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Human Intestinal Epithelial Cells Swell and Demonstrate Actin Rearrangement in Response to the Metalloprotease Toxin of *Bacteroides fragilis*

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Enterotoxigenic *Bacteroides fragilis* (ETBF) cells produce a 20-kDa heat-labile metalloprotease toxin which is potentially important in the pathogenesis of diarrhea associated with this infection. Previous studies indicate that subconfluent HT29/C1 cells treated with the *B. fragilis* toxin (BFT) develop morphologic changes with dissolution of tight clusters and apparent swelling. Such alterations suggest toxin-stimulated reorganization of the cellular cytoskeleton. The purpose of the current study was to evaluate the effect of BFT on actin microfilaments (F-actin) and cell volume. As assessed by fluorescent phallicidin staining which detects F-actin, BFT treatment of HT29/C1 cells resulted in redistribution of F-actin with loss of stress fibers, a floccular staining pattern, and cellular membrane blebbing without quantitative changes in F-actin fluorescence intensity. The F-actin redistribution was time and concentration dependent. In contrast to the cell shrinkage observed in response to the F-actin-depolymerizing agents cytochalasin D and *Clostridium difficile* toxin A, BFT stimulated an increase in HT29/C1 cell volume of 10 to 25% (compared with control cells) over a 24-h time course. Only 10 to 30 ng of BFT per ml was necessary to stimulate a maximal increase in HT29/C1 cell volume. The effect of BFT on cell volume was persistent and dependent on the proteolytic activity of BFT. In agreement with cell viability assays indicating that BFT did not injure HT29/C1 cells, intoxicated cells exhibited regulatory volume decrease, suggesting that toxin-treated cells remain physiologically dynamic. We conclude that BFT acts on the intestinal epithelial cell cytoskeleton to alter F-actin structure and to stimulate an increase in HT29/C1 cell volume. Although these two activities of BFT appear to be linked, the precise sequence of cellular events following intoxication of HT29/C1 cells with BFT remains unclear. We hypothesize that these F-actin and cell volume changes may lead to an alteration in tight junction function in the polarized intestinal epithelium, contributing to the pathogenesis of diarrhea in ETBF infections.

Enterotoxigenic *Bacteroides fragilis* (ETBF) strains have been associated with diarrheal illnesses in livestock and young children, with the predominant site of histopathology being the colon in animals (34, 35, 37, 39). Cell-free culture supernatants of this enteropathogen induced a secretory response in ligated ileal loops of lambs and calves, and the secretory activity was attributable to an ~20-kDa heat-labile protein (termed *B. fragilis* toxin [BFT]) (26, 29). In 1992, Weikel et al. developed an in vitro assay to detect BFT using the cloned human colonic epithelial cell line HT29/C1 (45). Distinct morphologic changes including loss of cell-to-cell attachments, rounding, apparent swelling, and, in some cases, pyknosis were observed in subconfluent cells treated with concentrated bacterium-free culture supernatants of ETBF strains. Purification of an ~20-kDa heat-labile protein toxin from culture supernatants of an ETBF strain indicated that HT29/C1 cellular changes were inducible with as little as 1 ng of purified BFT per ml (42). Subsequently, purified BFT (doses of ≥1 μg) was reported to stimulate secretion in rat, rabbit, and lamb ligated small and large intestinal segments (30).

The structural organization of epithelial cells is dependent on an ordered array of cytoskeletal filaments. Furthermore, dynamic changes in filamentous actin (F-actin) have been associated with the regulation of intestinal epithelial cell secretory responses (18–20, 40). Thus, the striking morphologic changes in HT29/C1 cells induced by BFT plus the observation that BFT cleaves monomeric (G) actin in vitro (23) suggest that this toxin alters the epithelial cell cytoskeleton by affecting, directly or indirectly, a cytoskeletal protein and that this effect might alter intestinal epithelial cell function.

The purpose of the present study was to evaluate the effect of BFT on F-actin and HT29/C1 cell volume. Our results show that BFT alters F-actin structure in a time- and concentration-dependent manner and increases cell volume in HT29/C1 cells without causing cell injury. In polarized intestinal epithelial cells, we postulate that these cellular changes may lead to perturbation of tight junction integrity and contribute to the pathogenesis of diarrhea in ETBF infections.

MATERIALS AND METHODS

Cell culture. HT29/C1 cells were obtained from Daniel Louvard (Institut Pasteur, Paris, France) and used at 20 to 32 passages after cloning from the parent HT29 line (13). The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 44 mM Na^+-bicarbonate, and 10 μg of human transferrin (Sigma, St. Louis, Mo.), 50 IU of streptomycin, and 50 μg of penicillin per ml. Cells were grown at 37°C in humidified 10% CO_2–90% air.

