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Human Colonic Epithelial Cells, HT29/C_1, Treated with Crude *Bacteroides fragilis* Enterotoxin Dramatically Alter Their Morphology

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***Bacteroides fragilis* has been associated with causation of diarrheal disease in livestock and humans. To date, conventional tissue culture and animal assays used to detect the biologic activity of bacterial enterotoxins have failed with enterotoxigenic *B. fragilis*. Although enterotoxigenic *B. fragilis* stimulates intestinal secretion in lamb and calf ligated intestinal loops, infant rabbits, and adult rabbits with ligated ceca, these animal systems are costly and complicated, which limits their usefulness for identification of enterotoxigenic *B. fragilis* strains. Using the cloned human colonic-epithelial-cell line HT29/C_1, we have developed an in vitro assay that is 89% sensitive and 100% specific in detecting enterotoxigenic *B. fragilis* strains as defined by the lamb ligated-intestinal-loop assay. Subconfluent HT29/C_1 cells treated with concentrated bacterium-free culture supernatants of enterotoxigenic *B. fragilis* strains develop specific and striking morphologic changes including loss of cell-to-cell attachments, rounding, swelling, and, in some cases, pyknosis. These morphologic changes are initially visible at 1 h after treatment and progress over at least the first 24 h. This tissue culture assay should prove useful in epidemiologic studies of enterotoxigenic *B. fragilis* and may facilitate basic studies to identify the *B. fragilis* toxin(s) and its mechanism of action.**

Diarrheal diseases are among the leading causes of morbidity and mortality for children worldwide (23, 25). Intensive studies to identify the etiologies of diarrheal disease in industrialized and developing countries frequently identify an enteric pathogen associated with only 50% or less of cases of diarrhea (6, 11). These data suggest that additional human enteric pathogens remain to be identified. In 1984, strains of *Bacteroides fragilis* associated with epidemic diarrheal disease in lambs and capable of stimulating a secretory response in ligated intestinal loops of lambs and calves were first described by Myers et al. (14). These bacteria, termed enterotoxigenic *B. fragilis*, were subsequently associated with diarrheal disease in calves, foals, piglets, and, most recently, humans (1, 2, 15, 16, 19, 20). Further investigations of the epidemiology of enterotoxigenic *B. fragilis* have been hampered by the lack of a simple assay for detection of toxin-producing bacteria. Although adult rabbits with ligated ceca and infant rabbits are readily infected (often resulting in lethal diarrheal disease) with enterotoxigenic *B. fragilis* (16–18, 20) and secretory responses to enterotoxigenic *B. fragilis* occur in lamb and calf ligated intestinal loops (14, 19), these animal models are not convenient for screening large numbers of *B. fragilis* isolates. Assays which have failed to detect enterotoxigenic *B. fragilis* include Y-1 adrenal and Chinese hamster ovary (CHO) cells; suckling mouse, adult mouse, hamster, pig, and rabbit ligated-ileal-loop assays; and the gene probe for cholera toxin or the heat-labile enterotoxin of *Escherichia coli* (19). The purpose of this investigation was to develop an in vitro assay to detect enterotoxigenic *B. fragilis* by using a human colonic-epithelial-cell line.

MATERIALS AND METHODS

B. fragilis isolates. The majority of our studies were done with enterotoxigenic *B. fragilis* 2-078382-3 (ATCC 43858; human) and 86-5443-2-2 (porcine) and nonenterotoxigenic *B. fragilis* 077225-2 (human). Isolates were identified as enterotoxigenic or nonenterotoxigenic on the basis of presence or absence of a secretory response in lamb ligated intestinal loops (14). Some studies were also done with 81 isolates (46 isolates of enterotoxigenic *B. fragilis* and 35 isolates of nonenterotoxigenic *B. fragilis*) obtained from the feces of children in a prospective study of diarrheal disease conducted from July 1986 until July 1988 in Whiteriver, Ariz., among the White Mountain Apache population (22). *B. fragilis* isolates grown (in an anaerobic chamber) on tryptose blood agar slants or in brain heart infusion broth were suspended in sterile defibrinated bovine blood and stored at −20 or −70°C until use.

Preparation of culture filtrates. *B. fragilis* isolates were grown in an anaerobic chamber on blood agar plates for 48 h; five or more colonies of a pure culture were then inoculated into brain heart infusion broth (B-D Microbiology Systems, Cockeysville, Md., or Difco brain heart infusion broth, Baxter Scientific Products, Columbia, Md.). Broth cultures were incubated anaerobically for 48 h at 37°C and then centrifuged at 12,000 × *g* for 30 min. The culture supernatants were concentrated approximately 20-fold with Amicon Centriprep 10 Concentrators (molecular weight cutoff, 10,000; Amicon, Danvers, Mass.) or with an Amicon stirred-

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cell apparatus 10 (PM-1 or PM-10 membranes with molecular weight cutoffs of 1,000 and 10,000, respectively) (12), filter sterilized, and stored at 4°C for up to 12 months.

Tissue culture assay. T84 cells were obtained from the American Type Culture Collection (Rockville, Md.). T84 cells were grown in plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) in a 1:1 mixture of Dulbecco modified Eagle's medium (GIBCO, Grand Island, N.Y.) and Ham's F-12 medium supplemented with 25 mM $NaHCO_3$, 13.4 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 90 μg of streptomycin per ml, 90 U of penicillin per ml, 5% newborn calf serum (Hyclone, Logan, Utah), and 5% fetal calf serum (Inovar Biologicals Inc., Gaithersburg, Md.). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air. Medium was changed three times weekly. For experimental use, subconfluent T84 cells were suspended by incubation with 0.025% trypsin–265 μM EDTA solution (GIBCO) and plated into LabTek slides (VWR, Bridgeport, N.J.).

