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Bacteroides fragilis Enterotoxin Induces Cytoskeletal Changes and Surface Blebbing in HT-29 Cells

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Certain strains of the anaerobic bacterium *Bacteroides fragilis* are known to produce an enterotoxin of about 20 kDa which is able to induce a fluid response in ligated intestinal loops and a cytotoxic response in HT-29 cells. It presents protease activity, belonging to a family of metalloproteases termed metzincins. In order to investigate the mode of action of the enterotoxin in cultured cells, we performed a study with HT-29 cells, using both fluorescence and electron microscopy. Treated cells underwent morphological changes, mainly consisting of the retraction of the cell body and the formation of numerous blebs on the cell surface. The microfilament system was reorganized, the F-actin being condensed as a ring at the cell periphery, whereas other cell organelles appeared to be unaffected. All these changes, clearly visible after 3 h of exposure to the toxin, were reversed within 24 h of treatment. By inhibiting the protease activity of the toxin with specific metal chelators, the cytoskeletal effects were also prevented. Thus, *B. fragilis* enterotoxin appears to act on cells by reversibly modifying the actin cytoskeleton, an effect probably dependent on its proteolytic activity.

The anaerobic bacterium *Bacteroides fragilis* constitutes about 1% of the normal bacterial flora in humans (10). However, it has also been isolated from humans with abscesses, soft tissue infections, and bacteriemias (28). Strains of *B. fragilis* associated with epidemic and endemic diarrheal diseases in lambs and capable of stimulating a secretory response in the ligated intestinal loops of lambs and calves (17) were also associated with endemic diarrheal diseases in humans (18, 25). These enterotoxigenic *B. fragilis* strains produce a toxin which, in addition to the fluid response in ligated intestinal loops, induces a cytotoxic response in a colon carcinoma cell line, namely, HT-29 cells (30). The enterotoxin (ET) is produced in vitro as an extracellular protein with a molecular mass of about 20,000 Da with no subunits as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29). Amino acid sequence studies of ET revealed a zinc-binding consensus motif characteristic of metalloproteases termed metzincins. Purified ET contains 1 g-atom of Zn^{2+} per molecule and is capable of hydrolyzing several proteins such as gelatin, actin, and fibrinogen and also of undergoing autodigestion (16).

Although a number of studies dealing with the cytotoxicity of ET have been recently reported (12, 26, 27), the mode of action of the toxin in cultured cells is still unknown. In order to gain more information about the cellular response to ET, we performed a morphological study by using both immunofluorescence and electron microscopy. HT-29 cells, derived from a human colon adenocarcinoma, were used because they are, so far, one of the most sensitive cell lines for ET (29, 30).

MATERIALS AND METHODS

Cell cultures. HT-29 cells were grown at 37°C in RPMI medium, supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, United Kingdom), 1% nonessential amino acids, 5 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). The subcultures were serially propagated after harvesting with 10 mM EDTA and 0.25% trypsin in phosphate buffer solution (pH 7.4).

Toxin, chemicals, and reagents. Highly purified ET was a generous gift from Richard J. Obiso and Roger L. Van Tassell, Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg. The toxin was purified as described previously (29) from *B. fragilis* VPI 13784. NH_4Cl was from Merck (Darmstadt, Germany). Monensin and 1,10-phenanthroline were obtained from Sigma Chemical Co. (St. Louis, Mo.). EDTA was purchased from BDH Laboratory Supplies (Poole, England).

Cell treatments. Forty-eight hours after being seeded on glass coverslips in 24-well plates (initial inoculum, 10^4 cells per ml), HT-29 cells were treated with different doses of ET (ranging from 1.5 ng/ml to 1.5 μg/ml). For all experiments, we used 15 ng of the toxin per ml, which is the concentration which causes almost 100% of morphological changes in HT-29 cells within 3 h. For inhibition studies, 1 mg of NH_4Cl per ml and 150 μg of monensin per ml were used, representing the minimum active dose causing no morphological changes. For experiments with metal chelators, ET was incubated with 1 mM EDTA or 1 mM 1,10-phenanthroline for 30 min at room temperature in a solution containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 0.14 M NaCl. As controls, untreated cells and cells exposed to the toxin were incubated with the same medium without chelators. After treatments, the cultures were prepared for fluorescence microscopy, transmission electron microscopy (TEM), and scanning electron microscopy (SEM). All the experiments were performed at least three times with triplicate samples for each datum.

Fluorescence microscopy. HT-29 cells were grown on 13-mm-diameter glass coverslips in separate wells (5×10^4 cells per well) in a 37°C incubator containing an atmosphere of 95% air and 5% CO_2. Following toxin treatments, both control and treated cells were fixed with 3.7% formaldehyde in phosphate buffer solution with 2% bovine serum albumin, for 10 min at room temperature. After being washed in the same buffer, the cells were permeabilized with 0.5% Triton X-100 (Sigma Chemical Co) in phosphate buffer solution for 10 min at room temperature. For F-actin detection, the cells were stained with fluorescein phallacidin (working dilution 1:500; Sigma Chemical Co.) at 37°C for 30 min. Finally, coverslips were washed and mounted with glycerol-phosphate buffer solution (2:1) and analyzed with a Nikon Microphot fluorescence microscope.

SEM. Control and treated cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min. Following postfixation in 1% OsO_4 for 30 min, the cells were dehydrated through graded ethanols, critical point dried in CO_2, and gold coated by sputtering. The samples were examined with a Cambridge 360 scanning electron microscope.

TEM. Control and treated cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min. Following postfixation in 1% OsO_4 for 30 min, the cells were dehydrated through graded ethanols and embedded with Agar 100.