BFT preparation. BFT was purified from VPI strain 13784 as previously described by Van Tassell et al. (42). Chelated BFT (to remove zinc from the active site of BFT) was prepared by treatment with 25 mM Na_4EDTA for 1 h, with subsequent dialysis (10 mM phosphate-buffered saline [PBS]; pH 7.4) to remove the excess EDTA (30). Comparison of the activity of BFT to chelated BFT by measurement of actin hydrolysis as previously described (23) indicated that this proteolytic activity of BFT was inhibited by >99% by chelation. By the HT29/C1 cell assay (25, 45), BFT was inactive at a concentration of 0.1 ng/ml, approximately 50% active at 1 ng/ml (i.e., causing morphologic changes in 50% of cells), and fully active (i.e., causing morphologic changes in 100% of cells) at

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10 ng/ml; chelated BFT was inactive at 1 ng/ml, approximately 50% active at 10 ng/ml, and fully active at 100 ng/ml.

F-actin staining. For time course experiments, subconfluent cells grown on plastic slides were incubated with 1 μg of BFT per ml for time points varying between 0.5 and 48 h. The cells were then washed in PBS, fixed in 3.7% formaldehyde for 10 min, and permeabilized with 0.5% Triton X-100 for 5 min. After air drying, rhodamine-phalloidin (Molecular Probes, Eugene, Oreg.) was applied to the slides for 30 min at room temperature in the dark. Rhodamine-phalloidin was removed by washing twice with PBS and once with double deionized water. Slides were mounted with a coverslip and examined for F-actin staining with a Zeiss microscope or by confocal microscopy.

For F-actin studies investigating concentration dependency, subconfluent cells grown on plastic slides were incubated with various concentrations of BFT for 24 h and then processed as described above.

For studies utilizing the fluorescent compound NBD [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]-phallicidin (Molecular Probes), subconfluent cells were incubated with 0.5 μM NBD-phallicidin for 3 or 6 h prior to preparation for cell volume measurements (see below) and/or examination by fluorescence microscopy.

Cell volume measurements. For cell volume experiments, subconfluent cells in plastic flasks were incubated with various concentrations of BFT for times of 1 to 24 h. The cells were then washed twice with Hanks' buffered salt solution without Ca^{2+} or Mg^{2+} (GIBCO, Gaithersburg, Md.) and incubated with Hanks' buffered salt solution containing 0.005% trypsin for 5 min in humidified 10% CO_2–90% air at 37°C. The flask was hit to deliver a lateral shearing force to release the cells from the plastic. Routinely, 5 to 10 ml of sodium medium containing 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 0.83 mM Na_2HPO_4, 0.17 mM NaH_2PO_4, 25 mM mannose (titrated to pH 7.4 with NaOH), 0.7 mg of ovomucoid trypsin inhibitor (Sigma), and 1 mg of bovine serum albumin (Sigma) per ml was immediately poured into the flask, and the cells were dispersed into a single cell suspension by gentle repeated pipetting. The cells were maintained in suspension by gently mixing in air at 37°C. The volume of suspended HT29/C1 cells was measured with a Coulter Counter (model ZM; Coulter, Hialea, Fla.) coupled to a computerized (CompuAdd 286/12; Austin, Tex.) pulse-height analyzer (Tennelec/The Nucleus, Oak Ridge, Tenn.). The measuring chamber of the Coulter Counter was maintained at 37°C with a feedback-controlled temperature controller (Digi-sense model 2186-20; Cole-Parmer) driving a hair dryer (Excellence 1500; Windmere Products). For measurement of cell volume, 1 ml of cell suspension was injected into 10 ml of Na^+ medium. Cell volume was measured immediately after trypsinization and 60 min later to allow for recovery from swelling induced by the effect of trypsin on HT29/C1 cells (33). Because cell volume measurements immediately after trypsinization and 1 h later revealed similar differences between control and BFT-treated cells, only the cell volume measurements 1 h posttrypsinization are presented. Each cell volume measurement represents averaged data from 10,000 to 40,000 cells collected over 8 s. By using histograms of cell number versus single cell volume, the computer automatically determined the centroid of the single cell volume distribution in femtoliters (10^{-15} liters [equivalent to cubic micrometers]). The instrument was calibrated with latex beads of known volume.

