strains from the Center for Vaccine Development, University of Maryland School of Medicine (25, 32), with well-established adherence patterns were used as the standard controls for three adherence patterns: (i) locally adherent (LA) EPEC strain E2348/69, serotype O127:H6, isolated from an infant diarrhea outbreak in England; (ii) aggregatively adherent (AA) EAggEC strain O42, serotype O44:H18, isolated from a child with acute diarrhea in Lima, Peru; (iii) an AA EAggEC hemolytic strain 17-2, serotype O3:H2, isolated from an infant with diarrhea in Chile; and (iv) diffusely adherent (DA) DAEC strain C1845, serotype O75:NM (nonmotile), isolated in Seattle, Wash., from a child with chronic diarrhea.

**HEp-2 cell adherence assay.** HEp-2 cell monolayers (50 to 70% confluence) were grown on circular 13-mm glass coverslips (BioWhittaker, Walkersville, Md.). Twenty microliters of bacterial cultures (2 × 10⁶ bacteria) was injected into each well, and the plates were incubated for 3 h in a humid, 5% CO₂ atmosphere. By the technique of Cravioto et al. (8) modified by Nataro et al. (32), culture medium was aspirated from the monolayers, which were washed four times with phosphate-buffered saline, fixed in 70% aqueous methanol for 5 min, stained with 10% Giemsa stain (Fisher, Pittsburgh, Pa.), and examined by light microscopy.

**Transmission electron microscopy.** Duplicate HEp-2 cell monolayers were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.25) for at least 1 h. The fixed monolayers were scraped from the glass surface with plastic pipettes, processed into agar blocks, and postfixed for 1 h in 1% OsO₄. Cells were washed and dehydrated through graded ethanol and propylene oxide and embedded in Spurr’s epoxy. Some 2.5% glutaraldehyde-fixed specimens were also block stained with 1% uranyl acetate during ethanol dehydration.

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**FIG. 1.** Light and electron microscopy of LA of the clinical isolate from patient B (A and B) and control EPEC strain E2348/69 (C and D) on HEp-2 cells. Light microscopy shows clusters of bacteria attached to cell surfaces, while transmission electron microscopy discloses the attaching-and-effacing lesions with villous effacement, cytoskeleton rearrangement, and pedestal formation. (A and C) Giemsa stain. Magnification, ×740; (B and D) Transmission electron microscopy. Magnification, ×30,000.
DNA probes. DNA probe analysis of the E. coli isolates was performed by a colony blot lift technique (12). DNA probes were labeled with the BIO-PRIME primer extension kit and detected by using the Blue-Gene system (both from Gibco/BRL, Gaithersburg, Md.). Probes specific for ETEC heat-labile toxin (29) and heat-stable toxin (oligonucleotide) (30), EPEC EAF (31) and Eae (16), EIEC pPS2.5 (41), EaggEC pCVD432 (2), DAEC pSLM852 (7), and EHEC pCVD419 (24) were used in this analysis. Probe hybridization was performed overnight at 42°C in a hybridization solution containing 45% formamide. After hybridization, filters were washed in 0.16× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.01% sodium dodecyl sulfate solution at 50°C as described by Gicquelais et al. (12).

Serotyping. Serotyping was performed at the Centers for Disease Control and Prevention by using standard procedures and sera.

RESULTS

LA. The isolate from patient B elicited an LA pattern, similar to that of EPEC control strain E2348/69 (O127:H6), when it was cultured with HEp-2 cells. Bacilli formed compact attachment plaques on the HEp-2 cells, but were not attached to the glass substrate (Fig. 1A and B). At the transmission electron microscopy level, the bacilli were observed to be attached preferentially to the supranuclear plasma membrane of the HEp-2 cells. Microvilli were effaced by firmly attached bacteria which were often situated in cupped ends of pedestal-like cell protrusions (Fig. 1B and D). For the attachment plaques, relatively uniform narrow gaps were found between the bacteria and the plasma membrane, and subplasmalemmal concentrations of actin-size thin filaments were found in the plaques. Bacteria were occasionally found within cytoplasmic vacuoles. Actin-rich plaques appeared to be maintained throughout internalization and could completely surround the intravacuolar bacteria. The strain from patient B was serotyped as O108:NM.

Despite the observation that the isolate from patient B gave a typical LA, EPEC-type phenotype when it was grown on HEp-2 cells, it did not hybridize with the eaeA and EAF probes.

AA. Five of the clinical isolates (those from patients A, C, D, E, and G) demonstrated AA to HEp-2 cells and the glass surface identical to those of the EAggEC O42 (O44:H18, hly mutant) and 17-2 (O3:H2, hly+ ) control strains (Fig. 2A and C). Transmission electron microscopy of the clinical isolates
and the control EAggEC strains showed bacteria loosely associated with the cell surface (Fig. 2B and D). Neither attachment plaques, pedestals, nor cytoskeleton rearrangements were observed. The strains from patients C to E were serotyped as O15:H18, O14:H10, and O153:H19, respectively, while the nonmotile strain from patient A agglutinated equally with O8 and O75ac antisera and, therefore, was serotyped as O8,O75ac:NM. The strain from patient G formed rough, dull colonies which were devoid of O antigen and therefore could not be serotyped.

Despite their typical EAggEC phenotype, none of these five clinical isolates hybridized with the AA probe.

