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Characterization of an Invasive Phenotype Associated with Enteroaggregative *Escherichia coli*

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Enteroaggregative *Escherichia coli* (EAggEc) strains are associated with persistent diarrhea in children in the developing world and exhibit a classic aggregative phenotype. We have demonstrated that EAggEc strains isolated from children with persistent diarrhea in Brazil, Bangladesh, and Pakistan also have the potential to be internalized by HeLa cells in the gentamicin protection assay. We have confirmed this phenomenon with transmission electron micrographs of bacteria engulfed by HeLa cells. We examined the mechanisms by which this process occurs. Staurosporine inhibited internalization of EAggEc strain 162 by 50% at a concentration of 0.1 μM. Genistein inhibited internalization of this same organism by 50% at a concentration of 50 μM. Cytochalasin D inhibited internalization by 50% at a concentration of 1 μg/ml. Staurosporine, genistein, and cytochalasin D inhibited the internalization of EAggEc strain 162 by HeLa cells in a dose-dependent manner. These data suggest that active cell processes such as signal transduction by protein kinase and/or tyrosine kinase may be involved in the internalization of EAggEc strain 162 by HeLa cells and that actin filaments and cytoskeletal structure may be important for this process.

Enteroaggregative *Escherichia coli* (EAggEc) strains have been a major cause of persistent diarrhea in children in the developing world and are recognized by their aggregative or "stacked-brick" pattern of adherence to HEp-2 or HeLa cells in culture (4–6, 12, 28). In addition, the aggregative adherence phenotype has been associated with a bundle-forming pilus, AAF/1, which is encoded by a 60-MDa plasmid and with the production of a heat-stable enterotoxin (EAST/1) which is also plasmid encoded (19, 20, 24). An oligonucleotide probe from the 60-MDa plasmid has been used to screen diarrheagenic *E. coli* strains from various geographic areas of the world and has identified 30 to 89% of the EAggEc strains identified in the tissue culture adherence assay (3). Volunteer studies have demonstrated that 1 of 19 adults developed diarrhea after ingesting large quantities of the EAggEc strain 17-2, from which the AAF/1-EAST/1 probe had been derived. However, 13 of those 19 volunteers developed antibodies to EAggEc strain 17-2, indicating that the volunteers were colonized by this organism (15, 17). These findings suggest that the mechanism of EAggEc pathogenesis is unclear and that additional factors may be required for the expression of the virulence phenotype by EAggEc strains. Whether cell signal transduction pathways are induced by the adherence of EAggEc strains to cells as they are by enteropathogenic *E. coli* (EPEC) adherence is an additional question that remains to be fully answered (2, 8). In this report, we demonstrate the potential of EAggEc strains to be internalized in HeLa cells. In addition, we present evidence for the involvement of cytoskeletal and cellular transducing elements in the process of invasion or bacterial mediated endocytosis by EAggEc strain 162.

MATERIALS AND METHODS

Bacterial strains. EAggEC strain 162 was isolated from a child in northeastern Brazil and characterized in a previous study (28). This strain fulfills the criteria for EAggEC by adherence to HEp-2 and HeLa cells in the characteristic cascading or stacked-brick-like pattern. In addition, this strain contains two plasmids: a large 60-MDa plasmid and a smaller 5-MDa plasmid. It does not contain *E. coli* heat-labile, heat-stable, or Shiga-like toxins by oligonucleotide probe or bioassay. Strain 162 is oligonucleotide probe negative for the *eaf* or *eae* genes coding for EPEC adherence and is also probe-negative for the genes which encode the invasion associated with enteroinvasive *E. coli*. Strain 162 is probe positive for the EAggEC AAF/1 pilus (data not shown). All other EAggEc strains used in this study are listed in Table 2; they originated from children with diarrhea in Brazil, Pakistan, or Bangladesh and exhibit the aggregative adherence phenotype.

