Intestinal Immune Response of Volunteers Ingesting a Strain of Enteroadherent (HEp-2 Cell-Adherent) Escherichia coli

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Enteroadherent Escherichia coli (EAEC) strains identified by adherence to HEp-2 tissue culture cells have been incriminated epidemiologically as important etiologic agents of diarrheal disease in both adult travelers and children in developing countries. One strain, JM 221, with no recognized E. coli virulence characteristics other than adherence to HEp-2 cells, caused diarrhea in 5 of 16 volunteers ingesting it. We studied the secretory immunoglobulin A (sIgA) responses to EAEC JM 221 of five volunteers with diarrhea and five volunteers who remained healthy after challenge. sIgA was extracted from stools obtained prechallenge and 7 days postchallenge. Total sIgA was standardized for all specimens. Specific sIgA titers were determined by dot blotting with the following JM 221 antigens: water-extractable surface antigens, whole cells, lipopolysaccharides, and outer membrane proteins. All five subjects who became ill had fourfold or greater rises in titers against each of the four antigens. The five subjects who remained healthy following challenge did not exhibit significant rises in titers to any JM 221 antigens, but their mean titers were significantly higher than the mean prechallenge titers of the volunteers with diarrhea, suggesting that high intestinal sIgA titers may be protective. The significant increases in intestinal antibody against JM 221 in the subjects who became ill is further evidence of the enteropathogenicity of EAEC strains.

HEp-2 cell adherence as a virulence feature of diarrheagenic Escherichia coli was first described among strains of enteropathogenic E. coli (EPEC) identified by serotype and implicated as causes of acute infantile diarrhea (2). Recently, HEp-2 cell adherence was recognized as a virulence feature of other E. coli strains not belonging to traditional EPEC serotypes (1, 5, 7, 8, 10). These HEp-2 cell-adherent E. coli strains have been referred to as enteroadherent E. coli (EAEC) and as enteroaggregative E. coli (6, 8, 12). EAEC strains have been incriminated as etiologic agents of diarrheal disease by epidemiologic studies, where these strains have been found more frequently in stool specimens from patients with diarrhea than in those from healthy controls (1, 5, 7, 8, 10). EAEC has been found in travelers and children with diarrhea in several developing countries (1, 5, 7, 8, 10). The frequency of occurrence of EAEC strains in stool specimens from patients with diarrhea has suggested that they could be important etiologic agents of infectious diarrhea (10). Entero-toxigenic E. coli was the only organism found more frequently than EAEC as an etiologic agent in our U.S. student population acquiring diarrhea in Mexico (8), and in one group of Mexican children with acute diarrhea during the summer of 1987, EAEC was the most commonly identified etiologic agent (10). A volunteer challenge study has confirmed that certain EAEC strains have the ability to cause diarrhea in humans (9). One strain, JM 221, caused diarrhea in 5 of 16 volunteers ingesting between 10⁸ and 10¹⁰ viable organisms. This E. coli had no recognized virulence characteristics of diarrheagenic E. coli other than adherence to HEp-2 tissue culture cells. The test strain did not produce heat-labile, heat-stable enterotoxins or Shiga-like toxins, it was not enteroinvasive, and it did not belong to any recognized EPEC serotype. Three patterns of HEp-2 cell adherence according to assay methodology have been described (12). JM 221 exhibited localized and aggregative adherence in the two currently utilized assays. In addition, the enteroadherence factor probe described for EPEC localized adherence did not hybridize with JM 221. A second EAEC strain in the same study exhibiting diffuse and aggregative HEp-2 cell adherence did not cause illness in volunteers experimentally challenged (9).

Since EAEC strains are relatively newly described agents of disease and their virulence characteristics other than various forms of adherence to tissue culture cells that are involved in pathogenicity for humans are not understood, we sought to determine if an EAEC strain shown to be pathogenic for volunteers elicits an intestinal immune response. Also, we sought to identify the antigens to which a secretory immunoglobulin A (sIgA) response is mounted.

MATERIALS AND METHODS

Volunteers. Specimens from adults challenged with HEp-2 cell-adherent E. coli JM 221 were used in the present study (9). All volunteers were healthy adults who were admitted to the General Clinical Research Center of the Methodist Hospital in Houston, Texas, in 1985. Eight volunteers ingested 7 × 10⁷ JM 221 organisms, and eight others ingested 1 × 10¹⁰ organisms of this strain after neutralization of their stomach acid. Prechallenge and postchallenge (day 7) stool specimens were collected and stored at −70°C. Of the 16 volunteers challenged, 5 developed diarrhea due to JM 221. Paired stool specimens from the five volunteers who developed illness and from five volunteers who remained healthy after challenge were used in this study.

