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Heterogeneity of Enteroaggregative *Escherichia coli* Virulence Demonstrated in Volunteers

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Enteroaggregative *Escherichia coli* (EAggEC) are diarrheal pathogens defined by aggregative adherence to HEp-2 cells. In an effort to identify pathogenic EAggEC isolates, four groups of 5 volunteers were fed 1 of 4 different EAggEC strains, each at a dose of 10^{10} cfu. Strain 042 caused diarrhea in 3 of 5 adults; 3 other EAggEC isolates (17-2, 34b, and JM221) failed to elicit diarrhea. A gene encoding enterotoxin EAST1 was found in strains 042 and 17-2 but not 34b or JM221; a 108-kDa cytotoxin was expressed in all 4 isolates. All 4 isolates showed a modest degree of gentamicin protection in HEp-2 cells. 17-2, 34b, and JM221 expressed the fimbrial antigen AAF/I; 042 did not express this fimbria as determined by immunogold electron microscopy and genetic probe hybridization.

Enteroaggregative *Escherichia coli* (EAggEC) are defined by their distinctive aggregating pattern of adherence to HEp-2 cells in culture [1]. Several case-control, prospective epidemiologic studies have associated EAggEC with diarrheal disease in children in the developing world, particularly in cases of persistent diarrhea (≥14 days) [2].

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The fundamental features of EAggEC pathogenesis are poorly understood, although molecular studies have identified both toxins and adherence fimbriae. Savarino et al. [3] have characterized an enterotoxin (called enteroaggregative ST or EAST1) related to the heat-stabile toxin of enterotoxigenic *E. coli*. Eslava et al. [4] reported the isolation of a 108-kDa protein in EAggEC supernatants that induces a destructive lesion in rat ileal loops; this lesion is similar to that described in a rabbit ileal loop model [1] and, to a lesser extent, in a gnotobiotic piglet model [5]. We have described a bundle-forming fimbria (designated aggregative adherence fimbria I [AAF/I]) on EAggEC isolates that correlates with the aggregative adherence (AA) phenotype in the HEp-2 assay [6, 7]. As yet, none of these phenotypes has been associated with diarrheal disease. Here we describe volunteer studies investigating the virulence of 4 EAggEC strains in humans.

Materials and Methods

Strains. The EAggEC strains used in this study have been described. 042 was isolated from a child with diarrhea in Lima, Peru [8]. It is AA probe–positive [9] and produces a very strong

"honeycomb" pattern of AA in the HEp-2 assay. 17-2 was isolated from a child with diarrhea in Chile [1]; 17-2 displays a "cytodetaching" pattern of AA, in which the HEp-2 cells are largely detached from the glass coverslip. 34b was isolated from a child with persistent diarrhea in India [10] (provided by M. K. Bhan, All India Institute for Medical Sciences, New Delhi). JM221 was isolated from an adult with diarrhea in Mexico and was shown to cause enteric symptoms with a low attack rate in volunteers [11] (provided by J. J. Mathewson, University of Texas Medical School, Houston). Both JM221 and 34b give typical AA to both HEp-2 cells and the glass coverslip. Strain HS is an *E. coli* control strain that is nonpathogenic in volunteers [12] and nonadherent and noninvasive to HEp-2 cells (unpublished data). E2348/69 is a virulent enteropathogenic *E. coli* strain shown to be invasive to HEp-2 cells [13].

Volunteer methods. Studies were done in the isolation ward of the Center for Vaccine Development, University of Maryland Hospital, using methods previously described [14]. The 4 EAggEC strains were grown in Luria broth at 37°C without shaking for 18 h. After this time, bacterial growth was harvested by centrifugation, washed, and resuspended in PBS. A single dose of 10^{10} cfu of each strain was administered after gastric neutralization with 2 g of sodium bicarbonate as described [14]. Subjects were followed for development of intestinal symptoms for 5 days; all stools shed during this period were collected, graded for consistency, and cultured for the challenge strain by use of an antibiotic resistance marker specific for each strain. Diarrhea was defined as the passage of a single liquid stool totaling at least 300 mL or two or more liquid stools totaling at least 200 mL over a 48-h period. Stools were graded according to our standard protocols: grade 1, solid; grade 2, soft but formed; grade 3, soft (takes shape of container); grade 4, liquid; grade 5, "rice water." Three colonies were picked from each stool culture for identification of the challenge strain by DNA probe. Beginning at 120 h after challenge (or 24 h after developing diarrhea), each volunteer was treated with a 5-day course of oral ciprofloxacin.

Western immunoblot analysis of volunteer sera was done as described [6].

Phenotypic assays. Immunogold electron microscopy (EM) using AAF/I antiserum was done as described [6]. Assay for EAST1 was done by DNA probe hybridization as described by Savarino et al. [3]. The 108-kDa toxin was assayed by concentrating culture supernatants of the 4 strains by ammonium sulfate precipitation, separating the supernatant proteins by SDS-PAGE, then doing immunoblot analysis with rabbit antisera raised against the purified toxin [4]. Details of this procedure will be published elsewhere. The presence of a reactive band at 108 kDa was considered to indicate a positive assay for presence of the toxin.

