Enteroaggregative *Escherichia coli* Produce Intestinal Inflammation and Growth Impairment and Cause Interleukin-8 Release from Intestinal Epithelial Cells

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Enteroaggregative *E. coli* (EAggEC) are emerging as an important cause of persistent diarrhea, especially in children in the developing world, yet the pathogenesis of EAggEC infection is poorly understood. In an ongoing prospective study of childhood diarrhea in an urban Brazilian slum, EAggEC are the leading cause of persistent diarrhea. Children from this study with EAggEC and persistent diarrhea had significant elevations in fecal lactoferrin, interleukin (IL)-8, and IL-1β. Moreover, children with EAggEC without diarrhea had elevated fecal lactoferrin and IL-1β concentrations. The children with EAggEC in their stool had significant growth impairment after their positive culture, regardless of the presence or absence of diarrhea. Finally, 2 EAggEC strains were shown to cause IL-8 release from Caco-2 cells, apparently via a novel heat-stable, high-molecular-weight protein. These findings suggest that EAggEC may contribute to childhood malnutrition, trigger intestinal inflammation in vivo, and induce IL-8 secretion in vitro.

In 1987, Nataro et al. [1] described a unique type of *Escherichia coli* isolated from children with diarrhea in Santiago, Chile. Like enteropathogenic *E. coli* (EPEC), these bacteria demonstrate mannose-resistant adherence to HEp-2 cells in tissue culture. However, unlike EPEC, they also adhere to each other and to the glass slide in what is classically described as a ''stacked-brick'' pattern. These bacteria were named ''enteroadherent-aggregative *E. coli,*'' although the name was subsequently shortened to enteroaggregative *E. coli* (EAggEC).

EAggEC have subsequently been identified among the leading causes of persistent diarrhea (i.e., illness >14 days) among children in developing countries. In contrast to acute diarrhea, in which dehydration is the major life-threatening complication, persistent diarrheal episodes carry additional morbidity due to prolongedly altered intestinal permeability and increased overall diarrheal burden, the long-term consequences of which remain to be fully appreciated [2, 3]. In one of the most carefully studied cohorts (in an urban shantytown in northeastern Brazil), EAggEC are the leading cause of persistent childhood diarrhea [4–6]. They have also been identified in patients with AIDS [7, 8] and in travelers to developing areas [9].

Despite its increasing importance, relatively little is known about the pathogenesis of EAggEC diarrhea. Pathogenic features identified thus far are unique aggregative adherence fimbriae (AAF), which mediate adherence in cell culture, invasion (which has been observed only in vitro) [10], and elaboration of three toxins: a small enterotoxin related to enterotoxigenic *E. coli* STa [11, 12], a heat-labile hemolysin [13], and a 108-kDa protein with cytotoxic effects in rat intestinal loops [14]. None of these has been definitively implicated as the leading pathogenic feature of EAggEC.

In this study, we attempt to define the pathophysiology of EAggEC infection in a well-defined cohort of children residing in an urban shantytown in northeastern Brazil. By measuring fecal inflammatory markers from infected children and controls, we sought to determine whether EAggEC induce elevated fecal lactoferrin (FLF) and proinflammatory cytokines and whether these organisms are associated with growth shortfalls even when diarrhea is not present. Moreover, we sought to identify a soluble factor from EAggEC strain 042 (shown to be pathogenic in healthy adult volunteers in the United States) [15] that causes release of the proinflammatory chemokine interleukin-8 (IL-8) from Caco-2 human intestinal epithelial cells in culture, suggesting a potential mechanism by which these organisms can produce intestinal inflammation and growth impairment or diarrhea.

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This research met all human experimentation guidelines of the US Department of Health and Human Services. Informed consent and cooperation were obtained from parents of all children involved.

R.L.G. holds a US patent for the fecal lactoferrin latex agglutination test used in this study.

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Materials and Methods

Patient population. The case and control subjects were obtained from a cohort of children in the Gonçalves Dias favela (shantytown) in Fortaleza, Brazil (described in [4–6]). A total of 186 children from the 405 households in the favela have been followed from birth to age 5, with thrice-weekly visits to document the presence of diarrhea (defined as ⩾3 loose or watery stools/day by the mother's report) or other illnesses. More details of this cohort have been described [5, 6]. Height and weight measurements were taken every 3 months. Stool samples were collected periodically from healthy children and during each episode of diarrhea. All stools were tested for the presence of common diarrheal pathogens as previously described [4–6]. One *E. coli* isolate from each stool sample was subcultured and tested for HEp-2 cell adherence and aggregative adherence (AA), diffuse adherence (DA), and attaching and effacing gene probes as described [16–18]. Strains defined as EAggEC included those *E. coli* demonstrating aggregative adherence on HEp-2 assay or hybridizing with the AA gene probe (or both). Children with positive stool cultures for EAggEC were classified into 3 groups: persistent diarrhea (PD; diarrhea >14 days), acute diarrhea (AD; diarrhea ⩽14 days), or no diarrhea (ND; free of diarrheal illness for 3 weeks before and after the positive stool culture). Control stools were from children in the same cohort free of diarrhea for 3 weeks before and after the sample and with no enteric pathogens isolated. For purposes of this study, children with exclusive EAggEC infection were those in whom an EAggEC strain was identified and tests for the other pathogens named above were negative.