Antigen preparation. Four different antigens were prepared from E. coli JM 221 (9). To prepare the whole-cell (WC) antigen, the test organism was grown in Trypticase soy broth overnight at 35°C. Cells were harvested by centrifugation and rinsed in phosphate-buffered saline (PBS). Protein concentration was determined (Bio-Rad, Inc.) and adjusted to 1 mg/ml. Water-extractable (WE) surface antigens associated with this strain were extracted by water and shaking (11). This extract was adjusted to 3 mg of protein per ml. Outer membrane proteins (OMP) were prepared with Sarkosyl (3), and this preparation was

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adjusted to 1 mg protein per ml. Finally, lipopolysaccharide (LPS) antigens were extracted from the strain by a phenol-water method (13) and LPS concentration was adjusted by weight after lyophilization to 0.5 mg/ml. These antigens were then used in the specific sIgA dot blot procedure.

**sIgA extraction.** sIgA was extracted by previously described methods (14, 15) from prechallenge and day 7 postchallenge stool specimens obtained from five volunteers with illness attributable to JM 221 and five who remained healthy after challenge. Briefly, approximately 2 g of feces was mixed with PBS, with aprotinin, thimerosal, and phenylmethylsulfonyl fluoride as enzyme inhibitors, incubated at 37°C for 30 min, and centrifuged at 1,200 × g for 20 min before the supernatant was collected. This procedure was repeated two more times without the incubation step. The supernatants were combined and centrifuged at 20,000 × g at 4°C for 1 h. The resulting preparation was then filtered through a 1.2-μm-pore-size membrane filter. The fecal extracts were then dialyzed first against several changes of distilled water for 24 h and for a second 24 h against m-pore-size membrane filter. The fecal extracts were then dialyzed for the incubation step. Thesupernatants were combined and centrifuged at 20,000 × g at 4°C for 1 h. After washing, thenitrocellulose was removed from the apparatus and incubated in a 1:500 dilution of peroxidase-conjugated goat anti-human IgA (alpha-chain specific) serum (Cappel Worthington, Malvern, Pa.) for 1 h at room temperature. Antibody-antigen complexes were visualized by the addition of Tris-HCl buffer with 0.03% 4-chloro-1-naphthol and 0.01% H2O2. The highest fourfold dilution with a visible reaction was considered the titer of that specimen. The total sIgA of all fecal extracts was standardized to the same concentration prior to determination of antigen-specific sIgA titers to allow comparisons among the specimens.

**Antigen-specific sIgA titrations.** The specific sIgA titers of the fecal extracts were also determined by using the Bio-Dot microfiltration apparatus (Bio-Rad) (14, 15). All determinations were done by placing a nitrocellulose sheet that had been soaked in 25 mM Tris–192 mM glycine buffer (pH 8.3) into the dot blot manifold. Fourfold dilutions of the fecal extracts were prepared in PBS. Fifty microliters of each fourfold dilution of the fecal extracts was placed in a well and allowed to filter through the nitrocellulose sheet. Then, three PBS rinses were done between steps and 100 μl of a buffer of 3% dry milk in Tris-HCl was filtered through the nitrocellulose to block any unbound sites. The nitrocellulose was then removed from the apparatus and incubated in a 1:500 dilution of peroxidase-conjugated goat anti-human IgA (alpha-chain specific) serum (Cappel Worthington, Malvern, Pa.) for 1 h at room temperature. Antibody-antigen complexes were visualized by the addition of Tris-HCl buffer with 0.03% 4-chloro-1-naphthol and 0.01% H2O2. The highest fourfold dilution with a visible reaction was considered the titer of that specimen. The total sIgA of all fecal extracts was standardized to the same concentration prior to determination of antigen-specific sIgA titers to allow comparisons among the specimens.

**RESULTS**

Table 1 shows the rises in intestinal titers of sIgA against different antigens prepared from EAEC JM 221. All of the volunteers who became ill had fourfold or greater rises between day 0 and day 7 in sIgA against all of the antigens tested. Volunteers ingesting this strain who remained healthy did not develop fourfold titer rises against WC, WE, or LPS antigens. One of the five had an sIgA response against OMP. The differences between the volunteer groups were statistically significant for all antigens. Absorption of two of the day 7 fecal extracts with whole cells of JM 221 reduced the specific titers of sIgA against this strain by 10-fold, showing the specificity of the fecal antibody responses being measured.

