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Bacteroides fragilis Toxin Rapidly Intoxicates Human Intestinal Epithelial Cells (HT29/C_1) In Vitro

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Enterotoxigenic *Bacteroides fragilis* strains associated with childhood diarrhea produce a 20-kDa protein toxin (BFT). Purified BFT causes striking morphologic changes in subconfluent human colonic epithelial cells (HT29/C_1). In a 3-h HT29/C_1 cell assay, the estimated half-maximal effective concentration of BFT was 12.5 pM, and morphologic effects were detectable as early as 30 min and nearly complete by 1.5 h. Concentrations as low as 0.5 pM could also cause intoxication, but morphologic changes were detectable only when the assay was extended to 18 h. The onset of this intoxication was concentration dependent and rapid, occurring within minutes (<7 min at 0.25 nM, <2 min at 2.5 nM). Notably, the onset of intoxication at 37°C became irreversible to washing within 2 min after exposure to BFT. Morphologic changes were completely inhibited by treatment of HT29/C_1 cells with BFT at 4°C but could be demonstrated by subsequent warming to temperatures of 15°C or higher after washing. The time required for the association of BFT with HT29/C_1 cells at 4°C was inversely correlated with concentration. Inhibitors of endosomal and Golgi trafficking (NH_4Cl and brefeldin A) prevented the intoxication of HT29/C_1 cells by *Clostridium difficile* toxin A and cholera toxin, respectively, but not by BFT. Agents altering microtubule structure did not affect the cellular activity of BFT. These data indicate that a purified toxin from *B. fragilis* strains associated with diarrhea rapidly and irreversibly intoxicates human intestinal epithelial cells (HT29/C_1) in a concentration- and temperature-dependent manner and that the process of intoxication may not involve internalization mechanisms utilizing microtubules or sensitive to pH or brefeldin A.

Diarrheal diseases are among the leading causes of morbidity and mortality for young children worldwide. However, in prospective studies, an enteric pathogen is identified in only about 50% of cases (13, 48). Although *Bacteroides fragilis* accounts for only 1% or less of the obligate anaerobes in normal colonic flora, *B. fragilis* is the predominant anaerobic pathogen isolated in intra-abdominal abcesses and from bloodstream infections (24, 34, 35, 41). A novel association between certain strains of this organism and diarrheal disease was discovered in 1983 during an outbreak of diarrhea in lambs (37). After initial sporadic human case reports, three case-control studies have shown an association between these enterotoxigenic *B. fragilis* (ETBF) strains and diarrhea in young children (43–45). ETBF may therefore be a hitherto unrecognized cause of human diarrheal disease (46). Interestingly, *B. fragilis* and *Clostridium* spp. represent the only anaerobes described to date which produce toxins with intestinal secretory activity.

ETBF strains were initially distinguished by the ability of their culture filtrates, and, therefore, an extracellular enterotoxic product, to stimulate secretion in lamb ligated intestinal loops. The extracellular toxin produced by ETBF, termed *B. fragilis* toxin (BFT), is a heat-labile zinc metalloprotease protein of approximately 20 kDa that may reside as a protoxin within the bacterial cell wall (32, 52). BFT has been purified (51) and the complete chromosomal gene (termed *bft*) has been sequenced (11), but its cellular target or mechanism of action is as yet unknown. In addition to stimulating secretion in lamb ligated intestinal loops, ETBF or BFT alters the morphology of intestinal epithelial cells in lamb, rabbit, and rat loops (38) and intestinal cell cultures (6, 36, 53). Purified BFT hydrolyzes G-actin, among other substrates, in vitro (32) and has recently been reported to alter F-actin distribution in HT-29 and HT29/C_1 cell cultures (6, 22).

The purpose of this investigation was to characterize the cellular action of BFT by studying its effect on the morphology of human intestinal epithelial cells (HT29/C_1). Recently a simple, sensitive, and specific in vitro assay using subconfluent HT29/C_1 cells was developed to identify toxigenic *B. fragilis* strains (36, 53) by light microscopy. In this colonic epithelial cell line, BFT causes striking morphologic changes consisting of loss of cell-cell attachments, rounding, swelling, and, in some cases, pyknosis (6, 22, 53). We used this morphologic assay to determine the effects of concentration, time, temperature, pH, and inhibitors of intracellular trafficking on the intoxication of HT29/C_1 cells by purified BFT.

