Localized Adherence and Attaching-Effacing Properties of Nonenteropathogenic Serotypes of Escherichia coli

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Traditional enteropathogenic Escherichia coli serotypes demonstrate a plasmid-mediated localized adherence in cultured HeLa or HEp-2 cells and induce an attaching-effacing intestinal lesion, both of which are considered pathognomonic and causes of diarrhea. This study describes three E. coli strains from infantile diarrhea which share these properties but belong to serotypes (O2:H2, O2:H25 and O15:H2) not considered enteropathogenic.

The term enteropathogenic Escherichia coli (EPEC) was coined by Neter et al. (17) for a collection of epidemiologically incriminated E. coli serotypes which, in many studies throughout the world, were isolated with significantly greater frequency from infants with diarrhea than from healthy controls. The World Health Organization's definition of EPEC includes the following serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (24).

It was only recently demonstrated that the majority of these serotypes adhere to HeLa and HEp-2 cells in culture, with the majority of strains showing a localized adherence (LA) and a few showing a diffuse adherence (20). Baldini et al. (2) showed that LA is mediated by an ~60-MDa plasmid, and the term EPEC adherence factor (EAF) was given to the gene product (1). A DNA probe constructed from EAF plasmid correctly identified LA+EPEC (16). Human volunteer studies suggested that EAF plasmid-associated product is an important virulence factor in the pathogenesis of EPEC diarrhea (13).

Ultrastructural studies of intestinal mucosa of naturally infected humans and experimentally infected animals suggested that EPEC produce diarrhea by an attaching-effacing (A/E) type of lesion (15, 22, 23). In this lesion, bacteria destroy the brush border of enterocytes and closely adhere to the apical surface, causing cupping and pedestal formation by the cell membrane and electron-dense fibrillar modifications in the terminal web areas beneath the attached bacteria. Bacteria occasionally are seen within phagosomes. There is evidence of inflammatory cell response in the lamina propria of affected mucosa (22).

In vitro studies by Knutton et al. (9, 11) suggested that EAF appears initially to facilitate contact with the mucosa, but chromosomally encoded factors are required to produce the A/E lesion. Subsequent in vivo studies confirmed this observation (21).

Studies in Peru and Brazil found that LA+EPEC serotypes were significantly associated with diarrhea, whereas LA+non-EPEC serotypes which were also encountered were isolated in equal frequency from both patients with diarrhea and controls (8, 14). However, in a study in Thailand (5), the vast majority of LA+non-EPEC serotypes were isolated from a significantly higher proportion of diarrheal cases than from controls.

In our studies on the role of diarrheagenic E. coli in the causation of diarrhea in Bangladeshi children, we isolated LA+EPEC serotypes from approximately 7% of 400 diarrheal children <1 year old (unpublished observations). We investigated the mucosal association of these serotypes in a rabbit ileal loop assay system to study their potential pathogenicity and role as diarrheal pathogens. The rabbit ileal loop assay system has been found previously to be suitable for studying the A/E properties of EPEC serotypes (15). An in vitro test for A/E lesions is the fluorescent actin staining (FAS) assay which uses tissue culture cells (10).

The three E. coli isolates studied were cultured from the stools of three children with watery diarrhea. Serotyping by O and H antisera by standard methods (3, 4) found that the isolates belonged to serotypes O2:H2, O2:H25, and O15:H2. No recognized enteric pathogens were isolated from these children. DNA probes were used to detect heat-labile enterotoxin (LT) and human and porcine heat-stable enterotoxins (STh and STp), E. coli enteroinvasiveness plasmid, EAF, diffuse adherence, and Shiga-like toxins I and II (SLTI and SLTII) in these serotypes as described previously (14, 18). The results were corroborated with bioassays. In bioassays, LT production was tested by the Y-1 adrenal tumor cell assay (19), ST production was tested by the sucking mouse assay (6), and SLTI and SLTII production was tested by the HeLa cell assay (7). The pattern of adherence to HeLa cells was tested in the presence of 0.5% D-mannose (14). All three E. coli serotypes were positive for LA by both DNA probe and tissue culture assays, but were negative for other diarrheagenic properties. Although E. coli strains belonging to the O15 serogroup have been described previously as toxigenic (12), E. coli O15:H2 in the present study was obviously not toxigenic.