Caco-2 cells were also obtained from the American Type Culture Collection. Cells were grown in 5% CO_2–95% air in a humidified incubator at 37°C in Dulbecco's modified Eagle's medium (Hazleton Biologics, Lenexa, Kans.) supplemented with 25 mM $NaHCO_3$, 10 mM HEPES, 50 μg of streptomycin per ml, 50 U of penicillin per ml, 1% nonessential amino acids, and 10% fetal bovine serum. Medium was changed three times weekly. For experimental use, cells were prepared as described above for T84 cells.

HT29 cells, C_1 clone, were obtained from Daniel Louvard, Institut Pasteur, Paris, France (7, 13). HT29/C_1 cells were grown in Dulbecco's modified Eagle's medium (Irvine Scientific, Santa Ana, Calif.) with 10% fetal bovine serum, 1 mM pyruvate, 44 mM $NaHCO_3$, 10 μg of human transferrin (Boehringer Mannheim) per ml, and penicillin (50 U/ml)-streptomycin (50 μg/ml) in 10% CO_2; medium was replenished 6 days/week. Cells were suspended 1:15 at 80 to 90% confluency (approximately once weekly) by using 0.005% trypsin–0.053 mM EDTA, plated into eight-well LabTek slides, and allowed to grow for 2 to 3 days. For all experiments, concentrated culture filtrates of *B. fragilis* were diluted directly into tissue culture media on the cells (final volume, 400 μl per well). Preliminary experiments determined that predilution of the enterotoxigenic *B. fragilis* filtrates led to rapid loss of toxin activity. Following the specified incubation time, the medium was aspirated from the cells, and the cells were fixed with 90% methanol and stained with Giemsa (Sigma, St. Louis, Mo.). Slides were read at ×100 magnification with standard bright-field light microscopy. In an additional experiment, the appearance of cells treated with an enterotoxigenic *B. fragilis* culture filtrate was assessed by using differential interference contrast (Nomarski) optics.

Other bacterial toxins. Purified *Clostridium difficile* toxin A and crude Shiga toxin (59 toxin) were gifts from Barbara Laughon, The Johns Hopkins University School of Medicine, and Alison O'Brien, Uniformed Services University of the Health Sciences, Bethesda, Md., respectively. Purified heat-stable enterotoxin of *E. coli* (STa) was obtained from Donald C. Robertson, University of Kansas, and prepared as described by Dreyfus et al. (4). Cholera toxin was purchased from Schwartz-Mann (Cleveland, Ohio).

RESULTS

Time course of effect of enterotoxigenic *B. fragilis* filtrates on intestinal epithelial-cell lines. Intestinal epithelial-cell lines have proven extremely useful as models for investigating the pathogenesis of infectious diarrheal diseases and for delineating the cellular mechanisms responsible for chloride secretion stimulated by bacterial enterotoxins (8, 10, 26, 28). To develop an in vitro assay for detecting enterotoxigenic *B. fragilis*, we chose to examine the effects of culture filtrates prepared from both enterotoxigenic and nonenterotoxigenic *B. fragilis* strains on three available human colonic epithelial cell lines, i.e., T84, Caco-2, and HT29/C_1. All three cell types polarize on substrate-coated filters and develop increased tissue resistances consistent with tight junction formation and analogous to intestinal tissue in vivo. The physiologic responses of T84 cells have been the best characterized (8, 10, 28). This cell line is a model of crypt cell function and secretes only chloride; brush border enzymes and sodium absorptive processes are absent. In contrast, Caco-2 and HT29/C_1 cells contain measurable brush border enzymes but also secrete chloride, which is consistent with a partially differentiated cell type midway along the villus-crypt axis (13, 26).

In initial experiments, the effect of enterotoxigenic *B. fragilis* and nonenterotoxigenic *B. fragilis* filtrates at a 1/4 dilution on T84, Caco-2, and HT29/C_1 cells was assessed after 3 h of exposure. As shown in Fig. 1A, untreated control subconfluent T84 cells form clusters containing cells of various sizes. The appearance of T84 cells was unaltered by treatment with a nonenterotoxigenic *B. fragilis* filtrate (Fig. 1B). However, T84 cells treated with an enterotoxigenic *B. fragilis* filtrate showed a change primarily at the edges of the clusters, with some cells detached from their neighbors, and were more pyknotic with apparent loss of cell cytoplasm; other T84 cells appeared to stretch (Fig. 1C). A similar experiment with Caco-2 cells revealed similar but less clear morphologic changes when the cells were treated with an enterotoxigenic *B. fragilis* filtrate (results not shown). In contrast, untreated control subconfluent HT29/C_1 cells formed uniform, smooth-edged clusters which remained unaltered by treatment with a nonenterotoxigenic *B. fragilis* filtrate (Fig. 1D and E respectively). Once treated with an enterotoxigenic *B. fragilis* filtrate, HT29/C_1 cells revealed a dramatic change in morphology, with dissolution of the tight clusters and distinct separation of the cells from each other (Fig. 1F). Similar morphologic changes in HT29/C_1 cells were observed with enterotoxigenic *B. fragilis* filtrates prepared from strains 86-5443-2-2 and 2-078382-3.