For all of the assays, strain CDC K12 was used as a noninvasive control; EPEC strain 2348 was used as a control invasion-positive strain.

All bacterial strains were stocked in nutrient broth and glycerol at −70°C. Strains were grown for the adherence and invasion assays for 18 h in Luria broth (LB) at 37°C. Bacteria were serially diluted after cell lysis, plated on LB agar, and grown overnight for quantification after the invasion assays.

Eucaryotic cells. HeLa cells (American Type Culture Collection) were grown and maintained in minimum essential medium (MEM; Gibco-BRL) supplemented with 10% (vol/vol) fetal calf serum (FCS) at 37°C with 10% CO_2.

Inhibitors. All inhibitors were purchased from Sigma. Stock solutions were prepared at the following concentrations in dimethyl sulfoxide and stored in aliquots at −20°C until use: staurosporine, 1 mM; genistein, 100 mM; cytochalasin D, 1 mg/ml; and colchicine, 2.5 mM. Prior to use, the inhibitors were diluted in MEM supplemented with 10% FCS and added to the HeLa cells.

Adherence assay. HeLa cells were seeded at a density of 10^4/ml in eight-chambered Lab-Tek slides (Nunc Inc., Naperville, Ill.) and incubated overnight in culture medium (MEM–10% FCS) at 37°C and 10% CO_2. On the day of the assay, the HeLa cells were refed and inoculated with 20 μl of overnight cultures of the specified bacterial strain which had been washed and rediluted in phosphate-buffered saline (PBS) to a density of 10^7 CFU/ml in culture medium containing 1% mannose. Cells and bacteria were incubated for 3 h at 37°C and 10% CO_2. The slides were then washed four times with PBS and fixed with methanol for 5 min (18). The chambers were then removed from the slide, and the monolayers were stained with Geimsa stain and examined under light microscopy. Each slide contained negative and positive controls.

Invasion assay. HeLa cells were seeded at a density of 10^5 per well in a 24-well plate and incubated 18 h in culture medium (MEM–10% FCS) at 37°C and 10% CO_2. Bacteria were grown overnight in LB broth at 37°C and diluted to a density of 10^8 cells per ml; 2.5×10^6 bacteria were added to each well containing HeLa cells, and the mixture was incubated for 2 h at 37°C with 10% CO_2. The cells were washed three times with PBS and incubated for an additional 2 h in culture medium containing 50 μg of gentamicin per ml to kill extracellular bacteria. The monolayers were lysed with 0.125% sodium deoxycholate, and serial dilutions were plated on LB agar for overnight incubation to determine the number of viable intracellular bacteria (11). When specific inhibitors were used, they were added to the culture medium and were present throughout the assay. Assays were done twice, each time in triplicate. Results are reported as the percentage

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TABLE 1. Invasion by EAggEC strains

Bacterial strain	Mean % of bacterial inoculum invading[a]:		
	HeLa cells	HT29 cells	T84 cells
162	12.0 ± 3.5	10.2 ± 1.6	8.2 ± 2.1
CDC K12	0.05 ± 0.08	0.0	0.0
2348	6.6 ± 1.7	ND	ND

[a] Mean of six assays (strains 162 and CDC K12) or two assays (strain 2348) ± standard deviation. ND, not determined.

of the original inoculum of bacteria that were internalized, calculated from the mean CFU of the bacterial strain which were internalized in the HeLa cells.

Electron microscopy. After the incubation period of the adherence and invasion assays but prior to cell fixation or lysing, HeLa cells with bacteria either adherent or internalized were trypsinized, washed and suspended in Karnofsky's fixative followed by 1% osmium tetroxide, and then embedded in Eponate plastic. Sections were stained with uranyl acetate and lead citrate and examined with a Hitachi 600 electron microscope.