The 3-h FAS assay was performed with HEp-2 cells as described previously (10), and all three serotypes were positive in this test.

The rabbit ileal loop assay was done by the procedure of Moon et al. (15). Nine- to ten-week-old New Zealand White rabbits were fasted overnight before ileal loop surgery. Six ligated loops, each approximately 10 cm long, were made in each rabbit. Each loop was inoculated with 1.0 ml of culture containing 10⁶ bacteria grown aerobically at 37°C for 24 h in

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FIG. 1. Hematoxylin-and-eosin-stained sections of rabbit ileal loop inoculated with negative control strain *E. coli* K-12 (A) and *E. coli* serotype O2:H2 (B and C). (A) Mucosa appears normal; there is no bacterial attachment to the villus tip. (B) Bacterial attachment to the surface of the epithelium is seen. (C) There is damage to the epithelial layer and large numbers of bacteria are seen penetrating epithelium and multiplying in probably dead cells. Inflammatory cells can also be seen in the lamina propria. Magnification, ca. ×410 for panels A, B, and C.

Trypticase soy broth (BBL). All strains, including a control nonpathogenic *E. coli* K-12 strain, were tested in two rabbits. The animals were sacrificed after 24 h. At autopsy, gross appearance of the loop including accumulation of fluid was noted, and sections were fixed in neutral buffered Formalin for morphological studies by light microscopy and transmission electron microscopy (22).

None of the three isolates induced fluid accumulation in the ileal loops. Histology, however, revealed foci of closely adherent bacteria on the mucosal surface. There were also cellular changes which included irregularity of the surface layer with attached bacteria and neutrophil infiltration of degenerated epithelium, lamina propria, and submucosa. All of these changes could be seen in the same loops. Some of these features are shown in Fig. 1B and C. No such changes were observed in the loops inoculated with *E. coli* K-12 (Fig. 1A).

Examination of the ultrathin sections of loops inoculated with all three patient isolates showed typical lesions associated with bacterial attachment to the surface of enterocytes with loss of the microvillus border. There was cupping of plasma membrane around bacteria, pedestal formation, and
increased electron density in the terminal web just beneath the site of bacterial attachment. Microvilli in between the sites of bacterial attachment were elongated. Intracellular bacteria were seen in phagolysosomes. Some of the changes are shown in Fig. 2. Each strain produced similar results in both rabbits.

Light and electron microscopic examinations of the lesions produced by the three nonenteropathogenic serotypes of *E. coli* in rabbit ileal loops showed that lesions were identical to those produced by traditional EPEC serotypes (15, 22, 23). Moreover, like traditional EPEC serotypes, they also produced LA in cultured HeLa cells. Studies to date have suggested that the two critical virulence factors for full pathogenicity of traditional EPEC are the abilities to produce LA and A/E lesions (9, 13, 21). Since the three nontraditional serotypes isolated from children with watery diarrhea also possessed these critical virulence factors, they should be considered as diarrheagenic as the traditional EPEC serotypes.

Knutton et al. (10) have shown recently that some of the non-EPEC serotypes were positive for A/E lesions by FAS assay. Our three non-EPEC serotypes which were positive for A/E lesions in gut loop were also positive for A/E lesions in the FAS assay. This suggests that the FAS test may also be used as a substitute test for the difficult in vivo gut loop test to screen non-EPEC serotypes for the ability to produce A/E lesions.

The traditional EPEC serotypes were initially identified in
Western countries by serotyping during investigations of epidemics of diarrhea. Perhaps strains other than the traditional serotypes have been causing both endemic and epidemic diarrhoeas in developing countries. Since methods to study these agents were not available, their role as causative agents of diarrhea would have been ignored. Case control studies based on demonstration of virulence characteristics need to be carried out to assess the role of nontraditional serotypes.

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