To increase the osmolality of the Na^+ medium from 310 to 400 mosM, tetramethylammonium chloride (TMACl), HEPES, and polyethylene glycol were added in appropriate amounts, and the osmolality of the medium was confirmed by an osmometer. These agents test for a change in membrane permeability to molecules with molecular weights of 75 (TMACl), 238 (HEPES), and approximately 3350 (polyethylene glycol).

Cell viability assays. (i) LDH. HT29/C1 cells grown for 3 to 5 days in 35-mm plastic dishes were treated with BFT (100 ng/ml or 1 μg/ml) for 18 h. After this incubation, the medium from the cells was removed and stored frozen at −20°C, until lactate dehydrogenase (LDH) activity was measured by spectrophotometric analysis (10). In each experiment, after removal of the tissue culture medium from untreated and/or experimental cells, total cellular LDH activity was measured in 0.1% Triton X-100 cellular extracts. The results shown are percentages of total LDH activity (micromoles per milliliter per minute) released.

(ii) Protein synthesis assay. Unless otherwise stated, subconfluent HT29/C1 cells grown for 3 to 5 days in 35-mm plastic dishes were treated with BFT (1 μg/ml) for 18 h in tissue culture medium supplemented with 1 μCi of [^{3}H]leucine per ml. After 18 h, the medium was removed, the cells were washed three times, and the amount of radioactivity present in 10% trichloroacetic acid cellular precipitates was determined by scintillation counting after solubilization with 2 N NaOH (44).

Other materials. Cytochalasin D was obtained from Sigma, and *Clostridium difficile* toxin A was purified as previously described (31) (gift of C. Pothoulakis).

Statistics. Statistical comparisons were made by the paired and unpaired Student *t* test. Results are means ± standard errors of the means.

RESULTS

Time course of effect of BFT on F-actin structure in HT29/C1 cells. In initial experiments, the effect of 1 μg of BFT per ml on F-actin structure in HT29/C1 cells was assessed after 0.5, 1, 2, 3, 6, 24, or 48 h of exposure ($n = 6$ for each time point). Figure 1A shows rhodamine-phalloidin staining of F-actin of untreated subconfluent HT29/C1 cells. These control cells show uniform, smooth-edged clusters and organized, linear-stress F-actin fibers. In contrast, cells treated with BFT reveal distinct separation from each other with membrane blebbing, loss of stress fibers with a floccular pattern of F-actin staining, and an increased cell globular appearance. These changes were noted by as early as 60 min, with the maximal effect evident by 24 h (Fig. 1B and C). These morphologic changes noted by fluorescence microscopy were compared with observations made via bright-field microscopy at similar time points with BFT-treated HT29/C1 cells following fixation and Giemsa staining (25, 45). By this technique, alterations in cell morphology were detected by 30 min, with a nearly maximal effect by 3 h, indicating that morphologic changes were appreciated earlier with Giemsa than with rhodamine-phalloidin staining (data not shown).

To further analyze the appearance of HT29/C1 cells after treatment with BFT, the cells were examined by confocal microscopy (data not shown). At the apical aspect of the cells, a loss of the peripheral localization of F-actin was observed in the BFT-treated cells compared with the control cells; at the basolateral pole of the cells, a floccular pattern of staining with loss of stress fibers after BFT treatment was confirmed. Of note, the position of the cellular nuclei shifted from the apical to the basal pole of the cells following BFT exposure.

Concentration dependency of the effect of BFT on F-actin structure in HT29/C1 cells. To assess the concentration dependency of the toxin effect on F-actin structure, subconfluent HT29/C1 cells were treated with 1, 10, or 100 ng or 1 or 3 μg of toxin per ml for 24 h and then processed in parallel for F-actin and Giemsa staining ($n = 3$ for each concentration). Reorganization of F-actin was noted by rhodamine-phalloidin staining initially at a concentration of 10 ng/ml, with a maximal change evident at a concentration of 1 μg/ml. By Giemsa staining, readily identifiable morphologic changes were noted with a 1 ng/ml concentration, with a maximal effect at 100 ng/ml (data not shown).

The effect of BFT on HT29/C1 cell volume. Earlier observations in our laboratory with Nomarski optics suggested that HT29/C1 cells swell in response to BFT (45). Figure 2 shows the time course of the BFT-stimulated increase in HT29/C1 cell volume. Cell volume increased 10 to 25% in BFT-treated cells compared with parallel untreated controls at each time point. The less-prominent increase in cell volume induced by BFT at the 3- and 6-h time points was attributed to an underlying increase in control population cell volumes secondary to nutrient uptake occurring after the addition of fresh medium to the cells at the beginning of the experiment. This nutrient response was not observed in toxin-treated cells (data not shown). By 18 to 24 h, control cell volumes stabilized and the percent increase in cell volume of toxin-treated cells versus controls was once again greater than 20%.