Figure 1 compares the geometric mean titers on day 0 and day 7 of the volunteers with diarrhea. There was an increase in the intestinal titers of sIgA against all test antigen preparations during the study. There was a statistically significant rise in geometric mean titer from the prechallenge specimens to those obtained on day 7 for three of the 4 EAEC antigens. There was a nonsignificant titer rise against the OMP preparation. No fourfold rises in the geometric mean sIgA titers for day 0 and day 7 stool extracts were seen for the group that did not develop diarrhea (Fig. 2). There was a noticeable difference in the day 0 titers of the two volunteer groups, with the group without illness showing the higher titers (Fig. 3). The geometric mean of intestinal sIgA titers for prechallenge fecal extracts of the healthy volunteers was higher than that of the volunteers who became ill had fourfold or greater rises between day 0 and day 7 in sIgA against all of the antigens tested.
who developed diarrhea for all antigens and significantly higher ($P < 0.001$) for the WC and WE antigens.

**DISCUSSION**

HEp-2 cell-adherent *E. coli* strains not belonging to traditional enteropathogenic serotypes have only recently been recognized as causes of illness. The nature of the immune response in EAEC infection has not been characterized. We have previously reported that 6 (46%) of 13 U.S. travelers to Mexico with diarrhea and a positive stool culture for EAEC had fourfold rises, by a tube agglutination test, in serum antibodies against the homologous EAEC strain (8). In the challenge study with one EAEC strain, no serum antibody rises were found among the volunteers, despite the development of clinical illness in 5 of 16 subjects (9). In this study, we found that infection with this EAEC strain elicited an intestinal sIgA response among the asymptomatic patients. We also found that preexisting high intestinal sIgA titers were associated with protection from acquiring EAEC-induced diarrhea. The geometric mean titers prior to challenge with the test EAEC strain were significantly higher for the five asymptomatic volunteers than for the five symptomatic patients. It is probable that mucosal immune responses are more common and more closely associated with protection than humeral antibodies in diarrheal diseases.

All of the antigens of JM 221 tested were shown to elicit an immune response. Preexisting high titers of intestinal sIgA against whole organisms and WE cell components loosely associated with the surface of this bacterium were associated with protection from symptomatic infection. Both of these antigen preparations consist primarily of surface-exposed components that have the initial interaction with the mucosal immune system. This probably explains the association between baseline antibody to WC and WE antigens and symptomatic disease. LPS of this strain elicited a fourfold or greater sIgA titer rise in volunteers who became ill, but preexisting intestinal antibody levels to LPS were not associated with protection. Our previous studies of sIgA immune response against other bacterial enteropathogens have shown by Western blot (immunoblot) that this response was largely directed at the LPS of the infecting organism (5, 14, 15). This apparent conflict probably has to do with methodology, since Western blots were not used in this study. Both WC and WE preparations are complex mixtures but contain a great deal of LPS.

These mixtures may elicit a more protective sIgA response than was seen against the highly purified LPS antigens tested alone.

There has been a great deal of confusion about the terminology and types of adherence patterns of HEP-2 cell-adherent *E. coli* not belonging to traditional EPEC serotypes. We have referred to these strains as EAEC on the basis of an assay that allows the recognition of diffuse and localized patterns of adherence. Other investigators have referred to this group of adherent *E. coli* as enteroaggregative *E. coli* on the basis of an adherence assay that allows the recognition of three adherence patterns: localized, diffuse, and aggregative (1). It has also been shown that the adherence phenotype is assay dependent (12). Aggregative EAEC strains are approximately 50% localized and 50% diffuse when tested in the assay we use. In the volunteer study (9), the EAEC strain that caused diarrhea among the volunteers was locally adherent and aggregative in the two adherence assays, while the EAEC strain that did not cause disease (JM 189) was diffusely adherent and aggregative by these assays. It is not clear at this time which EAEC strains have etiologic significance. We hope that further studies of patients naturally infected with different EAEC strains using the methodology developed here will help to clarify this point.

The methodology employed in this study has been used in previous studies to detect an intestinal immune response against other well-recognized bacterial enteropathogens (4, 14–16). Campylobacter jejuni, *Shigella sonnei*, enterotoxigenic *E. coli*, *Plesiomonas shigelloides*, and *Aeromonas* spp. have all been shown to elicit a significant intestinal sIgA response. We report here the use of the intestinal immune response in establishing the clinical importance of a newly described bacterial enteropathogen.

**REFERENCES**