MATERIALS AND METHODS

Cell culture. HT29 cells, C_1 clone, were obtained from Daniel Louvard (Institut Pasteur, Paris, France) and grown as previously described (53). For experimental use, cells were suspended 1:10 or 1:15 by using 0.005% trypsin–0.053 mM EDTA, plated onto 35-mm-diameter covered dishes or eight-well LabTek slides, and allowed to grow for 3 to 4 days until approximately 80 to 90% confluent.

Toxin purification. Toxin was purified from culture supernatant broth of enterotoxigenic *B. fragilis* 86-5443-2-2 as previously described (51). Analytical reverse-phase fast protein liquid chromatography (Pharmacia Inc., Piscataway, N.J.) was performed on the purified toxin preparation to assess the purity of the sample. Approximately 80 μg of BFT was injected onto a PepRPC reverse-phase column equilibrated with water–0.05% trifluoroacetic acid. Protein was eluted with a 0 to 100% gradient of acetonitrile–0.05% trifluoroacetic acid over 2 h. Eluted protein was detected by UV absorption at 226 nm. BFT eluted as a single sharp peak at approximately 41% acetonitrile. Purified BFT was maintained at −70°C in a 0.05 M Tris–0.18 M NaCl buffer (pH 7.5) until use. All experiments described were performed with a single batch of purified BFT.

HT29/C_1 cell assay. The HT29/C_1 cell assay was performed as described by Weikel et al. (53), with the following modifications. Because previous experi-

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ments in this laboratory had determined that fetal bovine serum attenuates toxin activity (3, 36), the cells were washed twice with Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (GibcoBRL, Grand Island, N.Y.) and replenished with 1 ml of the cell culture medium containing 0.1% bovine serum albumin (BSA; Sigma, St. Louis, Mo.) instead of fetal bovine serum on the day of experimental use. Stocks of purified BFT were thawed on ice and either added directly to the tissue culture medium or prediluted in cell medium containing 0.1% BSA to the desired concentration prior to addition to the cells. The cells were incubated with BFT for various times up to 3 h at 37°C in 10% CO_2 unless otherwise stated. A 3-h incubation was chosen because previous work had shown that this was the time point of maximum sensitivity of the HT29/C_1 cell assay for detecting BFT activity in culture supernatants of ETBF strains (53). Untreated controls were performed on each experiment day and handled exactly as the experimental cells. After the incubation period, the medium was aspirated and the cells were fixed with 90% methanol, dried in room air, stained with Giemsa (Sigma), and examined at a magnification of ×100 with standard bright-field light microscopy.

Toxin activity (defined as altered HT29/C_1 morphology, including cell rounding and cluster dissolution) was scored in blinded fashion, using a scale modified from previous work (53). A score of 4+ indicated rounding of 100% of cells in all clusters; 3+ indicated rounding in all clusters but sparing the central portion of large confluent areas; 2+ indicated rounding of only 50% of cells in each cluster; and 1+ indicated rounding of only the peripheral cells of clusters.

For time-of-onset experiments, at specified intervals after addition to cells (5 s to 30 min), the cell medium with or without BFT was aspirated, and the cells were washed twice with 37°C HBSS and replenished with fresh 37°C cell culture medium containing 0.1% BSA prior to further incubation in 10% CO_2 for 3 h unless stated otherwise. For periods of exposure of less than 15 min, the cells were kept on a 37°C warmer in room air.

For temperature experiments, the cell cultures were preincubated at 4, 15, 22, and 37°C for 30 to 60 min prior to BFT addition for 3 subsequent h at the same temperature. Temperature shift experiments were conducted by preincubating cells at 4°C and then adding cold, prediluted BFT for periods of 2 min to 3 h. The cells were then washed twice with ice-cold HBSS prior to incubation in fresh medium at 37°C for 3 h. No additional buffers were added to the medium. The pH of the medium ranged from 8.32 at 4°C to 8.03 at 37°C. Preliminary experiments had shown no change in toxin activity over a pH range of 5 to 9 at 22°C and 6 to 8 at 37°C.