We then examined the effect of an enterotoxigenic *B. fragilis* filtrate (1/4 dilution) on the morphology of HT29/C_1 cells over 24 h (n = 1 to 5 for different time points). Nonenterotoxigenic *B. fragilis* filtrates did not alter the morphology of HT29/C_1 cells at any time compared with either untreated control cells or cells treated with brain heart infusion broth alone. The first evidence of a change in morphology secondary to treatment with enterotoxigenic *B. fragilis* filtrate was noted after only 1 h (Fig. 2A). By 2 h, the dissolution of the cluster morphology of HT29/C_1 cells had progressed, and by 6 h, it was fully developed (Fig. 2B), with rounding and detachment of all cells in the monolayer from their neighboring cells. In most cases, cell morphologic changes after 6 h were more striking than those present after 3 h of exposure (Fig. 1F) to an enterotoxigenic *B. fragilis* filtrate. By 18 to 24 h of exposure to an enterotoxigenic *B. fragilis* filtrate, the normal cluster morphology was further disrupted; some cells were pyknotic and were noted to "line up" (Fig. 2C). Results were similar with filtrates prepared from enterotoxigenic strain 86-5443-2-2 or 2-078382-3. Because only a small amount of toxin-containing filtrates was

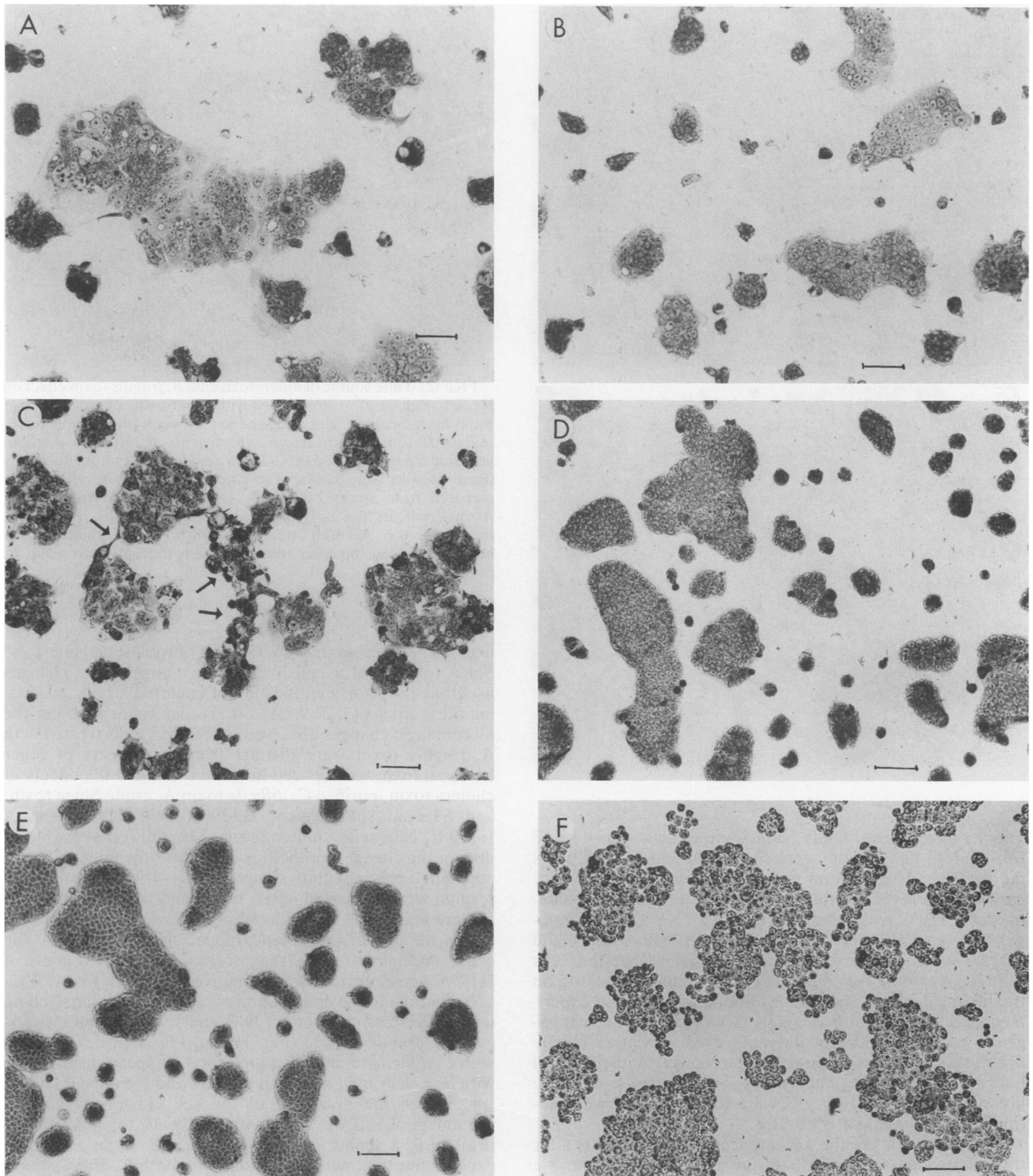
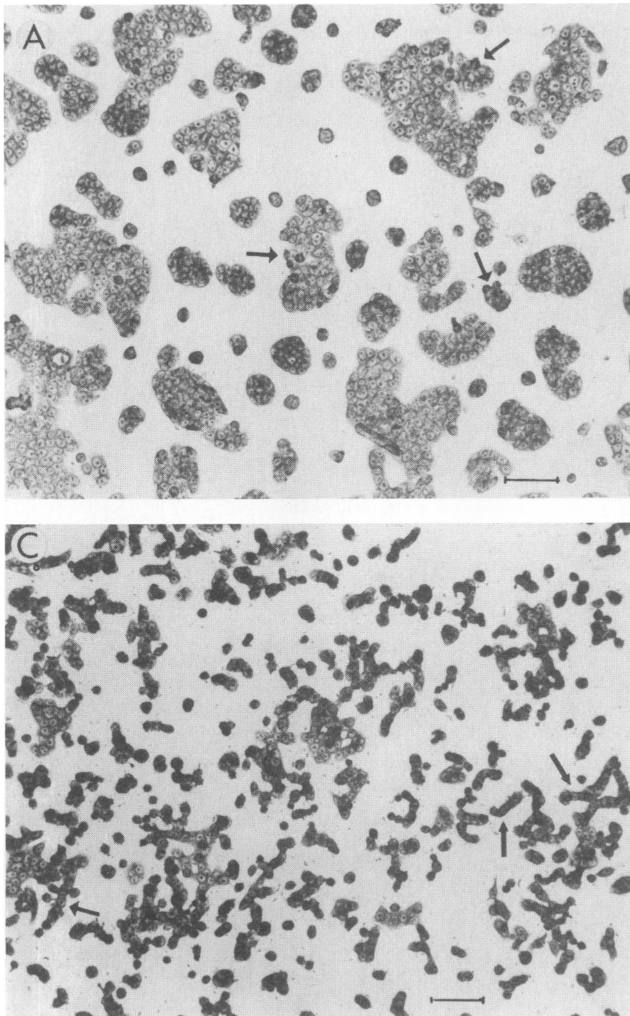