RESULTS

Internalization of HeLa cells by EAggEc strains. The adherence to and internalization of EAggEc strain 162 in HeLa cells are demonstrated in Fig. 1 and 2. In Fig. 1, the cells were trypsinized and fixed for the micrograph after 3 h of incubation, when they would normally be fixed for staining and examination by light microscopy. In Fig. 2, the cells were trypsinized and fixed after the 2-h incubation with gentamicin, when they would normally be lysed and serially plated for quantitative culture.

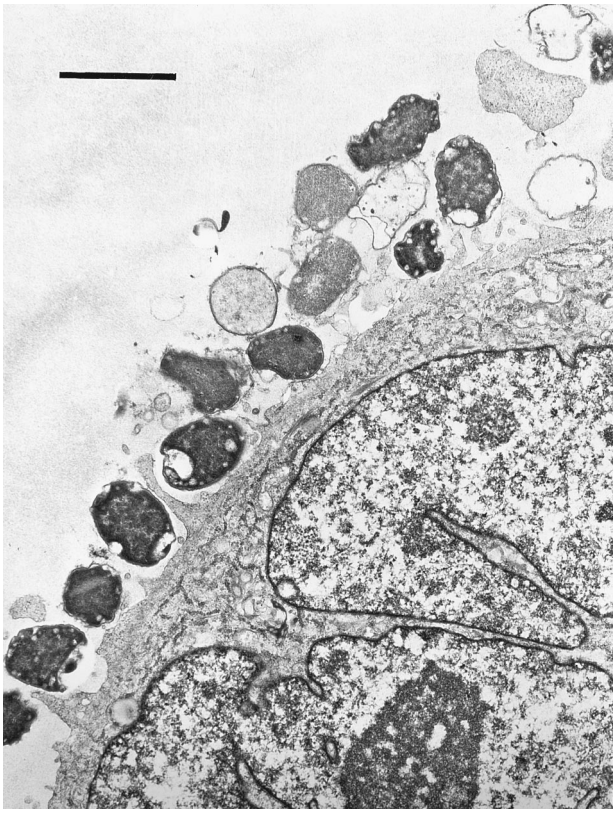

FIG. 1. Transmission electron micrograph of EAggEg strain 162 adherent to HeLa cells. Magnification is ×7,500; the bar represents 2 μm. The micrograph demonstrates the adherence process of the EAggEc strain 162 to HeLa cells and the initial phase of the internalization process, with the development of cellular extrusions which begin to surround the bacteria.

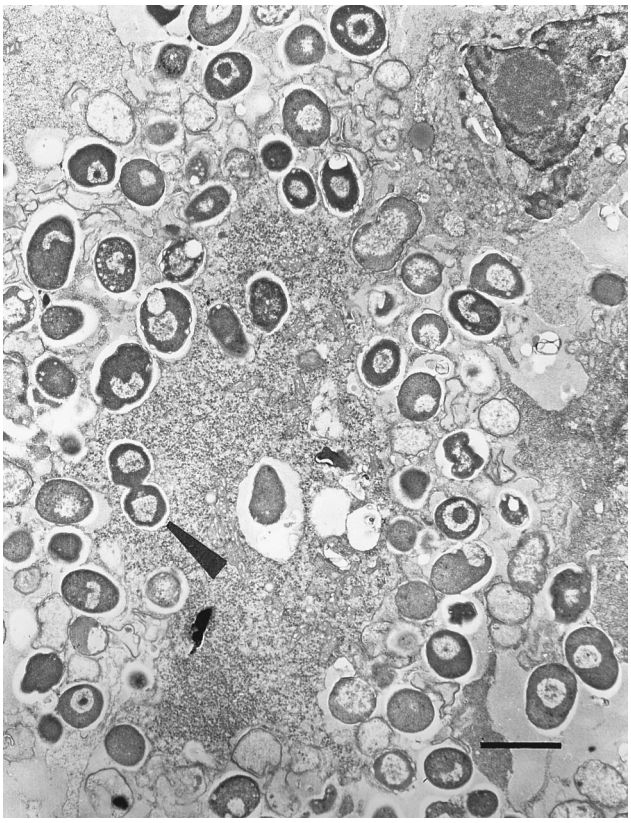

FIG. 2. Transmission electron micrograph of the internalization of EAggEc strain 162 within HeLa cells. Magnification is ×5,200; the bar represents 2 μm. Multiple organisms are demonstrated within the cell. The arrowhead indicates dividing organisms within the HeLa cell.