The reversibility of the BFT-induced increase in cell volume was examined. Subconfluent HT29/C1 cells were treated with 1 μg of BFT per ml for 18 h, with cell volume measurements made immediately as well as 1, 2, and 3 h following trypsinization. The BFT-induced volume increase was at least partially reversible, since volume measurements at each time point after the initial assessment showed values for the magnitude of regulatory volume decrease (16) that were similar in control and BFT-treated cells. For example, in one experiment, cell volumes immediately after trypsinization were 1,121 ± 5 and 1,450 ± 4 fl per cell in control and BFT-treated cells, respectively ($P < 0.001$), and the cell volumes 3 h after trypsinization

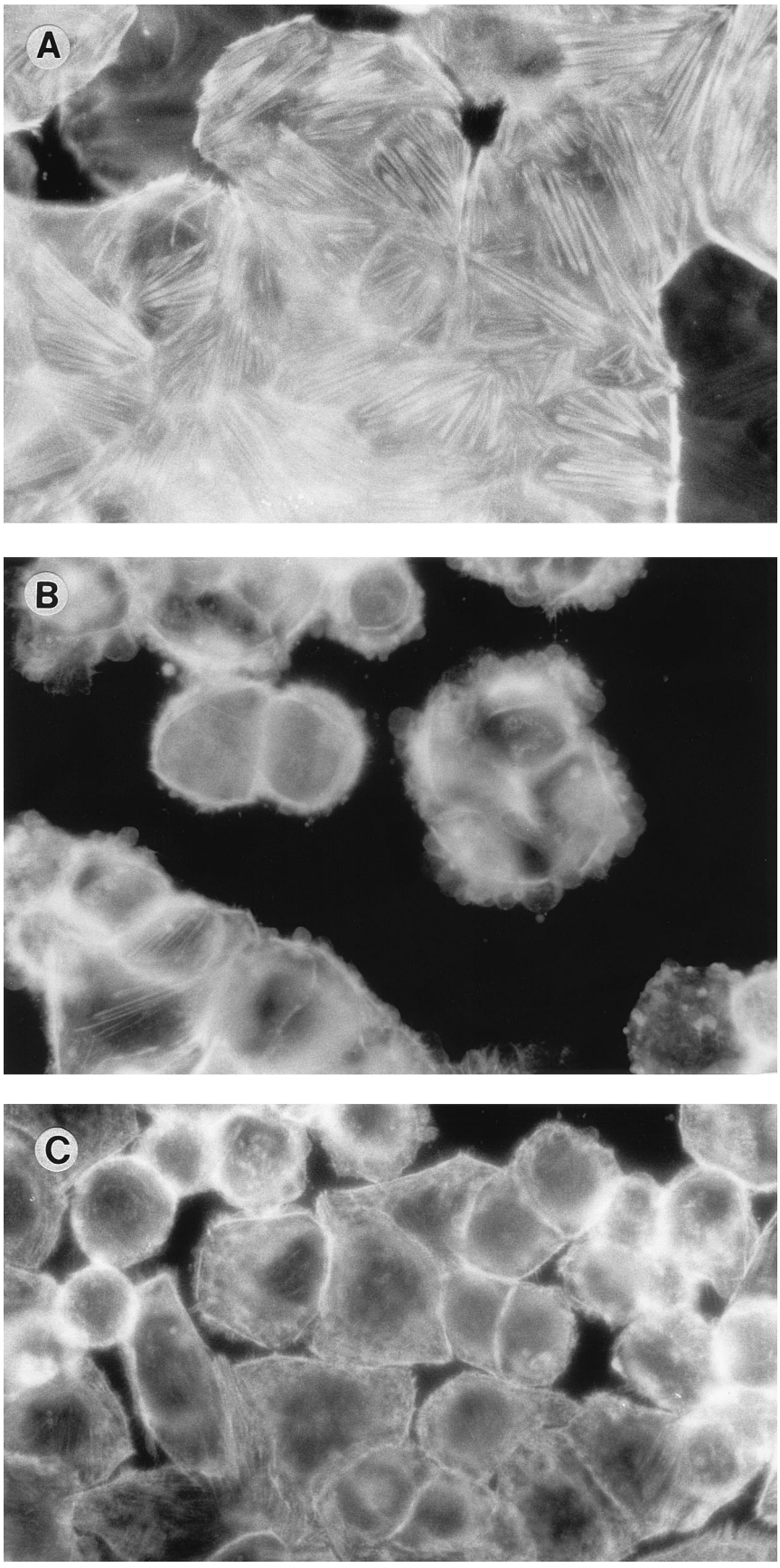

FIG. 1. Effect of BFT on F-actin structure in subconfluent HT29/C1 cells. Cells were stained with rhodamine-phalloidin and photographed at a magnification of ×100. (A) Untreated control cells; (B) cells treated for 2 h with 1 μg of BFT per ml; (C) cells treated for 24 h with 1 μg of BFT per ml. Control cells in smooth-edged clusters with organized stress fibers contrast with BFT-treated cells, which show membrane blebbing, loss of stress fibers, and a floccular pattern of F-actin staining.

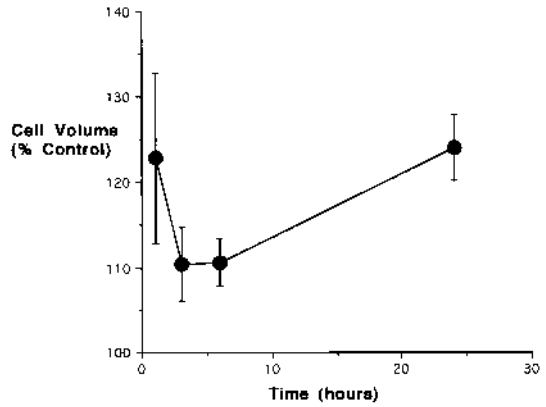

FIG. 2. Time course of the BFT-stimulated increase in HT29/C1 cell volume. Subconfluent HT29/C1 cells were treated with BFT (1 μg/ml) for 1 to 24 h prior to measurement of cell volume. Cell volume is increased 10 to 25% in BTF-treated cells compared with time-matched controls (mean change in cell volume at 1, 3, 6, and 24 h [BFT-treated cell volume minus control cell volume], 235 ± 94, 149 ± 64, 144 ± 31, and 301 ± 56 fl, respectively; $P < 0.001$ by comparison of BFT-treated and control cell volumes for each time point; $n = 5$ experiments).