FIG. 1. Effect of enterotoxigenic *B. fragilis* toxin on T84 and HT29/C_1 cells. Cells were stained with Giemsa and photographed at ×100 magnification. The scale marker on each photograph represents 100 μm. (A) Untreated control T84 cells. (B) T84 cells treated for 3 h with nonenterotoxigenic *B. fragilis* filtrate (1/4 dilution) prepared from strain 077225-2. (C) T84 cells treated with enterotoxigenic *B. fragilis* filtrate (1/4 dilution) prepared from strain 86-5443-2-2. Arrows indicate the alterations in cell morphology observed primarily at the border of cell clusters, with some cells appearing pyknotic or stretched. (D) Untreated control HT29/C_1 cells. (E) HT29/C_1 cells treated for 3 h with nonenterotoxigenic *B. fragilis* filtrate (1/4 dilution) prepared from strain 077225-2. (F) HT29/C_1 cells treated for 3 h with enterotoxigenic *B. fragilis* filtrate (1/4 dilution) prepared from strain 86-5443-2-2.



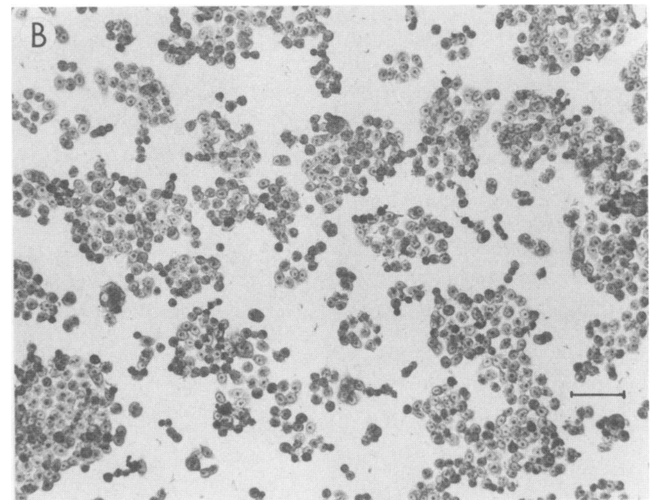


FIG. 2. Time course of enterotoxigenic *B. fragilis* toxin effect on HT29/C_1 cells. Cells were stained with Giemsa and photographed at ×100 magnification. The scale marker on each photograph represents 100 μm. Enterotoxigenic *B. fragilis* filtrate (1/4 dilution) prepared from strain 86-5443-2-2 was used for all experiments in this figure. Results were similar with enterotoxigenic *B. fragilis* filtrate prepared from strain 2-078382-3. (A) Cells treated for 60 min. Arrows indicate the early signs of altered morphology. (B) Cells treated for 6 h. (C) Cells treated for 18 h. Arrows indicate cells beginning to line up after treatment with the enterotoxigenic *B. fragilis* filtrate.

initially available for study, similar experiments (n = 1 to 8 for different time points) were conducted with toxin filtrates diluted 1/32. This dilution was chosen because preliminary experiments revealed no difference in the altered morphology of HT29/C_1 cells treated for 3 and 6 h with enterotoxigenic *B. fragilis* filtrate 86-5443-2-2 or 2-078382-3 at 1/4 and 1/32 dilutions. These experiments revealed that the cells fully recovered morphologically by 48 but not 24 h post-toxin treatment (data not shown). Additional time course experiments with T84 and Caco-2 cells (1/4 dilution of enterotoxigenic *B. fragilis* filtrate) did not reveal a similar, easily observable, progressive morphologic change. With these cell lines, the morphologic changes peaked at 3 to 6 h and were not sustained when the cells were treated with lower dilutions of toxin-containing filtrates.

To further analyze the appearance of HT29/C_1 cells after treatment with an enterotoxigenic *B. fragilis* filtrate, we examined the appearance of the cells by using differential interference contrast optics. As shown in Fig. 3, the cells treated with the toxin-containing filtrate appear rounded and also swollen, with evidence of loss of cell-to-cell attachments when this microscopic technique was used.

Comparison of effects of various bacterial toxins on HT29/C_1 cells. Treatment of several cell lines with bacterial toxins such as cholera toxin (CHO and Y-1 adrenal cell lines), *C. difficile* toxin A (WI38 lung fibroblast cell line), and Shiga toxin (HeLa cells) results in morphologic changes useful as tools for identification of bacterial strains producing these toxins (3, 5, 9, 21, 24). To determine whether the morphologic changes observed in HT29/C_1 cells treated with *B. fragilis* toxin were distinct from the effects of other bacterial toxins on this cell line, we examined the effects of cholera toxin, purified *C. difficile* toxin A, crude Shiga toxin, and STa on subconfluent HT29/C_1 cells. Treatment of HT29/C_1 cells with cholera toxin (1 μg/ml) for 24 h did not disrupt the cluster morphology except for the appearance of apparent spaces within some clusters (Fig. 4A). Similar results were observed after treatment of the cells with cholera toxin for 3 and 48 h. We speculate that these spaces within the clusters represent trapped fluid secreted by the cells. Preliminary data suggest that the cell volume of HT29/C_1 cells decreases in response to cholera toxin (12).