Stool analysis. Stool samples were tested for lactoferrin by latex agglutination (Leuko-Test; TechLab, Blacksburg, VA). Additional samples were frozen at −70°C for cytokine testing. Small aliquots were diluted 1:2 (wt:vol) in PBS containing 2.5 μg/mL leupeptin, 11 μg/mL aprotinin, and 0.5 m*M* 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma, St. Louis). After thorough mixing and centrifugation (15 min, 10,000 *g*), supernatants were removed and frozen at −70°C. Fecal supernatants were tested for IL-8, IL-1 receptor antagonist (IL-1ra), and IL-1β by ELISA (Quantikine IL-8 and IL-1ra; R&D Systems, Minneapolis; and CytElisa1β; CytImmune Sciences, College Park, MD). Several stool samples with elevated cytokine concentrations in the ELISA were retested with the omission of secondary antibody–enzyme complex to exclude a contribution of fecal peroxidase or phosphatase; all of these samples produced an optical density of <0.001.

Cell culture and IL-8 release. Caco-2 human colon carcinoma cells were provided by Cynthia Sears (Johns Hopkins University, Baltimore). Culture media and supplements were obtained from Life Technologies GIBCO BRL (Gaithersburg, MD) unless otherwise specified. Caco-2 cells were grown in a 5% CO_2 incubator at 37°C in Dulbecco's modified Eagle medium (#12100; Life Technologies) supplemented with 26 m*M* $NaHCO_3$, 1 m*M* sodium pyruvate, 1× nonessential amino acids, 1× penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively), and 10% fetal bovine serum (Hyclone, Logan, UT). Medium was changed twice weekly. Cells were passaged approximately biweekly by rinsing briefly in Ca^{2+}- and Mg^{2+}-free PBS and then incubating for 15 min at 37°C with 0.25% trypsin/2.65 m*M* EDTA in PBS. For IL-8 experiments, cells (passages 30–50) were plated at a density of 500,000/well in 24-well polystyrene plates (Costar, Cambridge, MA) and used at 5–14 days after seeding, at which time they were at least several days postconfluence. This high plating density was chosen to allow for rapid confluence and differentiation; nonadherent cells were removed by washing before each experiment.

For cytokine release experiments, 0.5 mL of MEM without fetal bovine serum was placed into each well, and varying amounts (usually 5–50 μL) of the bacterial culture or filtrate to be tested were added. The cells were incubated for the indicated times at 37°C (5% CO_2 incubator) and the medium removed and frozen at −70°C before testing.

Bacterial preparations. EAggEC 042 (O44:H18) and 17-2 (O3:H2), control *E. coli* K12 and HB101, EPEC E2348, and cholera vaccine strain CVD-110 were obtained from the Center for Vaccine Development (University of Maryland, Baltimore). EAggEC 042 (plasmid$^-$) and HB101:pJPN8 were prepared as previously described [16, 19]. Strain 042:3.4.14 (042 carrying a TnphoA insertion into the gene encoding the major fimbrial subunit of AAF/II) is to be described elsewhere (Nataro JP, unpublished data). The *Shigella flexneri* isolate was from a patient with diarrhea. For cytokine release experiments, bacteria were grown overnight in 1% tryptone/1% D-mannose (Difco, Detroit) with shaking at 200 rpm in a 37°C incubator. These conditions were chosen to duplicate the conditions under which our laboratory performs HEp-2 adherence assays.

Detection of IL-8 mRNA from Caco-2 cells. Caco-2 cells were grown in 25-cm^2 flasks for 10 days. Two hundred microliters of 0.22-μm-filtered cloudy overnight bacterial suspension (or 1% tryptone/1% mannose for the control) was added to 2 mL of fresh growth medium, and the cells were incubated for 21 h. The medium was removed, and total RNA was isolated by use of Trizol reagent (Life Technologies). Reverse transcription was done on 1 μg of RNA from each sample by the addition of 1 m*M* each dATP, dCTP, dGTP, and dTTP (Life Technologies), 20 U of RNase inhibitor (Boehringer Mannheim, Indianapolis), 100 pmol of random hexamers (Pharmacia, Uppsala, Sweden), 10 m*M* dithiothreitol (Life Technologies), and 1 μL of Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim) in first-strand buffer (Life Technologies) in a total volume of 20 μL for 1 h at 37°C. The reaction was terminated by a 10-min incubation at 97°C and rapid chilling on ice. For controls, the reaction was done identically on the same RNA samples with the omission of reverse transcriptase and on a sample with no RNA template. RNA isolated from Caco-2 cells treated with 1 μg/mL IL-1β (known to contain IL-8 mRNA) was used as a positive control.

The polymerase chain reaction (PCR) reaction was done by the addition of 10 μL of reverse transcription product to a solution containing 4 μL of 10× PCR buffer A (Fisher Scientific, Pittsburgh), 25 pmol each of 5′ and 3′ primers, and water to a total volume of 40 μL. The reaction was heated to 95°C for 1 min, and 0.5 μL of Taq polymerase (Fisher) was added. The reaction was cycled 30 times at 95°C for 1 min, 58°C for 1 min, and 72°C for 3 min. Ten microliters of each reaction product was electrophoresed in agarose, stained with ethidium bromide, visualized by UV light, and recorded with instant photographs. The primers were obtained from Life Technologies and had the following sequences: 3′-TCTCAGCCCTCTTCAAAAACTTCTC and 5′-ATGACTTCCAAGCTGGCCGTGGCT.