Consistently, 10 to 15% of the original inoculum of strain 162 is internalized in HeLa cells (Table 1). Strain 162 was also able to be endocytosed by T84 and HT29 intestinal cells, with 8 to 10% of the original inoculum internalized. We evaluated our library of EAggEc strains isolated from children with diarrhea in Brazil, Bangladesh, and Pakistan for the ability to be internalized in HeLa cells in the gentamicin protection assay (Table 2). Two of 8 (25%) of the Brazilian EAggEc strains tested, including strain 162-1, were invasive, 15 of 32 (47%) of the Bangladesh EAggEc strains tested were invasive, and 10 of 20 (50%) of the Pakastani EAggEc strains tested were internalized into HeLa cells. A strain was considered positive in this assay when more than 5% of the original inoculum was inter-

TABLE 2. Characterization of the internalization of EAggEc strains from various geographic locations into HeLa cells

Origin of EAggEc strain	No. of EAggEc strains tested	No. (%) of strains positive in the assay[a]
Brazil	8	2 (25)
Bangladesh	32	15 (47)
Pakistan	20	10 (50)
Overall	60	27 (45)

[a] An assay was considered positive when >5% of the original inoculum was internalized within the HeLa cells. Strain CDC K12 was used as a negative control, and EPEC strain 2348 with 6.6% of the original inoculum invading was used as a positive control.

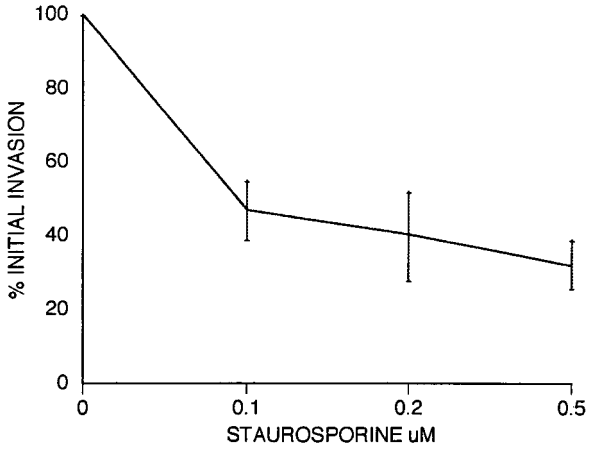

FIG. 3. Effect of staurosporine on internalization of EAggEc strain 162. The concentrations of staurosporine which were added to the cells 60 min prior to the addition of bacteria are shown on the horizontal axis. Staurosporine was present throughout the experiment. The data are presented as the means ± standard deviations for six assays. The baseline level of internalization of EAggEc strain 162 was considered 100% (vertical axis); the relative inhibition of that original level of internalization by the increasing concentrations of staurosporine is indicated on the vertical axis. EPEC strain 2348 served as a control in these assays; 74% of the initial inoculum invaded at 0.1 μM staurosporine, 40% invaded at 0.2 μM, and 35% invaded at 0.5 μM.