In contrast to the dissociation of cells and cellular swelling noted after treatment of HT29/C_1 cells with the enterotoxigenic *B. fragilis* filtrate (Fig. 1F and Fig. 3A), cells treated with *C. difficile* toxin A (2 μg/ml) for 3 h appeared shrunken, with less clear individual cell borders and the appearance of spicules on the periphery of the cell clusters (Fig. 4B). Treatment of HT29/C_1 cells with *C. difficile* toxin A for 24 h resulted in a similar morphologic change. In an additional experiment, treatment of the cells with both *C. difficile* toxin A and *B. fragilis* toxin resulted in a mixed morphologic change in the cell clusters, suggesting distinct pathways to cell damage for the two toxic effects in these cells.

In contrast to untreated cells grown for the same period (Fig. 4D), cells treated with Shiga toxin for 48 h did not grow, and the damage they sustained was consistent with the known effect of Shiga toxin on the inhibition of protein synthesis (Fig. 4C) (9, 20). No morphologic changes were

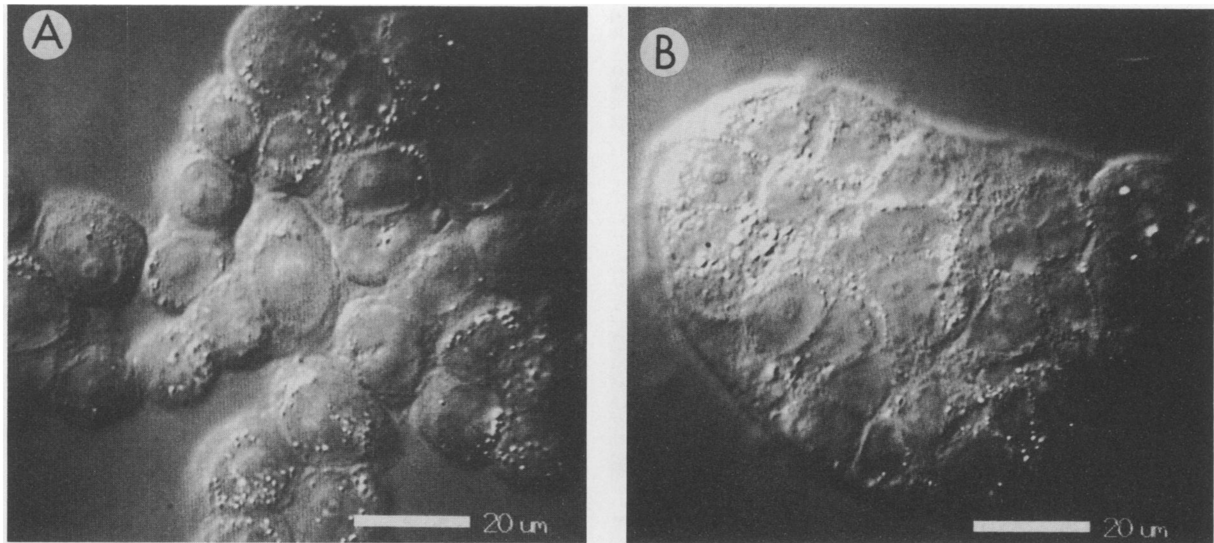


FIG. 3. Enterotoxigenic *B. fragilis* toxin-treated HT29/C_1 cells viewed with Nomarski optics. (A) Cells were treated for 6 h with enterotoxigenic *B. fragilis* filtrate prepared from strain 86-5443-2-2 (1/32 dilution); (B) untreated control cells. The photographs are at ×1,000 magnification.

observed in HT29/C_1 cells treated with Shiga toxin for 3 h. There were also no morphologic changes when HT29/C_1 cells were treated with STa for 3 h. Additional data in our laboratory indicate that HT29/C_1 cells do not respond to STa with a change in cellular cyclic-GMP levels either in the absence or the presence of the phosphodiesterase inhibitor isobutyl-methyxanthine (1 mM; data not shown). These data are consistent with HT29/C_1 cells lacking one or more cellular components necessary for a response to STa and distinguish this cell line from T84 cells or Caco-2 cells, which respond to STa with an increase in cyclic-GMP levels (10, 28; data not shown).

Evaluation of clinical isolates of *B. fragilis* for toxin production. To determine if the morphologic changes observed in HT29/C_1 cells with filtrates prepared from a few enterotoxigenic *B. fragilis* isolates would be observed with testing of larger numbers of clinical isolates, we tested 20-fold-concentrated filtrates prepared from 81 human isolates of *B. fragilis*. Testing was done at a 1/4 dilution of the sterile bacterial filtrates in HT29/C_1 medium for 3 h. All slides were read by two investigators (C.S.W. and F.D.G) without knowledge of the type of *B. fragilis* isolate being tested. All 35 nonenterotoxigenic *B. fragilis* filtrates were negative in the HT29/C_1 cell assay, i.e., produced no change in cellular morphology compared with untreated control cells. In contrast, 41 of 46 enterotoxigenic *B. fragilis* filtrates produced changes in the HT29/C_1 cells morphologically similar to those shown in Fig. 1F. Both investigators obtained the same results.