**DA.** The isolate from patient F displayed the same DA pattern to HEp-2 cells as the control DAEC strain, strain C1845 (Fig. 3A and C). Bacteria were randomly distributed over the surfaces of the cells and were intercalated into the...
microvilli (Fig. 3B and D). There were no attachment plaques, pedestals, or cytoskeletal rearrangements. Occasional bacteria were found within cytoplasmic vacuoles.

Despite the typical DA phenotype, the strain from patient F did not hybridize with either the DA or the other probes.

**Mixed adherence pattern.** The isolate from patient H showed a combination of the attaching-and-effacing LA, a stacked-brick AA pattern, and intercalated DA patterns, respectively (Fig. 4A to D). Bacteria were occasionally found in cytoplasmic vacuoles.

This strain was serotyped as O8, O75ac:NM, and DNA hybridization studies were negative for this strain.

**DISCUSSION**

This in vitro study indicates that *E. coli* strains isolated from the stools of patients with AIDS and chronic diarrhea in the United States display the same phenotypic patterns on HEp-2 cells as pathogenic enteric bacteria previously considered to be predominantly confined to patients with diarrhea in the developing world. The infection in this immunocompromised patient population appears to be predominantly of the colon, especially the right colon (35). Despite the similarities of the HEp-2 patterns of the clinical isolates to the patterns of known pathogenic strains, these clinical isolates lack their genotypes and belong to other serotypes. For example, the strain from patient B had an O108:NM serotype and elicited the classic attaching-and-effacing lesions with HEp-2 cells, but it was found to be *eaeA* DNA probe negative. This suggests that there are, as yet, unidentified factors associated with HEp-2 cell and enterocyte adherence phenotypes that allow bacteria, possibly nonpathogenic bacteria, even in the developing world, to be pathogenic for immunocompromised individuals, such as patients with AIDS.

EPEC has been known as an important enteropathogen for many years (22, 37, 48), and it is drawing increasing interest with the emergence of the large immunocompromised HIV-infected patient population. Autopsies of infants who have died during the initial stage of disease revealed dense films of

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**FIG. 4.** Light (A) and electron (B to D) microscopy showing mixed adherence pattern of the isolate from patient H on HEp-2 cells. While light microscopy predominantly shows AA, transmission electron microscopy shows LA (B), loose aggregative (C), and DA intercalated (D) patterns of interaction. (A) Giemsa stain. Magnification, ×1,100. (B to D) Magnification, ×25,000.
bacteri a adhering to damaged ileal and cecal mucosa (10, 21, 22). These strains display the same cytopathology in animal models (11), small intestinal biopsy specimens from infants with diarrhea (39, 46), and now, colonic biopsy specimens from AIDS patients with chronic diarrhea (35). Electron microscopic studies of EPEC strains demonstrate attaching-and-effacing lesions with pedestals and cytoskeletal rearrangements (28, 37, 45).

EAggEC, with its enterotoxin and AA pattern, has been associated with diarrhea in both children and adults and now in patients with HIV disease (5, 6, 14, 27, 33, 40, 47). EAggEC manifests the same characteristic stacked-brick AA pattern on the intestinal mucosa and urothelium that is seen on the cells and the glass substrate in the HEp-2 cell assay (19, 44, 49). The EAggEC control isolate and five clinical EAggEC isolates displayed the same loose association with the HEp-2 cells and a lack of cytoskeletal rearrangement, internalization, or intercalation. EAggEC AA resembles the so-called loose pattern of cytopathic enterocyte interaction that was demonstrated in colonial biopsy specimens from symptomatic AIDS patients, including three described here (patients A, D, and G) (35).

DAEC is receiving increased attention as a putative cause of diarrhea (13, 15, 23, 43). The DA HEp-2 cell growth pattern, characteristic of DAEC C1845, was encountered as the sole growth pattern for one clinical isolate. The bacteria were intercalated among microvilli and were occasionally internalized. Similar intercalation of bacilli with microvilli had been observed in colonial biopsy specimens from seven patients with AIDS and chronic diarrhea (35).

The apparent combination of three patterns (AA, DA, and LA) seen in the isolate from one patient could have several explanations. Some researchers have suggested heterogeneity of DAEC strains from children with diarrhea. Even among the cell DA-positive and probe-positive DAEC strains, LA-positive (clustered adherence) bacteria causing cell cytoskeleton rearrangement and actin accumulation, like typical EPEC isolates, have been found in experiments with HeLa cells (50, 51). However, EPEC attaching-and-effacing lesions have not been observed after attachment of the DAEC C1845 strain to the brush border of polarized monolayers of Caco-2 or T84 cells (3). The bacteria did cause elongation of microvilli and vesiculation of their tips.

An additional patient with AIDS and chronic diarrhea was recently found to have attaching-and-effacing bacteria in biopsy specimens showing ileocolitis (38). Initial PCR studies of the patient’s two E. coli isolates revealed an O111:NM serotype, eaeA probe-positive strain, a classical diarrheagenic LA strain for infants and young children, and a O130:H27 serotype, eagg probe-positive strain, an unusual EAggEC strain that is associated with diarrhea and that displays an AA pattern of interaction (38).

The finding of E. coli isolates from the stools of symptomatic patients with AIDS that attach to HEp-2 cells and the finding of attaching-and-effacing lesions in biopsy specimens suggest the possibility that these strains are the cause of the pathology (35). The fact that such a strain can be negative with the eaeA, eaf, and EHEC probes suggests the possibility of a novel attaching-and-effacing pathogen. At a recent consensus conference on EPEC (unpublished data), such organisms were hypothesized to exist and given the name atypical EPEC.

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REFERENCES