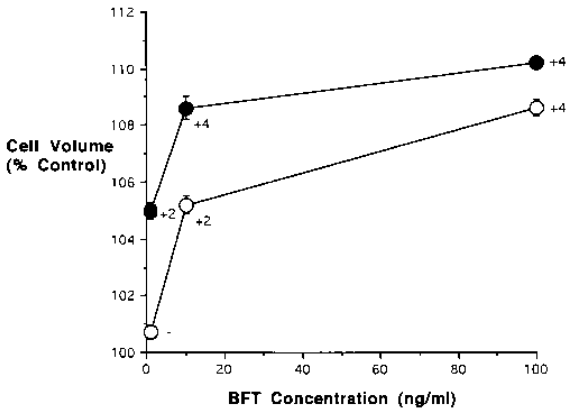

FIG. 3. Concentration dependency and effect of chelated BFT on HT29/C1 cell volume. HT29/C1 cells were incubated with BFT (closed circles) or chelated BFT (open circles) in parallel for 4 h prior to cell volume measurements ($n = 3$). An increase in cell volume after BFT treatment was detectable initially at a toxin concentration of 1 ng/ml ($P < 0.001$) and maximally at a concentration of 10 ng/ml. The cell volume increase stimulated by chelated BFT was significantly less at each concentration of toxin examined ($P < 0.005$). −, no change in HT29/C1 cell morphology observed by phase or light microscopy after BFT treatment; +4, morphology of 100% of the cells altered.

were 990 ± 2 and 1,256 ± 4 fl per cell in control and BFT-treated cells, respectively ($P < 0.001$ for control versus BFT-treated cells; $P < 0.001$ for early versus 3-h cell volume measurements for control or BFT-treated cells).

The concentration dependence of the cell volume increase after BFT treatment of HT29/C1 cells was examined at 4 and 18 h. At 4 h (Fig. 3), 1 ng of BFT per ml stimulated a significant increase in cell volume, with a maximal effect evident by 10 ng/ml. At 18 h (data not shown), cell volume increases were not evident at a concentration of 1 ng of BFT per ml and were maximal at 30 ng/ml, with a half-maximal effective concentration of 10 ng/ml.