Statistical analyses. Nonparametric or exact tests were used for all stool analyses because of violation of the assumption of

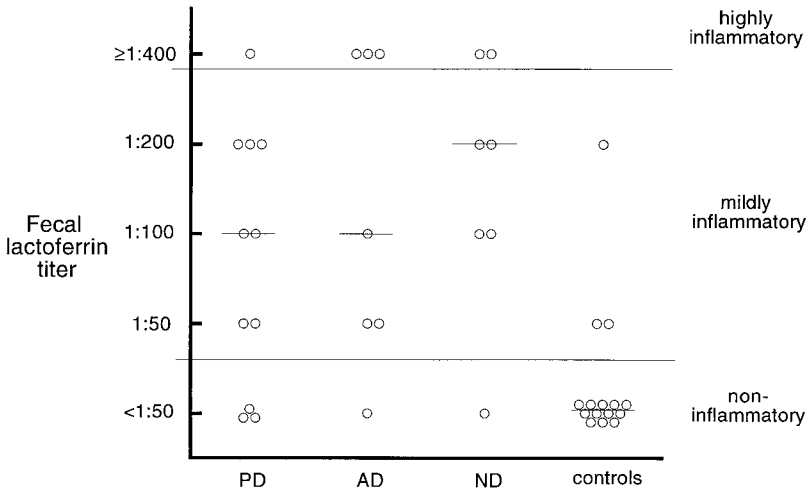

Figure 1. Fecal lactoferrin titers in Gonçalves Dias children. Stool was obtained from children with persistent diarrhea (PD) or acute diarrhea (AD) with EAggEC as only detected intestinal pathogen and from children free of diarrhea for 3 weeks before and after sample was taken (in prospective surveillance) who carried EAggEC exclusively (ND) or no detected intestinal pathogens at all (controls). Stools were diluted 1:50 in assay diluent and tested for latex agglutination. Samples positive at 1:50 were diluted 2-fold repeatedly until no agglutination was obtained. Horizontal lines indicate medians. Differences were statistically significant ($P = .001$, Kruskal-Wallis; $P < .01$ for each EAggEC group vs. controls, Mann-Whitney).

equal variances. FLF titers and cytokine concentrations were compared by the Kruskal-Wallis nonparametric group comparison, and when a significant difference ($P < .05$) was seen, this was isolated by Mann-Whitney U (Minitab 10.5 Power Mac). Growth data were analyzed by use of the Centers for Disease Control and Prevention Anthropomorphic Software Package (version 3.0) and expressed as number of SD units above or below the mean population growth (each Z score unit = 1 SD from the mean). Means of infected and uninfected children were compared by Student's t test. Unless otherwise stated, data are presented as mean ± SE; the highly skewed fecal cytokine values are reported as median (interquartile spread).

Results

EAggEC produce intestinal inflammation. Twenty-six stool samples with EAggEC and no other pathogens were obtained. Of these, 11 were from episodes of PD, 7 from AD, and 8 from ND. As shown in figure 1, FLF titers were elevated in most of these samples, regardless of the presence of diarrhea; in contrast, FLF titers were <1:50 in 12 of 15 controls (children in the study cohort with no intestinal pathogens isolated and no diarrhea for 3 weeks before and after the sample was taken). The differences were statistically significant ($P = .001$, Kruskal-Wallis; $P < .01$ for each EAggEC group vs. controls, Mann-Whitney). By way of comparison, FLF titers >1:400 are routinely seen in shigellosis, while titers of >1:50 are rare in healthy people [20].

Fecal IL-8 and IL-1β concentrations were significantly elevated in some of the sample groups compared with controls (figures 2 and 3; $P = .017$ for each, Kruskal-Wallis). Stools in the PD group had significantly more IL-8 than did stools of controls. Values are median (interquartile spread): 91.5 pg/mL (8, 2690) vs. 0 pg/mL (0, 14.6; $P = .013$, Mann-Whitney). There was a smaller but still significant elevation of fecal IL-8 in the AD group as well: 16 pg/mL (8, 88; $P = .049$). Stools from the ND group had no increase in IL-8: 3.0 pg/mL (0, 11.7). Stools from all 3 EAggEC groups had elevated IL-1β compared with stools from controls: PD = 676 pg/mL (110, 194,788; $P = .009$); AD = 172 pg/mL (23, 6879; $P = .088$); ND = 294 (63, 510; $P = .018$); controls = 29.9 pg/mL (0, 192.4). Of note, 5 patients in the PD group had extremely high IL-1β concentrations (25,200 to >950,000 pg/mL).

While IL-1ra was detectable in all 4 groups and did not differ significantly among them (not shown), the ratio of IL-1β to IL-1ra was significantly elevated in the EAggEC groups compared with controls, as shown in figure 4 ($P = .05$, Kruskal-Wallis). Medians (interquartile spreads) were 0.4 (0.1, 1.6), 0.163 (0.039, 1.139), 0.174 (0.089, 0.644), and 0 (0, 0.09) for PD, AD, ND, and control subjects, respectively ($P = .016$, .033, .047; Mann-Whitney). Moreover, children with EAggEC were significantly more likely than controls to have IL-1β/IL-

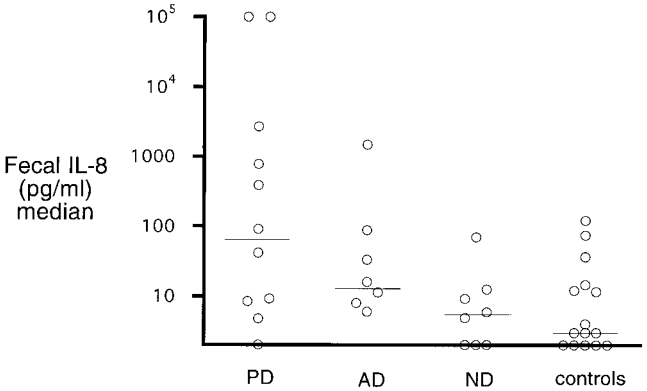

Figure 2. Fecal interleukin (IL)-8 concentrations in Gonçalves Dias children. Stools from controls or children with exclusive EAggEC infection and persistent diarrhea (PD), acute diarrhea (AD), or no diarrhea (ND) were diluted in PBS with protease inhibitors and tested for IL-8 by EIA. Difference among groups was statistically significant ($P = .017$, Kruskal-Wallis), as were differences between diarrheal groups and controls ($P = .013$ and .049 for PD and AD, respectively, Mann-Whitney). Horizontal lines indicate medians.