For inhibitor experiments, the cell cultures were preincubated for 30 to 60 min at 37°C in culture medium containing the inhibitory agent being tested: 30 mM NH_4Cl, 7.1 to 71 μM brefeldin A (2 to 20 μg/ml), or 0.1 mM chloroquine (all from Sigma). The medium was then replaced with medium containing 0.25 nM (5 ng/ml) BFT, 3.2 nM (1 μg/ml) *Clostridium difficile* toxin A (generously provided by C. Pothoulakis), or 11.7 nM (1 μg/ml) cholera toxin (Sigma) in addition to the inhibitor. The cells were fixed and stained after the specified intervals. Controls included cells exposed to culture medium containing inhibitor alone, each toxin alone, or neither. In further time-of-onset experiments, the medium containing inhibitor plus 0.25 nM BFT was aspirated at specified intervals (1 to 15 min), and the cells were washed twice with 2 ml of HBSS, replenished with fresh culture medium with or without the same inhibitor, incubated for 3 h at 37°C, fixed, and stained. A positive control within each experiment was replenished with medium containing BFT alone.

To evaluate the effect of microtubule stabilizers (taxol, 1 to 10 μM [Calbiochem, La Jolla, Calif.]) and destabilizers (nocodazole, 1 μM [Aldrich, Milwaukee, Wis.] and colchicine and vinblastine, 1 to 10 μM [both from Sigma]) on the activity of BFT, HT29/C_1 cells were incubated with these drugs for 2 h in serum-free medium followed by addition of BFT (1 or 10 ng/ml) for 3 h at 37°C prior to fixation and staining.

For pH experiments, HT29 medium containing 0.1% BSA without $NaHCO_3$ buffer was supplemented with three buffers: glycylglycine, morpholine propanesulfonic acid (MES) (both from Research Organics, Cleveland, Ohio), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Sigma) (15 mM each). Aliquots of this buffered medium were then pH adjusted to 5, 6, 7, 8, or 9 and filter sterilized. The HT29/C_1 assay was then conducted as described above.

In each experiment, variables were assessed in duplicate or triplicate and data are presented as the average score for each group of replicates. Each experiment was repeated 2 to 10 times.

RESULTS

Concentration dependency and time course of BFT effects on HT29/C_1 cells. BFT intoxicated and altered the morphology of HT29/C_1 cells in a concentration-dependent manner (Fig. 1). After a 3-h incubation, purified BFT consistently elicited a 4+ change in HT29/C_1 morphology (i.e., affected 100% of cells) at concentrations above 0.1 nM (2 ng/ml). The effects of BFT concentrations up to 25-fold higher than 0.1 nM were indistinguishable morphologically at 3 h suggesting that 0.1 nM was the maximal effective concentration for morphologic changes detectable by light microscopy. The estimated half-maximal effective concentration (EC_{50}) was approximately 12.5 pM. However, at very low concentrations of BFT, morphologic changes appeared to depend on the length of incubation. For example, incubation of cells with 0.5 pM or 2.5 pM elicited 2+ changes after prolonging the incubation of the standard HT29/C_1 assay to 18 h (data not shown). Based on these results, subsequent experiments were done with BFT at a concentration of 0.25 nM unless otherwise stated.

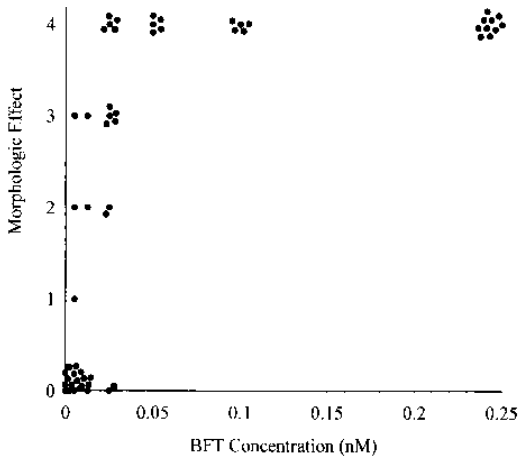

FIG. 1. Effects of different concentrations of BFT on HT29/C_1 cells. Subconfluent HT29/C_1 cells were treated with various concentrations of BFT for 3 h at 37°C. The morphologic effect of BFT on the cells was assessed semiquantitatively (1+ to 4+) as described in Materials and Methods. These data indicate that 0.1 nM (2 ng/ml) consistently alters the morphology of 100% of cells (4+ effect). The approximate EC_{50} of BFT on HT29/C_1 cells at 3 h is 12.5 pM. Each point represents one observation; data from nine experiments were pooled.