Because we observed that the potency of the filtrates to alter cell morphology varied between isolates (see below) and because the alteration in cell morphology progressed over 18 to 24 h (Fig. 2), we retested the five lamb-positive, cell-negative enterotoxigenic *B. fragilis* filtrates on HT29/C_1 cells for 6 and 18 h. This identified one more strain as positive, although the toxic effect on the cells was quite limited. We also retested filtrates prepared from these strains concentrated 40-fold. No further toxigenic *B. fragilis* strains were identified. Thus, testing of filtrates by using a 1/4 dilution and a 3-h incubation identified 89% of enterotoxigenic *B. fragilis* isolates as defined in the lamb ligated-intestinal-loop assay; additional measures to enhance the sensitivity of the assay detected only one more positive isolate.

We have observed that filtrates produced by some toxigenic *B. fragilis* strains were very potent, altering the morphology of virtually every cell; in contrast, other filtrates produced changes only in small clusters of cells or at the margins of cell clusters. To assess the variation in the toxic effect produced by filtrates prepared from 41 human enterotoxigenic *B. fragilis* strains, each filtrate was serially diluted up to 1/100 and tested on HT29/C_1 cells. The following data indicate the highest dilution showing a toxic effect on HT29/C_1 cells for each strain. At a 1/4 dilution, 1 strain was toxic; at a 1/16 dilution, 1 strain was toxic; at a 1/32 dilution, 2 strains were toxic; at a 1/64 dilution, 9 strains were toxic; and at a 1/100 dilution, 28 strains were toxic. These data confirmed the variable toxin activity in broth filtrates from different strains of enterotoxigenic *B. fragilis*.

DISCUSSION

Enterotoxigenic *B. fragilis* isolates were recently recognized as a possible cause of diarrheal disease in livestock and humans (1, 2, 14–16, 19, 20, 22). In the latter study, enterotoxigenic *B. fragilis* was isolated from 12% of persons with undifferentiated diarrhea and from only 6% of nondiarrheic controls ($P < 0.03$) and was distinctly correlated with age, with the highest isolation rates (20 to 24% in individuals with diarrhea versus 8 to 9% in controls) in children 13 to 36 months of age. However, the extent and importance of this potential pathogen in human diarrheal disease are unknown, and work to date has been hampered by the absence of a convenient in vitro assay for detecting and studying toxin-producing strains. Our results demonstrate that concentrated filtrates prepared from most strains of *B. fragilis* identified as toxin producers in the lamb ligated-intestinal-loop assay yield specific, readily identifiable morphologic changes in HT29/C_1 cells, a cloned human intestinal epithelial-cell line derived from a colon carcinoma.