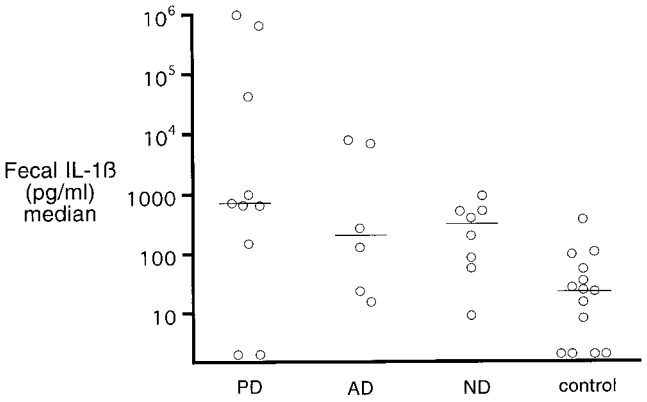

Figure 3. Fecal interleukin (IL)-1β concentrations in Gonçalves Dias children. Stools from controls or children with exclusive EAggEC infection and persistent diarrhea (PD), acute diarrhea (AD), or no diarrhea (ND) were diluted in PBS with protease inhibitors and tested for IL-1β by EIA. Difference among groups was statistically significant (P = .017, Kruskal-Wallis). Individually, PD and ND groups had statistically significant elevations in fecal IL-1β (P = .009 and .018, respectively, Mann-Whitney), while AD group fell short of .05 level (P = .088). Horizontal lines indicate medians.

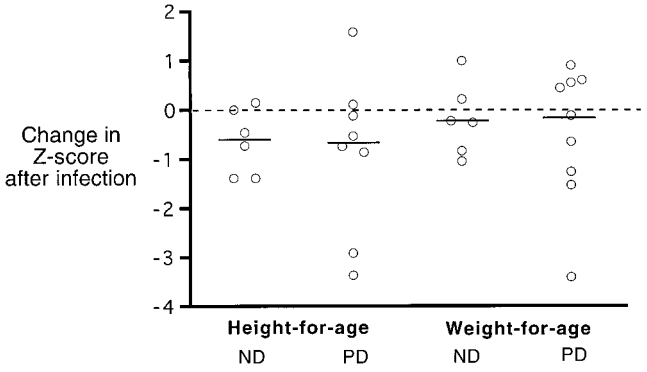

Figure 5. Growth impairment after EAggEC infection. Children with exclusive EAggEC infection (no other intestinal pathogens isolated) and either persistent diarrhea (PD) or no diarrhea (ND) had height and weight measurements taken 2–4 months before and 2–4 months after positive stool culture. Each height and weight measurement was compared with normalized growth curves to yield Z score (SD unit above or below mean). There was trend toward loss of height for age among both groups (P = .064 and .18 for ND and PD, respectively, paired t test).

1ra ratios of >0.01 (P = .007, .016, and .016 for PD, AD, and ND groups, respectively, by Fisher's exact test).

There were significant correlations between values of several of the fecal inflammatory markers from patients in this study.

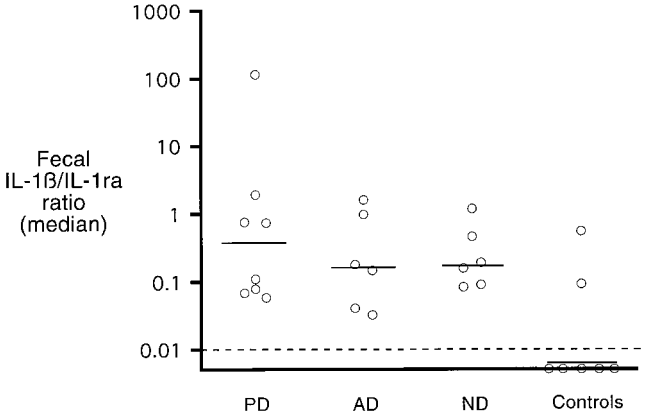

Figure 4. Ratios of interleukin (IL)-1β to IL-1 receptor antagonist (IL-1ra) in stool from children in Gonçalves Dias study. Stools from controls or children with exclusive EAggEC infection and persistent diarrhea (PD), acute diarrhea (AD), or no diarrhea (ND) were diluted in PBS with protease inhibitors and tested for cytokines by EIA. Children with EAggEC had significantly higher ratios than controls, presumably representing more IL-1 receptor activation in these groups (P = .05, Kruskal-Wallis; individual differences significant at P = .016, .033, .047 for PD, AD, ND vs. controls, Mann-Whitney). Children with EAggEC were also significantly more likely than controls to have ratios >.01 (P = .007, .016, and .016 for PD, AD, and ND groups, respectively, Fisher's exact test). Horizontal lines indicate medians.

FLF titer correlated with both IL-1β and IL-1β/IL-1ra (r = .520, .466; $P < .001$ and $< .01$, respectively; Spearman rank-order correlation). Moreover, fecal IL-8 and IL-1β were significantly correlated (r = .343; P = .021).