To determine the minimum incubation time required to detect morphologic changes in the HT29/C_1 assay, the cells were incubated with 0.25 nM BFT for intervals of up to 3 h (Fig. 2). Cell rounding and swelling, involving almost 50% of

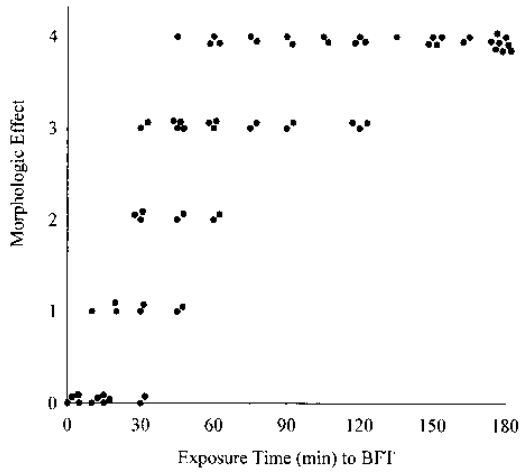

FIG. 2. Minimum incubation time required to detect BFT effects on HT29/C_1 cells. Subconfluent HT29/C_1 cells were treated with a fixed concentration of BFT (0.25 nM) for various incubation times at 37°C. Morphologic changes were initially detectable by 30 min of incubation (1+ effect), and 100% of cells (4+ effect) were affected in three of four experiments by 1.5 h of incubation. A 12-fold increase in BFT concentration to 3 nM decreased the time to detection of 4+ changes to 30 min (data not shown). Each point represents one observation; data from four experiments were pooled.

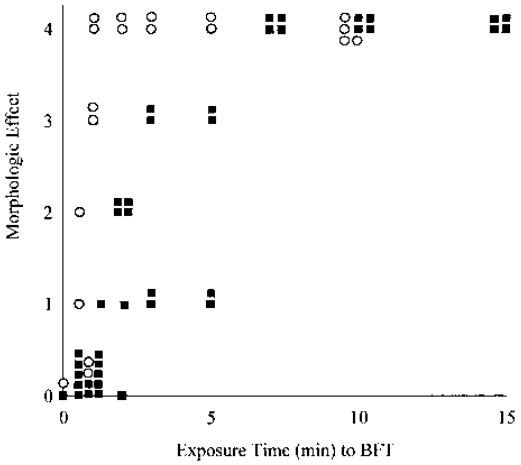

FIG. 3. Time to the onset of BFT intoxication of HT29/C_1 cells. Subconfluent HT29/C_1 cells were exposed to a fixed concentration of BFT for various times, followed by washing and incubation at 37°C with BFT-free culture medium. To detect the subsequent morphologic changes, cells were fixed and stained after a total incubation of 3 h (0.25 nM) or 45 min (2.5 nM) at 37°C. With 0.25 nM (squares), 100% of cells were intoxicated by 7 min of BFT exposure; with 2.5 nM (open circles), 100% of cells were intoxicated by approximately 2 min of BFT exposure. Each point represents one observation; data from eight experiments were pooled.