The HEp-2 adherence assay was done as described [15]. In vitro invasiveness of HEp-2 cells was assessed as described by Donnenberg et al. [13]. *E. coli* control strains for these assays were enteropathogenic *E. coli* E2348/69 (positive) and HS (negative).

DNA probe hybridization. All strains were tested for colony blot hybridization with the EAggEC probe as described [9]. Hybridization for AAF/I-homologous sequences was done using a 3.2-kb *Acc*I fragment from the AAF/I region 1 gene cluster as described [7].

Results

Volunteer studies. We previously fed EAggEC strain 17-2 to 19 community volunteers, at a dose of 10^{10} cfu [6]. Only 1 subject experienced diarrhea. The sera of 13 of the 19 revealed increases in antibody to a 14-kDa protein encoded by the 17-2 plasmid [6]. In preparation for the current study, we prescreened community volunteers for serologic evidence of antibodies to the 14-kDa protein by Western immunoblot. Of the 60 prescreened, 40 had antibodies; 17 of the 20 eligible seronegative volunteers agreed to return for an inpatient study.

In addition to the 17 who agreed to be enrolled in the inpatient study, 3 seropositive volunteers were recruited to bring the total to 20. Subjects were allocated into 4 groups of 5 each; each group received a different EAggEC strain. The 3 seropositive subjects were distributed to different groups. Subjects were given a single dose of 10^{10} cfu after gastric neutralization. The 17-2 isolate used for this study was isolated from the stool of the 1 patient in the previous study who had diarrhea.

Of the 20 subjects, only 4 experienced grade 3 (or looser) stools. All 4 were recipients of strain 042 ($P < .02$ by computer simulation); 3 of them met the case definition of diarrhea (714, 1306, and 1411 mL of liquid stool). The fourth subject who had grade 3 stools passed a total loose stool volume of 137 mL 1 day after challenge and experienced borborygmi at that time. The incubation period for the 4 subjects averaged 14.3 h (range, 7–22). The loose stools contained mucus in 2 of the 4 subjects but there was no gross blood or pus; none of the stools tested positive for blood or fecal leukocytes. No subject reported fever, headache, or other systemic symptoms. One 042 recipient had grade 3 or 4 stools for 7 consecutive days, including 6 days of diarrhea after antibiotics were initiated. This subject also complained of anorexia and abdominal gurgling. The 042 recipient who did not have grade 3 stools was without complaints; he was not seropositive for the 14-kDa protein before the study. None of the subjects fed 17-2, JM221, or 34b passed grade 3 or looser stools. None experienced systemic or enteric symptoms.

Stool culture with confirmation of EAggEC by DNA probe demonstrated intestinal colonization of all subjects by their respective challenge organism. All 20 subjects shed their organism by 24 h after inoculation; 17 continued to shed at 96 h, at which time antibiotic therapy was begun. The results of quantitative stool culture for the challenge strain and duodenal string culture are shown in table 1. Analysis of variance revealed that the mean peak excretion of strain 042 was significantly higher ($P < .05$) than those of JM221 and 17-2; 042 excretion was higher (but not significantly) than that of strain 34b.

Phenotypic studies. The 4 EAggEC strains were screened for the expression of phenotypes or genotypes that might

Table 1. Clinical and bacteriologic features of subjects fed EAggEC strains.

Strain (serotype)	Diarrheal attack rate	Mean peak excretion (cfu/g of stool)	Duodenal culture EAggEC-positive*
042 (O44:H18)	3/5	6.7×10^7	2/3
17-2 (O3:H2)	0/5	9.8×10^5	0/3
JM221 (O92:H33)	0/5	1.3×10^5	0/3
34b (O?:H11)	0/5	1.3×10^7	1/2

* Data reported only if pH of string >5.0.

correlate with the apparent difference in human virulence. All 4 strains were hybridized with a DNA probe sensitive and specific for the toxin EAST1 [3]. Strains 042 and 17-2 hybridized with a DNA fragment encoding the EAST1 gene (*astA*); JM221 and 34b did not hybridize with this fragment. All 4 strains were also tested for the production of the 108-kDa toxin described by Eslava et al. [4]. The supernatant of each strain was separated by SDS-PAGE and immunoblotted with polyclonal antiserum against this protein. All 4 had protein species of ~108 kDa that reacted with the toxin-specific antiserum (not shown).

Transmission EM was done on all 4 strains. All had multiple fimbrial morphologies, including rigid 7-nm-diameter fimbriae. Strains 17-2, 34b, and JM221 also had flexible, bundle-forming fimbriae on their surfaces that reacted with AAF/I antiserum by immunogold EM. Strain 042 expressed fimbriae of different morphologies on its surface (in agreement with studies reported by Vial et al. [1]); however, none of the 042 fimbrial structures reacted with AAF/I antiserum.