EAggEC are associated with growth impairment. The finding of elevated fecal inflammatory markers in children with EAggEC, even without overt diarrhea, raised the possibility that some of these children may have sequelae of persistent intestinal inflammation, most notably growth impairment. We therefore analyzed data on these children's growth, which were collected as part of the prospective surveillance. Of 14 subjects with PD due to exclusive EAggEC infection, 9 had paired measurements of height, weight, or both during the 3 months preceding and 3 months following the positive stool culture. Similar data were available for 6 of the subjects with EAggEC but no diarrhea. As shown in figure 5, these groups of children had evidence of growth impairment during this period, as determined by weight for age and height for age compared to standard growth curves. The PD group lost 0.86 ± 0.57 SD units (Z score) of height for age and 0.50 ± 0.46 units of weight for age (P = .18 and .3, respectively, paired t test), and the ND group lost 0.64 ± 0.24 units of height for age and 0.20 ± 0.30 units of weight for age (P = .064 and .5, respectively, paired t test). When combined, the entire group with EAggEC as the only pathogen had a statistically significant decline in height for age after their positive stool culture (0.76 ± 0.34 units; $P < .05$, paired t test).

Similar growth impairment was seen in the larger group of children as a whole with EAggEC in their stools (i.e., including those with other pathogens with or without diarrhea). As seen in figure 6, these children had significant declines in both weight for age (P = .02) and height for age (P = .001) during

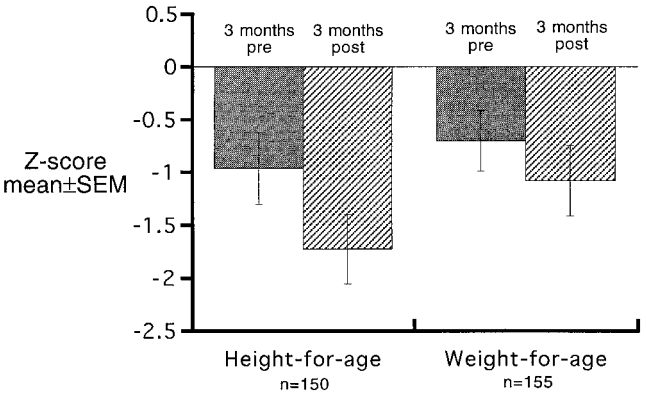

Figure 6. Growth measurements before and after positive EAggEC stool culture regardless of presence of diarrhea or other stool pathogens. Individual heights and weights were compared to normalized population growth curves. Children with EAggEC had impaired growth in height ($P = .001$) and weight ($P = .02$) after EAggEC infection (t test).

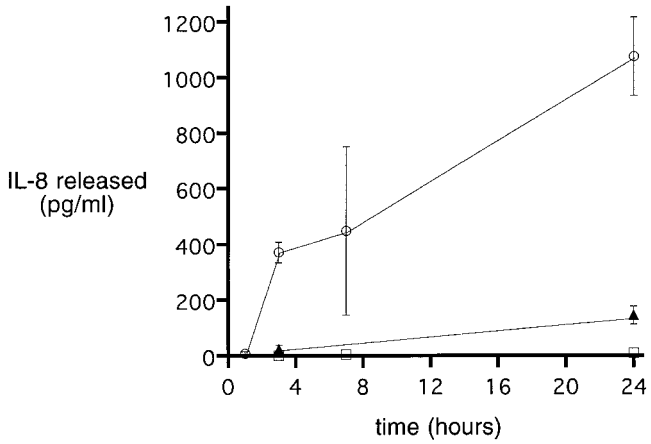

Figure 8. Time course of IL-8 release from Caco-2 cells: 50 μL of 0.22-μm filtrate from overnight bacterial culture in tryptone/mannose was added to 500 μL of medium, and supernatants were removed at times listed. Means and SEs are shown for EAggEC 042 (○), *Escherichia coli* K-12 or HB101 (▲), or tryptone/mannose alone (■).

the 6 months surrounding their positive EAggEC stool culture (paired t test).

EAggEC cause IL-8 release from Caco-2 cells. EAggEC, other intestinal pathogens, and control bacterial strains were assessed for their ability to release proinflammatory cytokines from Caco-2 cells in culture at 3 h. No IL-1β was detected in supernatants of Caco-2 cells exposed to these bacteria or their filtrates. Moreover, as shown in figure 7, control *E. coli* K12, CVD 110, EPEC strain E2348 (O127:H6), and *S. flexneri* did not cause significant IL-8 release from Caco-2 cells when grown to comparable broth culture turbidity to that of EAggEC 042 and 17-2. However, EAggEC strains 042 and 17-2 both released significant amounts of IL-8 from Caco-2 cells compared with *E. coli* K12 (296.75 ± 51.6 pg/mL, $P < .001$, and 74.4 ± 20.6 pg/mL, $P < .02$, respectively, Student's t test). These effects were reproduced by application of cell-free bacterial culture filtrates, suggesting that a soluble factor rather than cellular adherence was responsible for inducing the IL-8 release. Culture medium alone (tryptone/mannose) did not release detectable IL-8 at 3 h.