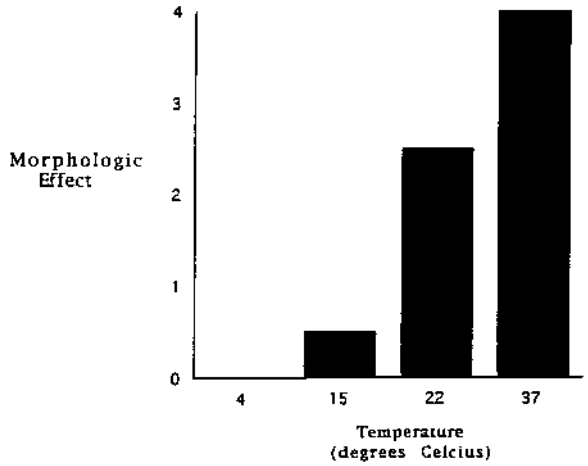

FIG. 4. Effect of temperature on BFT-induced morphologic changes in HT29/C_1 cells. Subconfluent HT29/C_1 cells (precooled for 30 to 60 min) were exposed to a fixed concentration of BFT, 1 or 3 nM, for 3 h continuously at 4, 15, 22, or 37°C prior to fixation and staining. Morphologic effects of BFT are completely inhibited at 4°C and partially inhibited at 15 and 22°C. Two experiments were performed in duplicate.

each cluster (2+ score), became detectable as early as 30 min. By 2.5 h, 100% of cells were affected (4+ score). This minimum incubation period to cellular intoxication was also concentration dependent because similar experiments using 3 nM BFT decreased the time to detection of 4+ morphologic changes to 30 min (data not shown). Thus, purified BFT rapidly alters HT29/C_1 cluster morphology in a concentration-dependent manner within 2.5 h.

Time of onset and reversibility of the effects of BFT on HT29/C_1 cells. The onset of HT29/C_1 cellular intoxication by BFT is extremely rapid. Only 7 min of exposure to 0.25 nM BFT was sufficient to elicit morphologic changes in 100% of cells (4+ score) after a subsequent 1.5- or 3-h incubation at 37°C (Fig. 3). Increasing the BFT concentration by 1 order of magnitude (to 2.5 nM) decreased the time to onset of intoxication of 100% of cells from 7 to 2 min. Thus, the rapidity of cellular intoxication by BFT is also concentration dependent. In fact, in pilot experiments, before BFT was routinely prediluted to the desired final concentration prior to addition to the cells, the direct addition of a small volume of concentrated (up to 30×) BFT stock for even 5 s before washing caused 4+ cellular effects only directly at the site of BFT addition, detected after a subsequent 3-h incubation. This effect could not be dispersed about the culture dish despite immediate vigorous mixing, nor could it be reversed by up to four washes. These data suggest that the effect of BFT on HT29/C_1 cell morphology is rapid and concentration dependent and that its onset is irreversible to washing within minutes or even seconds.

Effect of temperature on HT29/C_1 cell intoxication by BFT. The intoxication of HT29/C_1 cells by BFT is inhibited by lowered temperatures (Fig. 4). For these experiments, HT29/C_1 cells were precooled to 4, 15, or 22°C prior to the addition of 1 or 3 nM BFT for 3 h. In contrast to the striking morphologic changes seen in the standard assay at 37°C, BFT effects were completely inhibited at 4°C and partially inhibited at 15 and 22°C.

The inhibitory effect of 4°C was used to examine the interaction of BFT with HT29/C_1 cells more closely in temperature shift experiments (Table 1). These experiments showed that even at 4°C, this interaction was concentration dependent and could not be reversed by washing. During a 15-min exposure at 4°C, 0.25 nM BFT elicited no morphologic changes after subsequent warming and incubation, yet a 1 order of magnitude increase in concentration to 2.5 nM for 15 min caused intoxication of 100% of cells. Similarly, a 2-min exposure at 4°C to 2.5 nM was insufficient to intoxicate any cells, yet a twofold increase in concentration to 5 nM BFT for just 2 min caused intoxication of all cells. Thus, a concentration-dependent interaction was the first step in BFT intoxication of HT29/C_1 cells. Once this initial event had occurred (e.g., after only 2 min at 4°C for 5 nM BFT), intoxication could not be reversed by washing but still required subsequent incubation at 37°C to become detectable by light microscopy.