In animals, the major site of disease appears to be the

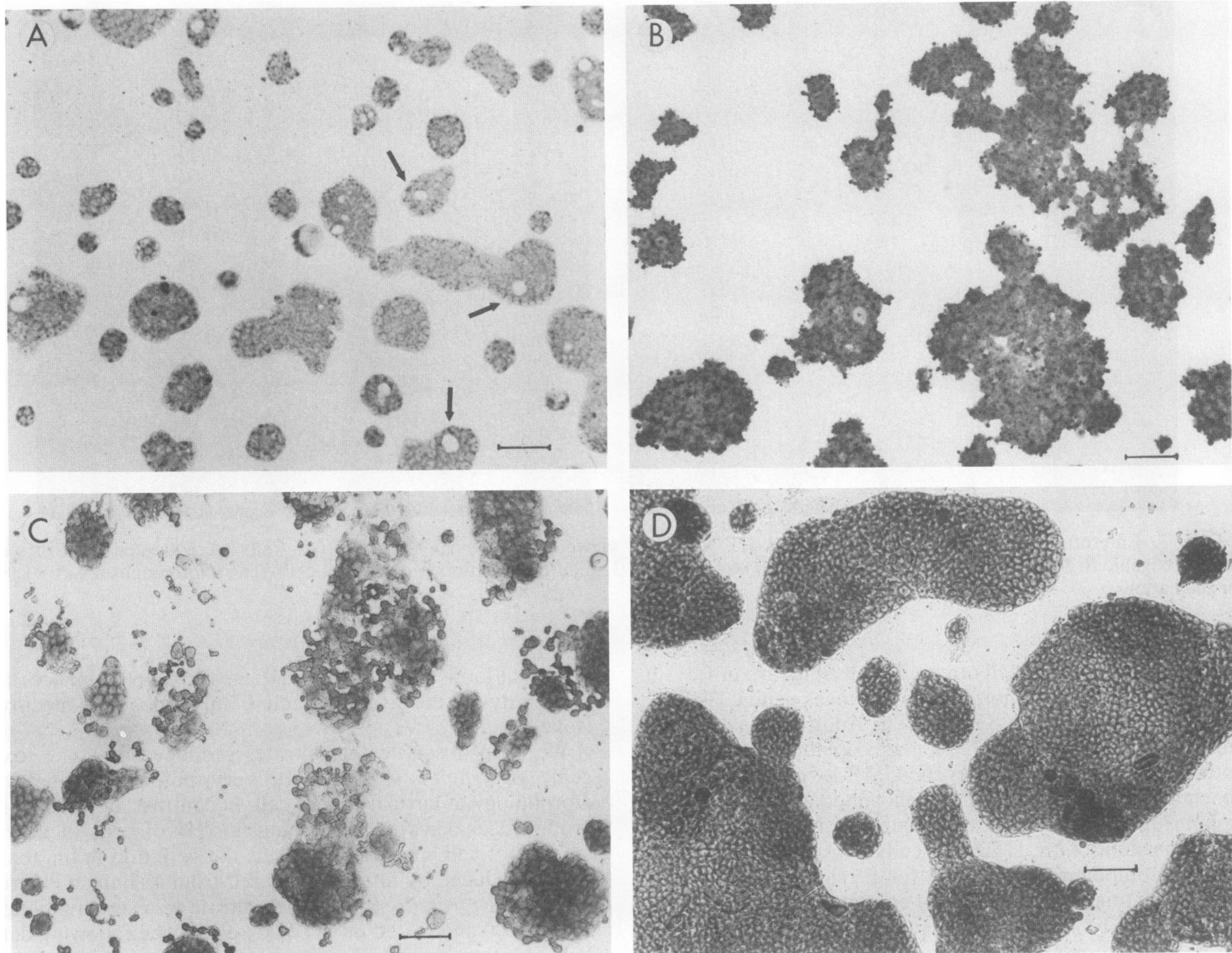


FIG. 4. Comparison of bacterial toxin effects on HT29/C_1 cells. Cells were stained with Giemsa and photographed at ×100 magnification. The scale marker on each photograph represents 100 μm. (A) Cells treated with cholera toxin (1 μg/ml, 24 h). Arrows indicate the appearance of "holes" within cell clusters. (B) Cells treated with *C. difficile* toxin A (2 μg/ml, 3 h). (C) Cells treated with Shiga toxin (2.5 × 10^3 50% cytotoxic doses per ml) for 48 h. (D) Untreated control cells grown for 48 h (compare with panel C).

colon, where changes in cellular architecture are most prominent at the tips of the villi (2, 18, 20). In vivo, intestinal epithelial cells appear to round and detach from their neighbors, a change reminiscent of that observed in HT29/C_1 cells. Although the precise nature of HT29/C_1 cells is yet to be defined, studies indicate that these cells contain microvillar enzymes and secrete chloride electrogenically but lack sodium absorptive processes (13). These data suggest that HT29/C_1 cells represent a cell type midway between crypt cells and terminally differentiated villous cells. Both these properties and the clonal nature of HT29/C_1 cells may explain why these cells show morphologic changes in response to toxin-containing *B. fragilis* filtrates more readily than do T84 cells, Caco-2 cells, and other cell lines tested.

To date, as our observations reported here and the work of others indicate, concentration of culture supernatants has been necessary to detect toxin activity (14, 19). This has been a consistent finding in the lamb ligated-intestinal-loop assay, in which unconcentrated culture filtrates do not cause secretion. However, in recent experiments, unconcentrated culture filtrates from certain enterotoxigenic *B. fragilis* strains are also active in the HT29/C_1 cell assay (27). Additional experiments indicate that as little as 15 min of predilution of the filtrates (i.e., dilution prior to placement of the filtrates on the HT29/C_1 cells) diminishes toxin activity and that storing filtrates concentrated only 10-fold for as little as 36 h also decreases their potency in altering HT29/C_1 cell morphology. The reason(s) for these observations is not known. One hypothesis is that the toxin is a multimeric molecule which is more stable when the culture supernatants are concentrated. Alternatively, unconcentrated culture supernatants may contain an inhibitor of toxin activity or an enzyme which degrades the toxin. Purification of the toxin and experiments examining the direct effect of enterotoxigenic *B. fragilis* and nonenterotoxigenic *B. fragilis* strains on the HT29/C_1 cells may help us understand these observations and identify the importance of a putative toxin in the pathogenesis of infections caused by enterotoxigenic *B. fragilis* strains. In addition, toxin purification will establish whether or not the lamb-active and cell-active toxins are identical.

Our experiments with human isolates of enterotoxigenic

B. fragilis suggest that the amount of toxin produced by individual strains identified as enterotoxigenic *B. fragilis* in the HT29/C_1 cell assay varies. Preliminary work with rabbits has also suggested that some strains of enterotoxigenic *B. fragilis* are more virulent than other strains. For example, certain strains of enterotoxigenic *B. fragilis* cause death due to diarrheal disease in young rabbits, whereas other strains do not (16, 18, 20). Not enough information is available to determine if the strains identified in our studies as "high toxin producers" because of a persistent toxic effect on HT29/C_1 cells after dilution are also strains more virulent in the rabbit model. In addition, since the optimal conditions for enterotoxigenic *B. fragilis* growth and toxin production have yet to be studied, it may be that the in vitro growth conditions presently used favor certain strains, thus causing the apparent difference in toxin production between strains.

In summary, our results demonstrate a reliable and specific in vitro assay system for the detection of most but not all strains of enterotoxigenic *B. fragilis* as defined by the lamb ligated-intestinal-loop assay. Some possible reasons why some strains of enterotoxigenic *B. fragilis* are falsely negative in the cell assay are that more than one secretory toxin is produced by diarrheagenic *B. fragilis* and that these strains are weak toxin producers under the in vitro conditions used. The disadvantage of the cell detection assay is that HT29/C_1 cells are costly to maintain, requiring 10% fetal calf serum for growth and medium replenishment 6 days weekly. We are presently investigating whether the assay can be performed in 96-well culture dishes instead of the 8-well LabTek slides used in the studies reported here. Despite these drawbacks, this assay should prove useful in epidemiologic studies to determine the extent and importance of enterotoxigenic *B. fragilis* infections as a cause of human diarrheal disease and should enable basic work on toxin purification and mechanism of toxin(s) action to progress.