Measurable IL-8 release from Caco-2 cells was barely detectable after 2 h of exposure to EAggEC 042 culture filtrates (<30 pg/mL) but increased sharply after 3 h and continued to increase for up to 24 h (figure 8). In contrast, filtrates of control *E. coli* K-12 released very little IL-8 at 3 h, even when grown to comparable bacterial numbers as EAggEC 042, as determined by colony counts, optical density readings, and/or man-

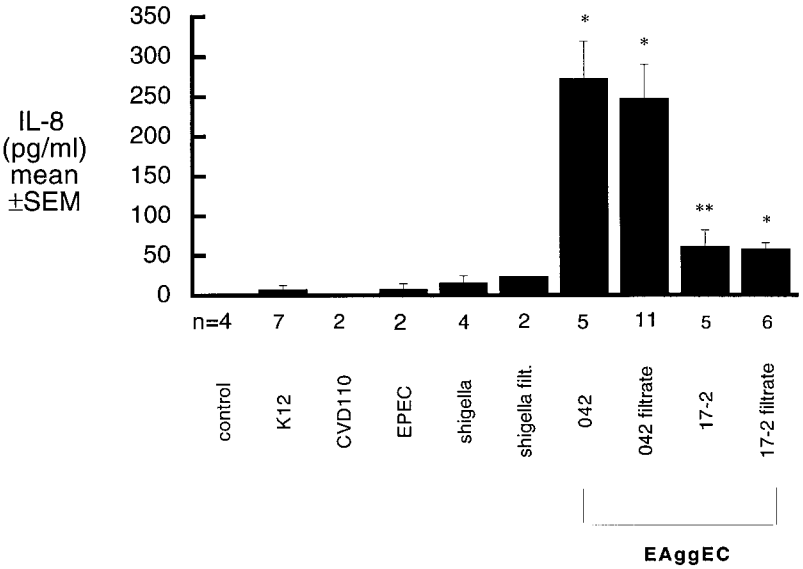

Figure 7. Interleukin (IL)-8 release from Caco-2 human colon carcinoma cells exposed to bacteria or bacterial filtrates: K12, control *Escherichia coli*; CVD-110, cholera vaccine strain; EPEC, enteropathogenic *E. coli*; shigella, isolate from patient with diarrhea; 042 and 17-2, EAggEC isolates. Cloudy overnight bacterial culture (25 μL) in 1% tryptone/1% D-mannose was applied to confluent Caco-2 monolayers in 500 μL of MEM; supernatants were removed after 3 h and tested for IL-8 by EIA. EAggEC strains 042 and 17-2 caused significant IL-8 release, but tryptone/mannose alone (control) and other enteric pathogens or nonpathogenic bacteria did not. IL-8 release was reproduced after incubation of Caco-2 cells with 0.2-μm filtrates of EAggEC culture. * $P < .001$; ** $P < .02$.

ual counting in a hemocytometer (mean ± SE = 24.7 ± 21.8 pg/mL vs. 400.3 ± 88.8 pg/mL for 042 in the same 3 experiments).

Subsequent analysis of this effect revealed the following observations. First, the effect of EAggEC 042 filtrates on Caco-2 cells was consistently abolished by treatment with either trypsin (Sigma; 10,000 U/mL; 20 μg/mL for 1 h at 37°C) or proteinase K (Amresco, Solon, OH; 100 μg/mL for 45 min at 42°C), even after subsequent protease inactivation with either 10% fetal bovine serum or soybean trypsin inhibitor (Sigma; 20 μg/mL). In contrast, IL-8 release was not inhibited by polymyxin B (10 μg/mL) or heating for 15 min at 95°C. Moreover, it was not present in filtrates after passage through 100,000 M_r filters (Centricon-100, Amicon; Beverly, MA). IL-8 release was not inhibited by coincubation with saturating concentrations (1 μg/mL) of IL-1ra, suggesting that paracrine or autocrine release of IL-1 is not responsible for the IL-8 release. Finally, the 108- and 116-kDa EAggEC cytotoxins described in [14] (provided by Carlos Eslava, Universidad Nacional Autonoma de Mexico, Mexico City) did not release measurable IL-8 from Caco-2 cells at concentrations >1 μg/mL, and polyclonal antiserum against these toxins (at concentrations up to 1 mg/mL) did not block the IL-8–releasing activity of 042 filtrates.

Reverse transcriptase–PCR analysis of RNA isolated from Caco-2 cells revealed a substantial increase in IL-8 mRNA after 21 h of treatment with filtrates of EAggEC 042 but not filtrates of *E. coli* K-12, compared with cells exposed to tryptone/mannose alone. As shown in figure 9, tryptone/mannose– or K-12–treated cells had barely detectable IL-8 mRNA, consistent with the lack of IL-8 release into culture supernatants. In contrast, cells treated with filtrates of EAggEC 042 had amplifiable IL-8 mRNA qualitatively as great as that seen with IL-1β. Moreover, Caco-2 cells exposed to filtrates of EAggEC 042 for 24 h, washed, and incubated in medium for 24 h more did not release elevated IL-8 compared with controls (data not shown). Together, these findings strongly suggest that the IL-8 release induced by EAggEC filtrates is not due to cell lysis with subsequent release of preformed IL-8, but rather to pretranslational activation of IL-8 synthesis.

In an attempt to localize the genetic site in 042 responsible for production of the IL-8–releasing factor, we tested the following bacteria for IL-8 release from Caco-2 cells: 042 p^- (cured of the AA plasmid), 042:3.4.14 (containing a TnphoA insertion into the AAF/II pilin gene on the AA plasmid), and HB101:pJPN8 (a nonpathogenic strain containing the wild type 042 AA plasmid). The first 2 of these were clearly negative in the HEp-2 assay, while the third was weakly positive (occasional aggregated bacteria seen, but on <20% of cells).