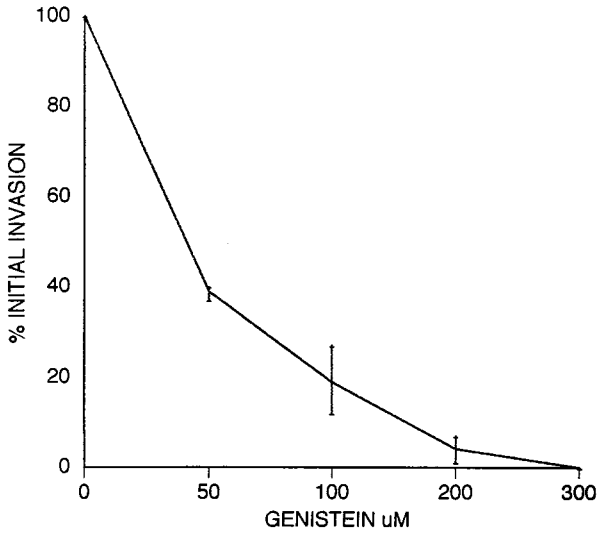

FIG. 4. Effect of genistein on the internalization of EAggEc strain 162. The concentrations of genistein that were added to the cells 30 min prior to the addition of the bacteria are shown on the horizontal axis. Genistein was present throughout the assay. The data are presented as the means ± standard deviations for six assays. The baseline level of internalization of EAggEc strain 162 was considered 100% (vertical axis); the relative inhibition of that original level of internalization by the increasing concentrations of genistein is indicated on the vertical axis. EPEC strain 2348 served as a control for these assays; 90% of the original inoculum invaded at 50 μM genistein, 45% invaded at 100 μM, 3.0% invaded at 200 μM, and 2% invaded at 300 μM.

nalized. Of these strains, only the strains from Pakistan were probed for AAF/1; 9 of the 10 strains which were internalized were probe positive. CDC K12 was used as a negative control; no colonies were recovered from invasion assays after overnight incubation for this strain. EPEC strain 2348 was used as a positive control.

Inhibition of internalization of EAggEc strain 162 by HeLa cells. Four agents known to inhibit various cytoskeletal and signal transducing events were used to dissect the internalization process. The effect of staurosporine on the invasion of strain 162 into HeLa cells was examined, and the results are shown in Fig. 3. When HeLa cells were pretreated for 60 min with staurosporine at concentrations of 0.1 to 0.5 μM before infection with strain 162, invasion was inhibited as a function of increasing staurosporine concentration. Fifty percent inhibition of invasion was observed at 0.1 μM staurosporine (Table 3).

The effects of genistein on the internalization of EAggEc strain 162 are shown in Fig. 4. When genistein was used to pretreat HeLa cells for 30 min before infection with strain 162 at concentrations of 50 to 300 μM, internalization of EAggEc strain 162 was inhibited in a dose-dependent manner. Fifty percent inhibition observed at 50 μM (Table 3), and a maximum inhibition of 99% was achieved at a concentration of 300 μM.

Similarly, the effects on the inhibition of internalization of EAggEc strain 162 as a function of increasing cytochalasin D concentration after pretreatment of the HeLa cells with the drug for 30 min prior to are shown in Fig. 5. Concentrations of 1 to 20 μg of the drug per ml were used, with 50% inhibition occurring at 1 μg/ml (Table 3). Inhibition of internalization occurred in a dose-dependent manner, and 97% inhibition of invasion was achieved at 20 μg/ml.

Pretreatment of HeLa cells for 30 min with various concentrations of colchicine did not reveal any effect of colchicine on

TABLE 3. Effects of various classes and concentrations of inhibitors on internalization of EAggEc strain 162 by HeLa cells

Inhibitor	Concn	% Inhibition
Staurosporine	0.1 μM	50
Genistein	50 μM	50
Cytochalasin D	1 μg/ml	50
Colchicine	0.25–100 μM	0