To determine whether the proteolytic activity described for BFT (23) was required to stimulate the increase in cell volume, the effects of various concentrations of chelated BFT on HT29/C1 cell volume were compared in parallel with unchelated BFT after a 4-h incubation (Fig. 3). Chelated BFT stimulated significantly smaller increases in cell volume at every concentration evaluated. When compared by either light or phase microscopy, concentrations of BFT which stimulated a measurable increase in cell volume were associated with detectable visual differences in cell morphology. For example, both the morphologic changes and cell volume increases stimulated by 10 ng of chelated BFT per ml were approximately equal to those measured with 1 ng of unchelated BFT per ml, indicating that chelation of BFT inhibited the cell volume increase by 90%.

To determine if the increase in cell volume was due to membrane damage with possible pore formation, the osmolality of the Na^+ medium was increased as described in Materials and Methods. An increase in the osmolality of the bathing media should induce regulatory volume decrease in the BFT-treated cells if they remain physiologically responsive and have intact membrane transporters. Alternatively, if a pore permissive to the osmolyte has formed, the cells should swell further. At 10 min following exposure to 400 mosM Na^+ medium containing either TMACl, HEPES, or polyethylene glycol, BFT-treated cells shrank (indicating intracellular osmolyte or salt loss) in a manner identical to that of the control cells. For example, in Na^+ medium, the volumes of control and BFT-treated cells were 1,118 ± 13 and 1,212 ± 1 fl per cell, respectively, and in TMACl medium, the volumes of control and BFT-treated cells were 970 ± 5 and 1,045 ± 4 fl per cell, respectively ($P < 0.001$; control or BFT versus TMA-control or TMA-BFT, respectively). These results were identical 45 and 90 min after exposure to medium containing the osmolytes (data not shown). However, at all time points examined, BFT-treated cells remained swollen to the same degree compared with control cells, in agreement with a persistent toxin effect on cell volume ($P < 0.001$; TMA-control versus TMA-BFT).

Examination of the relationship of cell volume and F-actin. Because BFT has been reported to cleave monomeric (G) actin in vitro (23), suggesting that BFT may hydrolyze F-actin in cells, two approaches were utilized to examine whether changes in F-actin might be linked to the increase in cell volume. First, the effect of actin depolymerization on cell volume was examined by treatment of the cells with cytochalasin D (5 μg/ml) or *C. difficile* toxin A (1 μg/ml). Cytochalasin D terminates actin polymerization by inhibiting the addition of monomeric (G) actin molecules to the barbed ends of F-actin filaments (17), and *C. difficile* toxin A has been reported to monoglucosylate Rho proteins in nonintestinal epithelial cell lines, causing depolymerization of F-actin (14). Rho proteins are small GTP-binding proteins involved in the regulation of actin filament assembly. In contrast to the results with BFT, treatment of HT29/C1 cells with either cytochalasin D ($n = 4$) or *C. difficile* toxin A ($n = 3$) for 3 h resulted in morphologic changes in HT29/C1 cells, characterized by cell surface spicule formation (reference 44 and data not shown) and cell shrinkage (1,226 ± 68 versus 1,110 ± 45 fl per cell, control versus cytochalasin D [$P \leq 0.02$]; 1,184 ± 68 versus 1,041 ± 39 fl per cell, control versus *C. difficile* toxin A [$P \leq 0.04$]).

Second, HT29/C1 cells were loaded with NBD-phallicidin to stabilize F-actin prior to treatment with BFT. NBD-phallicidin has been reported to diminish cyclic AMP-stimulated chloride secretion in monolayers of intestinal epithelial cells (T84) by stabilizing F-actin and inhibiting the F-actin-regulated Na/K/2Cl cotransporter (essential to transepithelial chloride secretion) (18–20, 40). Despite comparable loading of both control and BFT-treated cells with NBD-phallicidin at both time points examined, NBD-phallicidin did not inhibit the cell volume increase (1,102 ± 2 versus 1,188 ± 2 fl per cell, control

TABLE 1. Effect of BFT on HT29/C1 cell viability

Treatment group	% of total LDH released[a]	18-h [^{3}H]leucine uptake[b]	3-h post-BFT [^{3}H]leucine uptake[b]
Control	4.1 ± 0.7	126,718 ± 18,208	13,152 ± 847
BFT[c]			
100 ng/ml	7.4 ± 3[d]		
1 μg/ml	11.5 ± 2.1[e]	164,306 ± 22,213[e]	24,068 ± 633[e]

[a] For a BFT concentration of 100 ng/ml, four experiments performed in duplicate; for a BFT concentration of 1 μg/ml, three experiments, with two to eight replicates per experiment. Results are means ± standard errors of the means.
[b] Two to three experiments performed in duplicate or triplicate. Results are mean counts per minute per dish (± standard errors of the means).
[c] HT29/C1 cells were treated with BFT for 18 h with the indicated toxin concentrations.
[d] Not significant versus paired controls.
[e] $P < 0.001$ (control versus BFT conditions).

versus BFT-treated cells; 1,099 ± 2 versus 1,207 ± 4 fl per cell, control versus BFT-treated cells loaded with NBD-phallicidin) or the morphologic changes (data not shown) stimulated by BFT. By confocal microscopy with constant detector gain and imaging settings, no difference in the average whole-cell F-actin fluorescence intensity was identified when control and BFT-treated cells loaded with NBD-phallicidin were compared (84 ± 11 versus 80 ± 3 arbitrary fluorescence units, control versus BFT-treated cells).