As shown in figure 10, overnight culture filtrates of these bacteria released significantly less IL-8 from Caco-2 cells (in 20–24 h of incubation) than did wild type 042 (P = .001, Kruskal-Wallis). Interestingly, while the plasmid-cured 042 released somewhat less IL-8 than did the wild type (505.3 ± 70.1 vs. 806.1 ± 169.6 pg/mL; P > .1, Mann-Whitney), the other 3 isolates released significantly less (85.5 ± 26.2, 156.5 ± 23.3, and 168.4 ± 28.8 pg/mL for 042:3.4.14, HB101:pJPN8, and HB101 WT, respectively; P = .006, .003, and .011, Mann-Whitney). These same results were obtained regardless of the colony counts of the cultures before filtration. Interestingly, higher amounts of IL-8 were released from Caco-2 cells exposed overnight to filtrates of 042:3.4.14 that were concentrated by 50% or 100% ammonium sulfate precipitation but not similarly concentrated filtrates of *E. coli* K-12 (data not shown).

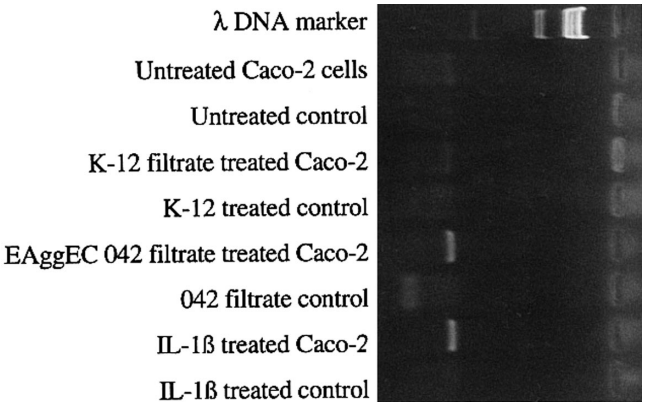

Figure 9. Interleukin (IL)-8 mRNA expression from Caco-2 cells. Cells grown in 25-cm^2 flasks were exposed to 1% tryptone/1% D-mannose (untreated) or filtrates of overnight cultures of *Escherichia coli* K-12 or EAggEC 042 in tryptone/mannose. Total cellular RNA was isolated and 1 μg of RNA from each sample was reverse-transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase. For controls, all reagents except reverse transcriptase were added to reaction. RNA (1 μg) isolated from IL-1β–treated Caco-2 cells was run in parallel as positive control. Reverse transcription products were amplified with 30 cycles of PCR using Taq polymerase and published IL-8–specific primers. PCR product (10 μL) was run in each lane of agarose gel, visualized with ethidium bromide and UV light, and photographed with instant film. Band of expected size (~289 kb) was strongly visible in lanes corresponding to 042 or IL-1β–treated Caco-2 cells.

These preliminary findings suggest that the active component in 042 filtrates is a novel high-molecular-weight, heat-stable protein encoded on the bacterial chromosome but released in reduced amounts when the plasmid is removed or altered. Further analysis of this factor is underway.

Discussion

EAggEC are being increasingly recognized as an etiologic agent in persistent diarrhea, both in children in developing areas and in patients with AIDS in developed countries. However, they remain dramatically underreported, since their identification requires either gene probing (which may fail to recognize as many as half or more of isolates) or the unwieldy HEp-2 adherence assay [4]. To complicate matters, they are frequently identified in ''asymptomatic'' children (i.e., those without overt diarrhea) in developing areas (15%–31% in Bra-

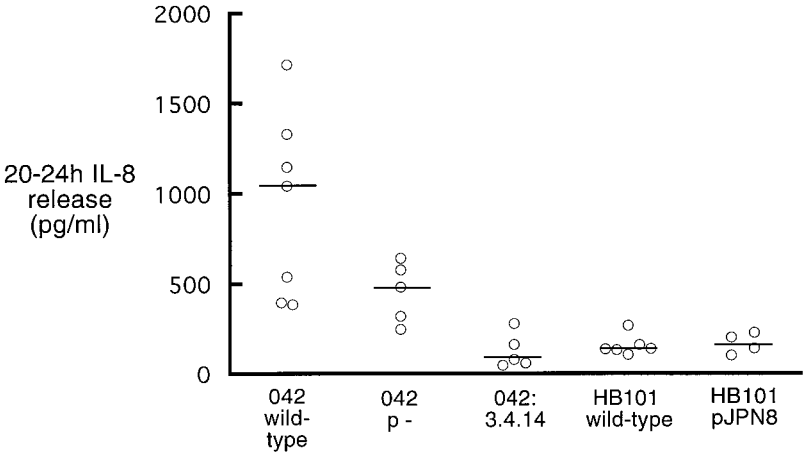

Figure 10. Interleukin (IL)-8 release from Caco-2 cells exposed to filtrates of plasmid-cured EAggEC 042 (042 p^-), 042 with fimbrial gene TnphoA disruption (042:3.4.14), nonpathogenic *Escherichia coli* HB101, and HB101 carrying 042 AA plasmid (HB101:pJPN8). Bacteria were grown in 1% tryptone/1% D-mannose overnight to roughly equivalent colony counts, and 0.2-μm filtrates were applied to Caco-2 cells in growth medium for 20–24 h. Supernatants were tested for IL-8 by EIA. Difference among groups was highly significant (P = .001, Kruskal-Wallis). Individually, 042:3.4.14, HB101, and HB101:pJPN8 released significantly less IL-8 than did 042 (P = .006, .003, .011, respectively), but 042 p^- did not (P = .144).

zil and 6.5%–9.9% in India) [4, 21] or in ''asymptomatic'' travelers returning from these areas [9]. However, a careful analysis of potential malnutritional or bowel-disruptive effects of EAggEC short of overt diarrhea has not been reported previously.