Effects of inhibitors of cellular trafficking on BFT activity. To investigate whether endocytosis and intracellular vesicular trafficking of BFT were necessary for the intoxication of HT29/C_1 cells, we tested the effects of three inhibitors of such trafficking: ammonium chloride, brefeldin A, and chloroquine. At concentrations previously shown to inhibit the actions of other toxins (6, 7, 10, 42), none of these agents inhibited the

TABLE 1. Time and temperature dependence of BFT intoxication of HT29/C_1 cells[a]

BFT (nM [ng/ml])	Morphologic changes		
	2 min	15 min	3 h
0.25 (5)	ND	0	4+
2.5 (50)	0	4+	4+
5 (100)	4+	ND	ND

[a] Subconfluent HT29/C_1 cells were precooled to 4°C for 30 to 60 min and then exposed to prediluted BFT at 4°C for 2 min, 15 min, or 3 h, as indicated. BFT was then washed off twice with 2 ml of cold HBSS. One milliliter of 37°C toxin-free culture medium was added, and cells were incubated for 3 h at 37°C followed by fixation and staining. As the concentration of BFT was increased, less time was required for BFT to irreversibly associate with the HT29/C_1 cells at 4°C. In one experiment, cells were exposed to 0.25 nM (5 ng/ml) BFT for 1.5 h at 4°C; however, no morphologic changes were noted after washing and a subsequent 3 h of incubation at 37°C (data not shown). Four experiments were performed in duplicate or triplicate for each assay condition. ND, not done.

effect of BFT (0.25 nM) on cell morphology. However, the onset of intoxication by BFT appeared to be slightly delayed from 7 to 15 min by chloroquine (data not shown). In contrast, the morphologic changes in HT29/C_1 cells induced by *C. difficile* toxin A (3.2 nM for 3 h causing cell shrinkage and spiculation at the periphery of cell clusters) and by cholera toxin (11.7 nM for 6 h creating vacuoles in cell clusters) (4, 53) were inhibited only by NH_4Cl and brefeldin A, respectively (data not shown), consistent with previous reports in both intestinal and nonintestinal cell lines (6, 10, 23, 25, 39). Drugs which alter microtubule structure (i.e., nocodazole, vinblastine, colchicine, and taxol [see Materials and Methods and references 1, 5, and 9]) were also examined to determine if these agents interfered with or altered the morphologic response of HT29/C_1 cells to BFT (1 or 10 ng/ml). These agents did not alter the response to BFT at any of the concentrations tested. None of the inhibitors alone altered HT29/C_1 morphology.

Effect of pH on HT29/C_1 cell intoxication by BFT. The optimal pH for metalloproteolytic action of BFT on G-actin and azocoll in vitro is reported to be 6.5 (32). To determine the optimal pH for the intoxication of HT29/C_1 cells by BFT, the cells were incubated for 3 h with 0.025 to 0.5 nM BFT diluted in medium with pH adjusted to 5, 6, 7, 8, or 9. Full morphologic effects of BFT were seen at all concentrations and at every pH except 5 (data not shown). The extremes of pH did not affect the morphology of untreated cells after 3 h. BFT itself is reportedly stable in vitro at pH 5 to 10 (51). Therefore, with this method, there does not appear to be an optimal pH for toxin activity detectable by morphologic examination at the cellular level.

DISCUSSION

Little is known about the cellular mechanism of BFT action. Two reports have demonstrated that the metalloproteolytic activity of BFT is related to cellular morphologic changes in vivo and in vitro. Obiso et al. demonstrated a >90% inhibition of HT-29 cellular morphology, the fluid response in the ileum or colon of lambs, rabbits, and rats, and no tissue damage in assays using BFT chelated with EDTA or 1,10-phenanthroline (38). Moncrief et al. also reported inhibition of HT-29 cell intoxication in assays using chelated BFT (32), a finding that we have also observed in HT29/C_1 cells in assays using a low concentration of chelated BFT (22). Although this group has also demonstrated the hydrolytic effect of BFT on purified G-actin in vitro (32), whether BFT hydrolyzes one or more cytoskeletal proteins of living cells has not been directly tested.