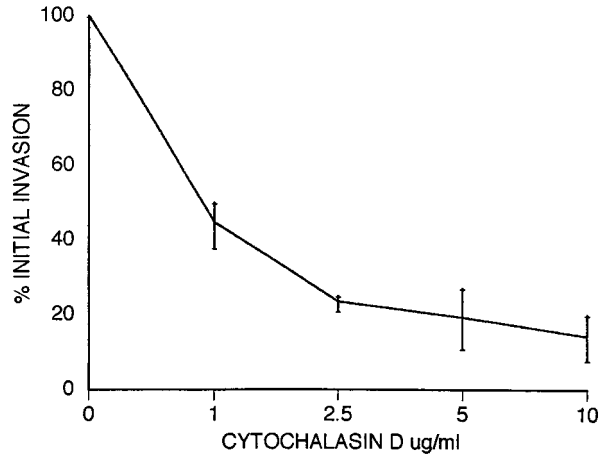

FIG. 5. Effect of cytochalasin D on internalization of EAggEc strain 162 in HeLa cells. The concentrations of cytochalasin D used to treat cells are shown on the horizontal axis. Cells were treated with cytochalasin D for 30 min prior to addition of the bacteria, and the drug was present throughout the assay period. The data are presented as the means ± standard deviations for six assays. The baseline level of internalization of EAggEc strain 162 was considered 100% (vertical axis); the relative inhibition of that original level of internalization by the increasing concentrations of cytochalasin D is indicated on the vertical axis. EPEC strain 2348 served as a control in these assays; 10% of the original inoculum invaded at 1.0 μg/ml, 0.5% invaded at 2.5 μg/ml, and 0.3 and 0.1% invaded at 5 μg/ml and 10 mg/ml, respectively.

the process of bacterial internalization for EAggEc strain 162. At concentrations of 0.25 to 100 μM, no changes in the internalization of strain 162 were seen (Table 3).

In all of these experiments, the cells remained viable by trypan blue exclusion throughout the concentration ranges of the drugs used.

DISCUSSION

With increasing sophistication in understanding the pathophysiologic mechanisms of virulence of bacteria which are enteric pathogens, there appear to be distinctions in the mechanisms which bacteria use to invade epithelial cells (9, 16). While invasion has not been reported to be a virulence factor for the EAggEc strains, strains have been reported to be protected in the gentamicin invasion assay (6, 19).

EPEC strains initiate epithelial cellular signal transduction mechanisms to promote their own uptake into cells. Drugs that inhibit phosphorylation by protein kinases can block the uptake and internalization of these bacteria by eucaryotic cells (21, 22, 25). It has been shown that tyrosine phosphokinase (TPK) inhibitors such as staurosporine and genistein block invasion of several enteric pathogens, including *Yersinia*, *Shigella*, *Salmonella*, and enteroinvasive *E. coli* strains, which utilize active bacterial invasins to enter cells, as well as of the EPEC strains, which most likely initiate the internalization process through a series of attachment steps (7, 21–23).

In this study, we have been able to demonstrate that a high percentage of EAggEc strains from a variety of geographic locations are internalized by HeLa cells. The strain which we have studied most intensively, 162, is able to invade a variety of cell lines, including two intestinal cell lines. That we have visualized bacteria dividing after internalization and that strains are able to invade intestinal cell lines as well as other epithelial cell lines suggest that this is not a mere epiphenomenon. We have also been able to demonstrate that signal transduction pathways including the protein kinases are used in this process. Both staurosporine and genistein were effective in inhibiting the internalization of EAggEc strain 162 into HeLa cells, implying a role for TPKs and possibly protein kinase C (PKC) in this internalization process.

Staurosporine is a potent inhibitor of different classes of protein kinases, including PKC, cyclic AMP-dependent protein kinase, and also some TPKs (25). Genistein, a more specific TPK inhibitor, blocks the binding of ATP to TPK, thereby rendering the enzyme inactive and unable to phosphorylate tyrosine residues (1). Both PKCs and TPKs are localized in focal contacts associated with integrins and may participate in transducing an internalization signal that is generated upon the binding of an invasin-like protein to its receptor (13, 14).