Some strains of EAggEC are known to cause diarrhea [15], but the pathophysiology of EAggEC diarrhea and the virulence traits that enable the organism to cause diarrhea in some people but not others remain poorly understood. Moreover, it is not clear whether EAggEC adherence itself is pathogenic (as with EPEC) or whether some soluble factor must be delivered by adherent bacteria. As noted earlier, several groups have identified toxins as potential virulence traits for EAggEC, but a convincing link between toxin and disease (such as for *Clostridium difficile* or enterotoxigenic *E. coli*) has not been proven.

The present studies were designed to test the hypothesis that EAggEC produce an inflammatory diarrhea, as determined by elevations in fecal inflammatory markers. There is substantial precedent for this type of investigation. Fecal lactoferrin has been shown to be more sensitive for the presence of intestinal inflammation than microscopy for fecal leukocytes, since the latter degrade rapidly with storage and can lose their characteristic morphology [22, 23]. Elevated FLF is the best clinical predictor for *C. difficile* colitis in hospitalized inpatients [24] and is the best available predictor for culture-positive bacterial enteritis [25]. FLF has also been used as a simple marker of intestinal inflammation in inflammatory bowel disease, both to follow disease activity and to distinguish inflammatory bowel disease from irritable bowel syndrome or other noninflammatory conditions [26].

In addition to FLF, fecal concentrations of the proinflammatory cytokines IL-8 and IL-1β were measured. Previous work has established that inflammatory enteritides such as shigellosis [27, 28] and inflammatory bowel disease [29] are associated with increases in fecal cytokine concentrations, which are often undetectable in healthy controls. We have also recently reported elevations in fecal IL-8 and lactoferrin in subjects who developed diarrhea after administration of the cholera vaccine strain CVD-110 [30]. Finally, we measured the ratio of IL-1β to IL-1ra in stool as a potential marker for proinflammatory activity. In most studied systems, a 10- to 100-fold excess of IL-1ra is required to saturate IL-1 receptors and prevent their activation by IL-1 [31]. While it is not clear whether similar ratios are useful to measure in stool (if, for example, the two compounds are degraded at different rates), it is notable that we found markedly elevated IL-1/IL-1ra ratios in many of our subjects with EAggEC but not in controls. Further studies may help to validate the usefulness of the IL-1/IL-1ra ratio in this setting.

This study demonstrates that EAggEC are associated with evidence of intestinal inflammation, which is occasionally as severe as that seen with invasive diseases such as shigellosis. Interestingly, FLF titers were elevated even in children with EAggEC without diarrhea. Moreover, even these ''asymptomatic'' infections were associated with growth impairment. These findings suggest that EAggEC may be an even more significant pathogen than was previously suspected, since many children labeled as ''controls'' in published epidemiologic surveys may actually have had subclinical infections with potentially serious sequelae. Moreover, this finding raises the possibility that malnutrition and poor growth may in many cases be due to previously unrecognized or unsuspected infectious agents. In the case of EAggEC, this effect may be due to intestinal inflammation or to the thick mucus gel with which they are associated in animal models or human intestinal explants, which could theoretically impair absorption of nutrients [19, 32].

Given the degree of intestinal inflammation seen in children with EAggEC, we postulated that these bacteria could produce some of their pathophysiology by inducing proinflammatory cytokine release from intestinal epithelial cells. Several reports have demonstrated up-regulation of these cytokines from T84 or Caco-2 intestinal epithelial cells stimulated by *Salmonella* or *Yersinia* species or *Entamoeba histolytica,* but invasion and lysis were believed to be the critical events leading to this up-regulation [33, 34]. There are no published reports of cytokine

release from intestinal epithelial cells caused by adherent, noninvasive organisms, although pulmonary epithelial cells do release IL-8 after stimulation with culture filtrates of *Burkholderia cepacia* [35]. Moreover, cytokine release from intestinal epithelial cells has been demonstrated after treatment with *C. difficile* toxin A [36].

We demonstrate here for the first time that a noninvasive enteric pathogen can release IL-8 from Caco-2 cells and, more importantly, that this IL-8 release is due to production of a novel heat-stable, high-molecular-weight protein rather than to adherence of intact organisms. It appears that this protein is encoded on the bacterial chromosome but may depend on an intact virulence plasmid for appropriate synthesis or release; moreover, delivery may be enhanced by adherence of the organisms. In vivo, both adherence and toxin production may be required (as is the case for other bacteria, such as enterotoxigenic *E. coli* or *Vibrio cholerae*).

The strong correlation between fecal IL-1β concentrations and IL-1β/IL-1ra ratios and the other inflammatory mediators (FLF and IL-8) raises the possibility that the effect of EAggEC in vivo might involve activation of IL-1 receptors, and a postulated release of IL-8 from Caco-2 cells via effect of IL-1 in subcellular compartments that was not detected in culture supernatants and not inhibitable by IL-1ra cannot be excluded. Further studies will be required to elucidate the signaling pathways involved and to clarify the importance of proinflammatory cytokine release in the pathophysiology of EAggEC infection.

In summary, we have found evidence that EAggEC produce an inflammatory enteritis in children that is associated with growth impairment, even in the absence of diarrhea. Part of the pathophysiology of EAggEC infection may be due to proinflammatory cytokine release from the intestinal epithelium stimulated by EAggEC or their toxins. These findings also raise the possibility that some persistent diarrheal pathogens may be even more important causes of early childhood morbidity in developing areas than was previously recognized.

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