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REFERENCES

1. **Border, M., B. D. Firehammer, D. S. Shoop, and L. L. Myers.** 1985. Isolation of *Bacteroides fragilis* from the feces of diarrheic calves and lambs. J. Clin. Microbiol. **21:**472–473.
2. **Collins, J. E., M. E. Bergeland, L. L. Myers, and D. S. Shoop.** 1989. Exfoliating colitis associated with enterotoxigenic *Bacteroides fragilis* in a piglet. J. Vet. Diagn. Invest. **1:**349–351.
3. **Donta, S. T., H. W. Moon, and S. C. Whipp.** 1974. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. Science **183:**334–336.
4. **Dreyfus, L. A., J. C. Fratz, and D. C. Robertson.** 1983. Chemical properties of heat-stable enterotoxins produced by enterotoxigenic *Escherichia coli* of different host origins. Infect. Immun. **42:**539–548.
5. **Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman.** 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **10:**320–327.
6. **Guerrant, R. L., L. V. Kirchhoff, D. S. Shields, M. K. Nations, J. Leslie, M. A. De Sousa, J. G. Araujo, L. L. Correia, K. T. Sauer, K. E. McClelland, F. L. Trowbridge, and J. M. Hughes.** 1983. Prospective study of diarrheal illnesses in northeastern Brazil: patterns of disease, nutritional impact, etiologies, and risk factors. J. Infect. Dis. **148:**986–997.
7. **Huet, C., C. Sahuquillo-Merino, E. Coudrier, and D. J. Louvard.** 1987. Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. J. Cell Biol. **105:**345–357.
8. **Huott, P. A., W. Liu, J. A. McRoberts, R. A. Giannella, and K. Dharmsathaphorn.** 1988. Mechanism of action of *Escherichia coli* heat stable enterotoxin in a human colonic cell line. J. Clin. Invest. **82:**514–523.
9. **Keusch, G. T., A. Donohue-Rolfe, and M. Jacewicz.** 1982. *Shigella* toxin(s): description and role in diarrhea and dysentery. Pharmacol. Ther. **15:**403–438.
10. **Levine, S. A., M. Donowitz, A. J. M. Watson, G. W. G. Sharp, J. K. Crane, and C. S. Weikel.** 1991. Characterization of the synergistic interaction of *Escherichia coli* heat stable toxin and carbachol. Am. J. Physiol. **261:**G592–G601.
11. **Loosli, J., K. Gyr, H. Stalder, G. A. Stalder, W. Vischer, J. Voegtlin, M. Gasser, and B. Reichlin.** 1985. Etiology of acute infectious diarrhea in a highly industrialized area of Switzerland. Gastroenterology **88:**75–79.
12. **Montrose, M. H. (The Johns Hopkins University School of Medicine).** 1991. Personal communication.
13. **Montrose-Rafizadeh, C., W. B. Guggino, and M. H. Montrose.** 1991. Cellular differentiation regulates expression of cystic fibrosis transmembrane conductance regulator mRNA and Cl^- transport in human intestinal cells. J. Biol. Chem. **266:**4495–4499.
14. **Myers, L. L., B. D. Firehammer, D. S. Shoop, and M. M. Border.** 1984. *Bacteroides fragilis*: a possible cause of acute diarrheal disease in newborn lambs. Infect. Immun. **44:**241–244.
15. **Myers, L. L., and D. S. Shoop.** 1987. Association of enterotoxigenic *Bacteroides fragilis* with diarrheal disease in young pigs. Am. J. Vet. Res. **48:**774–775.
16. **Myers, L. L., D. S. Shoop, and T. D. Byars.** 1987. Diarrhea associated with enterotoxigenic *Bacteroides fragilis* in foals. Am. J. Vet. Res. **48:**1565–1567.
17. **Myers, L. L., D. S. Shoop, and J. E. Collins.** 1990. Rabbit model to evaluate enterovirulence of *Bacteroides fragilis*. J. Clin. Microbiol. **28:**1658–1660.
18. **Myers, L. L., D. S. Shoop, J. E. Collins, and W. C. Bradbury.** 1989. Diarrheal disease caused by enterotoxigenic *Bacteroides fragilis* in infant rabbits. J. Clin. Microbiol. **27:**2025–2030.
19. **Myers, L. L., D. S. Shoop, B. D. Firehammer, and M. M. Border.** 1985. Association of enterotoxigenic *Bacteroides fragilis* with diarrheal disease in calves. J. Infect. Dis. **152:**1344–1347.
20. **Myers, L. L., D. S. Shoop, L. L. Stackhouse, F. S. Newman, R. J. Flaherty, G. W. Letson, and R. B. Sack.** 1987. Isolation of enterotoxigenic *Bacteroides fragilis* from humans with diarrhea. J. Clin. Microbiol. **25:**2330–2333.
21. **O'Brien, A. D., and R. K. Holmes.** 1987. Shiga and Shiga-like toxins. Microbiol. Rev. **51:**206–220.
22. **Sack, R. B., L. L. Myers, J. Almeido-Hill, D. S. Shoop, W. C. Bradbury, R. Ried, and M. Santosham.** Submitted for publication.
23. **Synder, J. D., and M. H. Merson.** 1982. The magnitude of the global problem of acute diarrhea disease: a review of active surveillance data. Bull. W.H.O. **60:**605–613.
24. **Taylor, N. S., G. M. Thorne, and J. G. Bartlett.** 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect. Immun. **34:**1036–1043.
25. **Walsh, J. A., and K. S. Warren.** 1979. Selective primary health care: an interim strategy for disease control in developing countries. N. Engl. J. Med. **301:**967–974.
26. **Watson, A. J. M., S. Levine, M. Donowitz, and M. H. Montrose.** 1991. Kinetics and regulation of a polarized Na^+/H^+ exchanger from Caco-2 cells, a human intestinal cell line. Am. J. Physiol. **261:**G229–G238.
27. **Weikel, C. S., F. D. Grieco, and L. L. Myers.** 1991. Production and partial purification of a *Bacteroides fragilis* enterotoxin. Program Abstr. 27th Joint Conf. Cholera Relat. Dis., p. 25–28.
28. **Weikel, C. S., C. L. Spann, C. P. Chambers, J. K. Crane, J. Linden, and E. W. Hewlett.** 1990. Phorbol esters enhance the cyclic GMP response of T84 cells to the heat-stable enterotoxin of *Escherichia coli* (STa). Infect. Immun. **58:**1402–1407.