Our results demonstrate that purified BFT is a potent enteric toxin which alters subconfluent HT29/C_1 morphology in a concentration-, time-, and temperature-dependent manner. Although only a crude estimate of BFT's potency is possible due to the qualitative nature of the HT29/C_1 cell assay, the EC_{50} in the 3-h HT29/C_1 cell assay is approximately 12.5 pM. Despite not being directly comparable, the EC_{50}s of other diarrheagenic toxins are severalfold higher when examined by physiologic assays in another human colonic epithelial cell line, polarized T84 monolayers, where the EC_{50} of cholera toxin is 0.05 to 0.5 nM (23, 39), that of *Escherichia coli* heat-stable toxin (ST_a) is 40 to 100 nM (18, 27), that of *C. difficile* toxin A is 0.5 nM (16), and that of *C. difficile* toxin B is 1.5 nM (15). Interestingly, when the 3-h HT29/C_1 assay is prolonged to 18 h, concentrations of BFT over 1 order of magnitude more dilute than the estimated EC_{50}, i.e., 0.5 pM, can elicit similar morphologic changes. This latter result is potentially consistent with the reported description of BFT as a metalloprotease (32). If this enzymatic activity of BFT is important to its cellular activity, the delayed morphologic changes observed with a very low concentration of BFT may reflect amplification over time of the toxin's biologic effect by cellular intoxication with relatively few molecules of this enzymatically active bacterial toxin.

One of our most striking findings is that BFT is able to intoxicate HT29/C_1 cells in only minutes, if not seconds, at 37°C, depending on the concentration of BFT used to treat the cells. Furthermore, the onset of this rapid intoxication of HT29/C_1 cells is irreversible to washing. A similar rapid, irreversible onset of action has been described for two other anaerobic bacterial enteric toxins, *C. difficile* toxin A and the *Clostridium perfringens* enterotoxin. Ligated rabbit ileal loops exposed to 10 nM (3 μg/ml) *C. difficile* toxin A for only 5 min before washing showed the same subsequent secretory responses and histologic changes as found upon exposure for 2 h (26). Other investigators also found that in ligated rabbit ileal loops, a 5-min exposure to 50 μg of toxin A followed by washing (recovering 40 μg of toxin A, with a presumed irreversible association of the remaining 10 μg) resulted in the same histologic damage as exposure to 10 μg of toxin A for 12 h (31). *C. perfringens* A enterotoxin (CPE) also appears to associate rapidly and irreversibly with Vero cell membranes and rabbit intestinal brush border membranes. The first three events in CPE intoxication (specific receptor binding, membrane insertion, and membrane complex/pore formation) apparently occur within 5 min (28). Of these three events, the characteristics of the insertion step, which is essential for cytotoxicity (14), parallel those that we have observed for BFT action in that this step occurs within 2 min and is, like for BFT, temperature insensitive and irreversible to washing. BFT, however, is not lethal to cells by the tetrazolium blue method (51), [^{3}H]leucine uptake, or lactose dehydrogenase release (22). Furthermore, despite the rapid, irreversible onset of action of BFT reported here, the effects of BFT on HT-29 and HT29/C_1 cells are reversible over time (6, 22). The mechanism(s) accounting for the apparent cell recovery is unknown.

The rapidity and irreversibility of the onset of BFT effect on HT29/C_1 cells suggests at least three possible interactions: (i) specific binding to a receptor and subsequent signal transduction; (ii) interaction with the cell membrane in a manner resistant to washing, for example, by membrane insertion or complex formation with membrane molecules; and/or (iii) rapid internalization after binding. To address the first possibility, additional experiments such as cellular binding assays will be necessary to discern if the rapidity and irreversibility of BFT association with HT29/C_1 cells are due to binding to a specific receptor. Preliminary investigations have not yet documented a role for cyclic nucleotide- or calcium-mediated signalling in altering BFT's action on HT29/C_1 cells (3).

The second possibility, membrane insertion, is of particular interest in light of recent sequencing of the complete BFT gene (11). The predicted amino acid sequence at the carboxy terminus of the mature 186-residue toxin protein suggests that BFT may contain an amphipathic domain. With nearly all of the hydrophobic residues of this 20-residue region on one side of an alpha helix, multimers of BFT could form a pore- or channel-like structure in the lipid bilayer. This mode of action has been invoked for three other bacterial enteric toxins with predicted amphipathic regions: the accessory cholera enterotoxin (Ace) (50), *Staphylococcus aureus* delta toxin (30), and the second *E. coli* heat-stable enterotoxin (ST_b) (8, 47). Membrane-active toxins, however, need not intrinsically have amphipathic regions, since recent data about CPE suggest that pore formation may also be due to amphipathic structures

created after the toxin complexes with eukaryotic membrane proteins (28).