Invasion of HEp-2 cells has been shown to be a feature of enteropathogenic *E. coli* pathogenesis [13] and was thus hypothesized to be a potential distinguishing property of EAggEC strains. All 4 EAggEC strains were tested in the gentamicin protection assay. Overnight broth culture (3 μL) was added to confluent HEp-2 monolayers, and gentamicin survival was measured after a 3-h incubation (figure 1). Strains 042, 17-2, and JM221 showed similar levels of gentamicin protection, less than that elicited by enteropathogenic *E. coli* but significantly more than seen with the negative control, *E. coli* HS. However, strain 34b elicited significantly less invasiveness in this model than did the other strains.

Discussion

The mechanism of EAggEC diarrhea is as yet unknown. We used human volunteer experiments in an attempt to establish a model for this infection and to determine a pathogenic strain for use in further studies. A preliminary study of strain 17-2 resulted in only 1 case of mild diarrhea among 19 challenged subjects. In a second study, presented here, we prescreened volunteers for prior experience with EAggEC by assaying for antibodies to a 14-kDa EAggEC protein, fed them a 17-2 isolate obtained from the stool of the subject who developed diarrhea in the previous study, and prepared the inoculum in static Luria broth culture to maximize the AA expression of the isolates [6]. Despite these modifications, none of 5 subjects experienced any enteric symptoms when fed 17-2. Like 17-2, 2 other EAggEC strains also failed to elicit enteric illness. In contrast, however, 3 of 5 subjects experienced diarrhea when challenged with strain 042, and a fourth subject developed milder gastrointestinal symptoms within a day after challenge. The short incubation period of the illness, as well as the lack of fever, fecal blood, or fecal leukocytes, suggest that 042 diarrhea was more likely secretory than invasive in mechanism. One subject experienced 7 full days of diarrhea, despite being treated with ciprofloxacin after the first day of symptoms. This illness was similar in other respects to the more brief illness experienced by the other 042 recipients but is notable because of the association of EAggEC with persistent diarrhea. We have found that diarrhea of this duration is unusual in volunteer models of other pathogens.

The difference in virulence of EAggEC strains is not readily explained. Several putative virulence characteristics, including HEp-2 cell invasiveness (which was modest for all strains) and production of enterotoxins EAST1 and 108-kDa toxin, were not found to be specifically associated with strain 042. It is possible that any or all of these features may be necessary but not sufficient for EAggEC pathogenicity. Nevertheless, it is significant that the presence of AAF/I, the ability to stably colonize the human intestine, and the ability to elaborate one or more toxins were apparently not enough to confer pathogenicity in adults.

Phenotypes that were found to be specific for strain 042 were its very strong (honeycombed) adherence phenotype

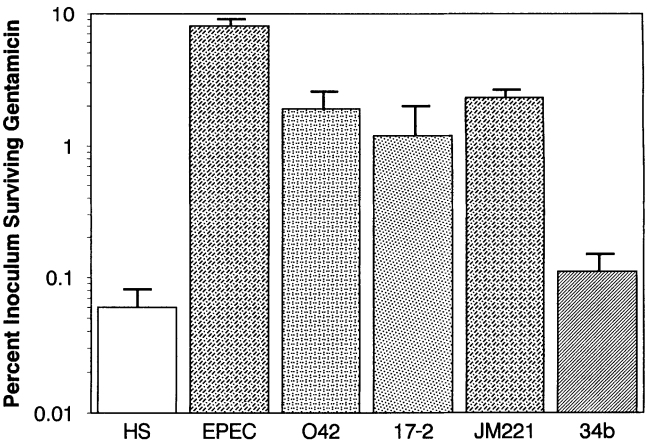

Figure 1. Gentamicin protection exhibited by EAggEC strains. Each strain was tested 9 times; bars represent % of inoculum surviving gentamicin treatment (mean ± SE). Enteropathogenic *E. coli* (EPEC) E2348/69 and *E. coli* HS were used as positive and negative controls, respectively.

and lack of expression of AAF/I. EM of 042 reveals the presence of other fimbrial structures that do not bind AAF/I antiserum; insertion mutagenesis of 042 has resulted in the identification of a novel fimbrial structure mediating AA in this strain, which we have designated AAF/II (unpublished data). AAF/II is thus a candidate for a necessary virulence determinant in EAggEC.

Epidemiologic studies in the developing world frequently fail to find an association of even established pathogens with diarrheal disease. This may be in part because of the high prevalence of asymptomatic excretion of known pathogens in populations that are exposed to a high pathogen load. However, heterogeneity in the virulence of established pathogens exists, and we have demonstrated this phenomenon with enteropathogenic *E. coli* strains [12] and enterotoxigenic *E. coli* (unpublished data). Identification of virulent strains helps to focus investigation on such isolates and their distinctive pathogenetic mechanisms. Our data suggest that adherence/colonization factors warrant attention as potentially important EAggEC virulence determinants.

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