The effect of ETBF toxin on HT29/C1 cell viability. Cell viability in subconfluent HT29/C1 cells treated with BFT was assessed by analyzing intracellular LDH release and the ability of the cells to synthesize new proteins (Table 1). In paired experiments, no increase in the release of intracellular LDH was detected after treatment with BFT (100 ng/ml or 1 μg/ml) for 18 h. Of note, uptake and incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable proteins was significantly stimulated in cells treated for 18 h with 1 μg of BFT per ml compared with untreated controls. To further examine whether the cells treated with 1 μg of BFT per ml were viable after 18 h of treatment, [^{3}H]leucine uptake was measured for 3 h following cellular intoxication with BFT for 18 h. These results indicated that protein synthesis was stimulated in BFT-treated cells even after prolonged treatment with a high concentration of BFT.

DISCUSSION

ETBF is a recently recognized enteropathogen associated with secretory diarrhea in livestock and children. Biopsies of colonic and ileal tissue in animal models have revealed prominent changes in cell architecture primarily at the tips of villi which include cell rounding and detachment from neighboring cells (1, 5, 27, 28, 30). These morphologic cellular changes are similar to what is observed in HT29/C1 cells after toxin exposure (4, 25, 45). The results in this paper demonstrate that the toxin secreted by ETBF organisms induces HT29/C1 intestinal epithelial cell morphologic changes in a time- and concentration-dependent manner, at least in part by altering the cellular distribution of F-actin (Fig. 1 and 2). These results are consistent with the recent report that BFT is a metalloprotease and cleaves monomeric (G) actin in vitro (23). Changes in F-actin detected in the cloned HT29 cell line (HT29/C1) progressed over 24 h and persisted for as long as 48 h after exposure to BFT (Fig. 1 and data not shown). Alterations in the F-actin network of intestinal epithelial cells in response to enteric bacteria (e.g., *Salmonella* species, enteropathogenic *Escherichia coli*, *Shigella* spp., and *Listeria monocytogenes* [3, 15, 24, 36, 32, 43]) or toxins (e.g., toxins A and B of *C. difficile* [9, 10] and the heat-stable enterotoxin [STa] of *E. coli* [19]) is a rapidly evolving discipline in bacterial pathogenesis which was recently termed cellular microbiology (2). Thus, BFT represents another bacterial toxin potentially useful in discerning mechanisms controlling actin dynamics.

Our experiments also suggest that standard light microscopy detects morphologic changes earlier and at a lower toxin concentration than studies using rhodamine-phalloidin to stain F-actin. Although the biologic significance of this observation is unknown, these results do indicate the utility and sensitivity of light microscopic examination of HT29/C1 cells to identify the activity of the toxin produced by ETBF organisms. Donelli et al. (4) reported that uncloned HT29 cells treated with 15 ng of BFT per ml reverted to a normal phenotype after 24 h but did not report on the reversibility of BFT's action at higher concentrations. In our laboratory, additional time course experiments with light microscopy suggest that HT29/C1 cells treated with BFT at a concentration of 600 ng/ml or less regain nearly normal morphology over 72 h (data not shown). However, abnormal morphology persists in cells treated with higher concentrations of toxin. Given that the BFT preparations used in the studies reported by Donelli et al. and herein are from the same source (i.e., gifts of the laboratory of Tracy Wilkins), these data suggest that the effect of BFT on cell morphology is reversible at lower concentrations of BFT and that the epithelial subclone, HT29/C1, is more sensitive to BFT than the parental HT29 cell line.