Cytochalasins, especially cytochalasin D, have been known to cause severe alterations in cellular morphology and surface topology which may interfere with adherence. Since cytochalasin D disrupts the assembly of actin filaments, it is possible that these filaments are involved in the internalization process. It has been shown that actin filaments are important in cellular adherence in other systems (10). Plausible roles for microfilaments and cellular surface topology in the invasion or internalization process could include providing access to putative receptor sites for an invasin-like ligand(s) or mediating a conformational changes in the cytoskeletal structure of the epithelial cell which would then facilitate endocytosis. At this point, it becomes necessary to attempt to assign precise functions for PKCs, TPKs, and actin microfilaments in the EAggEc-mediated invasion process.

The data presented in this study suggest that EAggEc strain 162 is capable of inciting HeLa cells and other epithelial cells to engulf the adherent aggregative bacteria and that this process is inhibited by drugs which interfere with signal transduction (via PKCs and TPKs) and actin polymerization. In addition, we have been the first to demonstrate that the internalization of EAggEc strains by HeLa cells occurs with a significant percentage of EAggEc strains isolated from various locations around the world, using the standard gentamicin protection assay. While these studies suggest that internalization of EAggEc strains by epithelial cells may be a property which is important for the enteric virulence associated with EAggEc strains, it is not possible to definitively establish virulence traits by studying in vitro invasion with cultured cell lines.

EAggEc strains have been routinely associated with persistent diarrhea, but there are few other data to characterize the clinical picture of children with these debilitating diarrheal illnesses. In a classical clinical study by Cravioto et al., 33% of the EAggEC isolates were obtained from children with persistent diarrhea that was also bloody (6). The authors of this study reported that two of the EAggEC strains isolated from children with bloody diarrhea had invasive ability, although no further details were given. In 71 of the episodes of bloody diarrhea in this study, an EAggEC strain was the sole isolated pathogen. No other studies which have documented the association of EAggEc strains with persistent diarrhea have provided clinical descriptions of the diarrhea to further assess the association of bloody diarrhea with EAggEc strains. However, in in vitro studies, Vial was able to demonstrate hemorrhagic changes in rabbit ileal loops with an EAggEc strain (27).

The results from the adult volunteer studies published to date are unsatisfying since a significant number of subjects did not develop diarrhea associated with EAggEc after ingesting large concentrations of bacteria (15, 17). The EAggEc strain used in these studies has not been shown to be invasive, although it manifests aggregative adherence, is the source strain for AAF/1, and is known to contain the secretory toxin EAST/1. Additional volunteer studies found that 4 of 20 subjects who received an inoculum of an EAggEc strain developed diarrhea or loose stools; these four subjects all received the same 042 strain which was also shown to be internalized in HEp-2 cells at 1 to 3%. In the same study, however, other strains, including 17-2, which were internalized in HEp-2 cells at 1 to 3% did not cause diarrheal disease (19). Whether the lack of diarrhea in these adult volunteer studies is related to an immunity acquired with age or whether an additional virulence factor may be required for these agents to be fully pathogenic is not known. The lack of a reliable animal model has made it difficult to assess the virulence factors necessary to produce diarrhea by EAggEC (26, 28). Further clinical studies and animal studies to evaluate the nature of diarrheal disease caused by these organisms may be of assistance.

The data in this study do not confirm that the ability of EAggEc strains to be internalized by HeLa cells is a virulence factor of the EAggEc strains, although this property is present on a significant number of strains from various geographic locations. It is likely that the internalization seen with EAggEc strains in our study is similar to that seen with the EPEC strains. It is possible that the attachment and subsequent engulfment of EAggEc is be a strategy for the aggregative bacteria to evade expulsion from the intestine and thus be present to contribute to persistent diarrheal disease, although it is also possible that the internalization of these bacteria also contributes to pathogenesis of diarrheal disease. Further work to delineate the full range of virulence factors of the EAggEc

strains and the molecular pathophysiology of the diarrheal illnesses caused by these strains is necessary.

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