To investigate the third possible interaction mentioned above, cellular internalization, we used two methods (low temperature and chemical inhibitors) which can alter the cellular uptake and processing of bacterial toxins. First, we observed that the ability of BFT to intoxicate HT29/C_1 cells (i.e., cause morphologic changes) is temperature dependent. Since most intracellular processes, including receptor-mediated endocytosis and vesicular trafficking, are inactive at 4°C, it is not surprising that the morphologic expression of BFT toxicity is completely inhibited at this temperature. However, we further observed that the interaction of BFT with HT29/C_1 cells is still concentration dependent at 4°C. This finding suggests that a surface membrane- and concentration-dependent event such as specific receptor ligand binding or membrane insertion (reported to occur for CPE at 4°C [29]) occurs at low temperatures. Since Moncrief et al. (32) have shown that the metalloproteolytic action of BFT on G-actin and azocoll in vitro is minimal at 4°C, it is unlikely that this enzymatic property of BFT is involved in the initial surface membrane interaction. Nevertheless, the temperature-sensitive proteolytic activity of BFT has been shown to be important in both its cellular activity and enterotoxicity (32, 38). In contrast, although the proteolytic activity of BFT was pH sensitive in vitro (32), the activity of BFT on HT29/C_1 cell morphology was largely pH insensitive (except at pH 5.0). The meaning of this discrepancy is unknown.

We also used a second approach to examine whether intracellular trafficking is part of the mechanism by which BFT intoxicates HT29/C_1 cells. Certain chemical inhibitors of receptor-mediated endocytosis and vesicular trafficking which either alkalinize certain intracellular compartments (NH_4Cl and chloroquine) or disrupt Golgi apparatus function (brefeldin A) have been previously shown to inhibit the intoxication of intestinal and nonintestinal cell lines by other enterotoxins (7, 10, 17, 21, 23, 25, 39). None of these agents prevented the intoxication of HT29/C_1 cells by BFT. Furthermore, in time course experiments, NH_4Cl and brefeldin A had no effect on, and chloroquine only minimally delayed, the onset of cellular intoxication within 15 min of exposure to BFT. In contrast, NH_4Cl, but not chloroquine or brefeldin A, inhibited the morphologic effects of *C. difficile* toxin A, a known cytoskeleton-altering toxin which acts by monoglucosylating the Rho protein (19) and whose action is inhibited by NH_4Cl and chloroquine in fibroblasts (17). Similarly, agents which alter microtubule structure (1, 5, 9) did not appear to affect the cellular activity of BFT. These findings corroborate a recent report in which NH_4Cl inhibits the F-actin rearrangements in HT29 cells caused by *C. difficile* toxin A but not BFT (6). In this same study, the organization of tubulin in parental HT29 cells was not modified by BFT. Thus, pH or brefeldin-sensitive or microtubule-dependent intracellular pathways may not be important in BFT intoxication of HT29/C_1 cells, but an alternative novel mechanism of internalization may exist.

One possibility is that BFT is internalized by caveolae, nonclathrin-coated plasmalemmal vesicles involved in the receptor-mediated transepithelial uptake of small macromolecules (33, 40, 49). The function of these vesicles is relatively temperature insensitive compared to internalization by clathrin-coated pits in the classic endocytic pathway (2) but could still account for a concentration-dependent surface receptor-ligand interaction at 4°C followed by internalization at 37°C and a cascade of events leading to morphologic changes.

In summary, we have shown that a novel toxin produced by *B. fragilis* strains associated with childhood diarrhea can alter the morphology of a human intestinal epithelial cell line, HT29/C_1, in a concentration-, time-, and temperature-dependent manner. Most notably, higher concentrations of BFT rapidly and irreversibly intoxicate intestinal epithelial cells apparently without utilizing microtubules or an acidified or Golgi-related vesicular compartment. Future studies to explore the mechanisms by which BFT alters the biology and physiology of enterocytes may provide clues to how this toxin may contribute to the pathogenesis of diarrhea.

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