Treatment of HT29/C1 cells with BFT also causes a concentration- and time-dependent increase in the cell volume of HT29/C1 cells. Our data suggest that low concentrations of BFT stimulate a relatively rapid onset of cell swelling, with a significant increase in cell volume detectable by 1 h of toxin exposure. Time points prior to 1 h (15, 30, or 45 min) did not show a consistent BFT-induced increase in cell volume (data not shown). The meaning of the slight shift to the left in the concentration dependency of BFT cell volume activity at 4 versus 18 h is not clear but is potentially consistent with early cell recovery. Although BFT stimulated a persistent increase in cell volume (at least 3 h in duration following extensive washing of the cells to remove BFT), this effect was at least partially reversible, with cells exhibiting a regulatory volume decrease similar to that of controls. Furthermore, BFT treatment did not lead to cellular injury (Table 1). These data indicate that BFT-treated cells remain in a dynamic state. The majority of the stimulated increase in cell volume is dependent on the proteolytic activity of BFT. However, chelation of BFT inhibits its ability to hydrolyze G actin by >99% in vitro, raising the possibility that a small portion of the biological activity of BFT is not sensitive to chelation. Restitution of the activity of BFT with exogenous zinc during the course of the experiments is not likely, because no zinc was present in the buffers used and previous attempts to directly reconstitute chelated BFT with 100 μM $ZnCl_2$ were only partially successful (30).

Although BFT may increase cell volume by specifically affecting one or more HT29/C1 ion transporters (e.g., via its proteolytic activity), an alternative hypothesis is that BFT may increase HT29/C1 cell volume by creating an ion channel in the cell as previously described, for example, for the *Staphylococcus aureus* delta toxin (22), the *Clostridium perfringens* enterotoxin (21, 41), and the adenylate cyclase toxin of *Bordetella pertussis* (12). Our data examining the permeability of BFT-treated HT29/C1 cells to osmotically active agents indicate that BFT does not create a pore in HT29/C1 cells, which can accommodate a molecule with a molecular weight of 75. Additional experiments will be necessary to identify if pore forma-

tion to smaller ions occurs. Of note, although it has been proposed that several enteric bacterial toxins may act by creation of an ion pore, this mechanism of action has not yet been linked to secretion by intestinal epithelial cells (38).

The relationship between F-actin and cell volume changes has been shown to be complex in other epithelia or cells with specific actin pools linked to different stages of cell volume regulation (7, 8). Thus, two crucial questions are (i) whether the increase in cell volume stimulated by BFT is mechanistically related to the observed changes in F-actin arrangement in HT29/C1 cells and (ii) what the order of onset of these cellular effects of BFT is. The data in this report do not resolve these questions. In our studies, treatment of HT29/C1 cells with cytochalasin D or *C. difficile* toxin A to pharmacologically dissociate the cellular F-actin structure caused cell shrinkage, not swelling. Although the proteolytic activity of BFT would also be expected to dissociate F-actin structure, we were unable to detect quantitative changes in F-actin measured using fluorescent phalloidin compounds, suggesting either that actin is not a substrate for BFT in vivo or that actin oligomers potentially resulting after BFT hydrolysis are of sufficient length to still bind phalloidin. Interestingly, treatment of HEp-2 cells for 24 h with *E. coli* cytotoxic necrotizing factor toxin 1 increases both F-actin rich structures and cell volume (6). Experiments utilizing NBD-phallicidin to stabilize F-actin also had no impact on the BFT-stimulated increase in cell volume. These results suggest either that (i) remodeling of microfilament actin is not linked to the volume increase stimulated by BFT or that (ii) NBD-phallicidin cannot stabilize F-actin to the activity of BFT. The latter possibility is suggested by the fact that the morphologic changes caused by BFT did not appear inhibited by NBD-phallicidin. One hypothesis to explain these data is that a localized pool of actin very sensitive to BFT and affected early in BFT cellular intoxication may be crucial to the volume change measured. This pool of F-actin may not be detectable by fluorescent phalloidin staining, in agreement with our results, in which higher concentrations of BFT were necessary to modify F-actin structure when it was assessed by this technique. This hypothesis is supported by recent observations suggesting specialized cellular functions for actin isoforms such as beta-actin, which is not detected by phalloidin staining (11).

In summary, these experiments demonstrate that BFT induces swelling and F-actin rearrangement in HT29/C1 human colonic epithelial cells without inducing cell injury. These activities are largely dependent on the proteolytic activity of BFT. We postulate that these biologic activities may contribute to the histopathology of the colon noted in experimental disease and to the secretory diarrhea noted in animals and humans infected with ETBF strains. Additional studies utilizing BFT and *C. difficile* toxin A which stimulate polar opposite effects on HT29/C1 cell volume are warranted to examine in detail the linkage of specific actin alterations and cell volume changes in intestinal epithelial cells. Characterization of the effects of BFT on the physiology of polarized intestinal epithelia in vivo and in vitro will be necessary to understand the importance of these observations to disease pathogenesis.

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