RESULTS

The morphological changes caused by ET are time and dose dependent. The morphological changes caused by ET in HT-29 cells were studied by using fluorescence microscopy, SEM, and TEM. As viewed by SEM, while control cells appeared organized in smooth-edged clusters with numerous microvilli (Fig. 1a), cells exposed to 15 ng of *B. fragilis* ET per ml for 3 h showed an evident retraction and the formation of numerous

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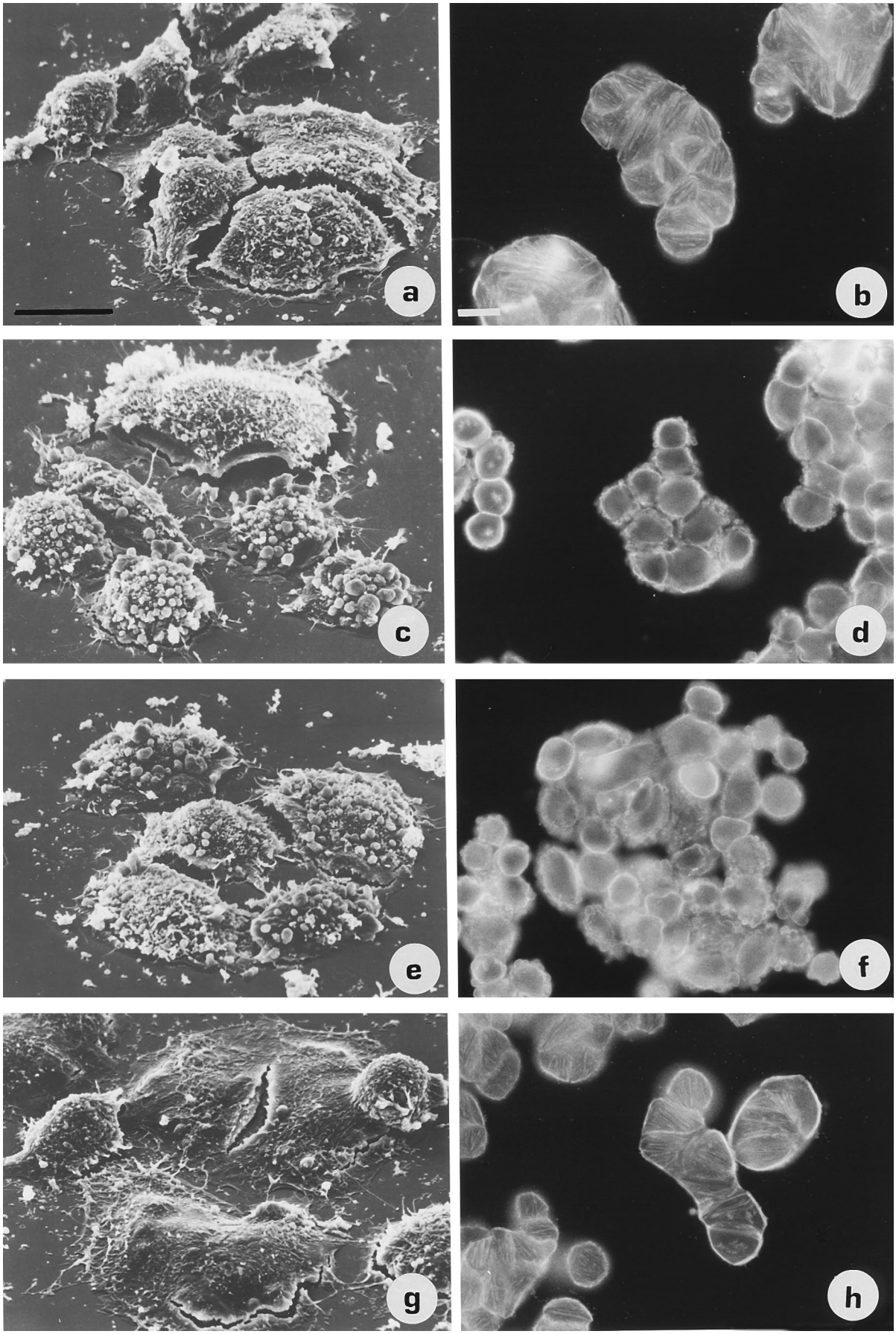
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FIG. 1. *B. fragilis* ET causes rounding up, surface blebbing, and F-actin rearrangement in HT-29 cells. Scanning electron micrographs of control cells (a), cells treated with ET for 3 h (c), cells treated with ET for 6 h (e), and cells treated with ET for 24 h (g). Fluorescence micrographs of cells stained with fluorescein phallacidin for F-actin detection. (b) Control cells; (d) cells treated with ET for 3 h; (f) cells treated with ET for 6 h; (h) cells treated with ET for 24 h. Bar, 10 μm.

blebs on the cell surface (Fig. 1c). The same phenotype was detectable when HT-29 cells were treated with ET for 6 h (Fig. 1e). After 24 h of exposure to the toxin, the cells reverted to the phenotype typical of the control cells (Fig. 1g). Since such alterations in surface morphology are always accompanied by changes in the organization of the microfilament system, we stained HT-29 cells with fluorescein phallacidin for F-actin detection. Whereas control cells presented well-organized stress fibers (Fig. 1b), cells exposed to 15 ng of ET per ml for 3 and 6 h showed a complete disappearance of stress fibers, F-actin being condensed as a ring at the cell periphery (Fig. 1d and f, respectively). Furthermore, these cells displayed numerous surface blebs positive for F-actin. According to SEM observations, cells exposed to ET for 24 h presented a phenotype

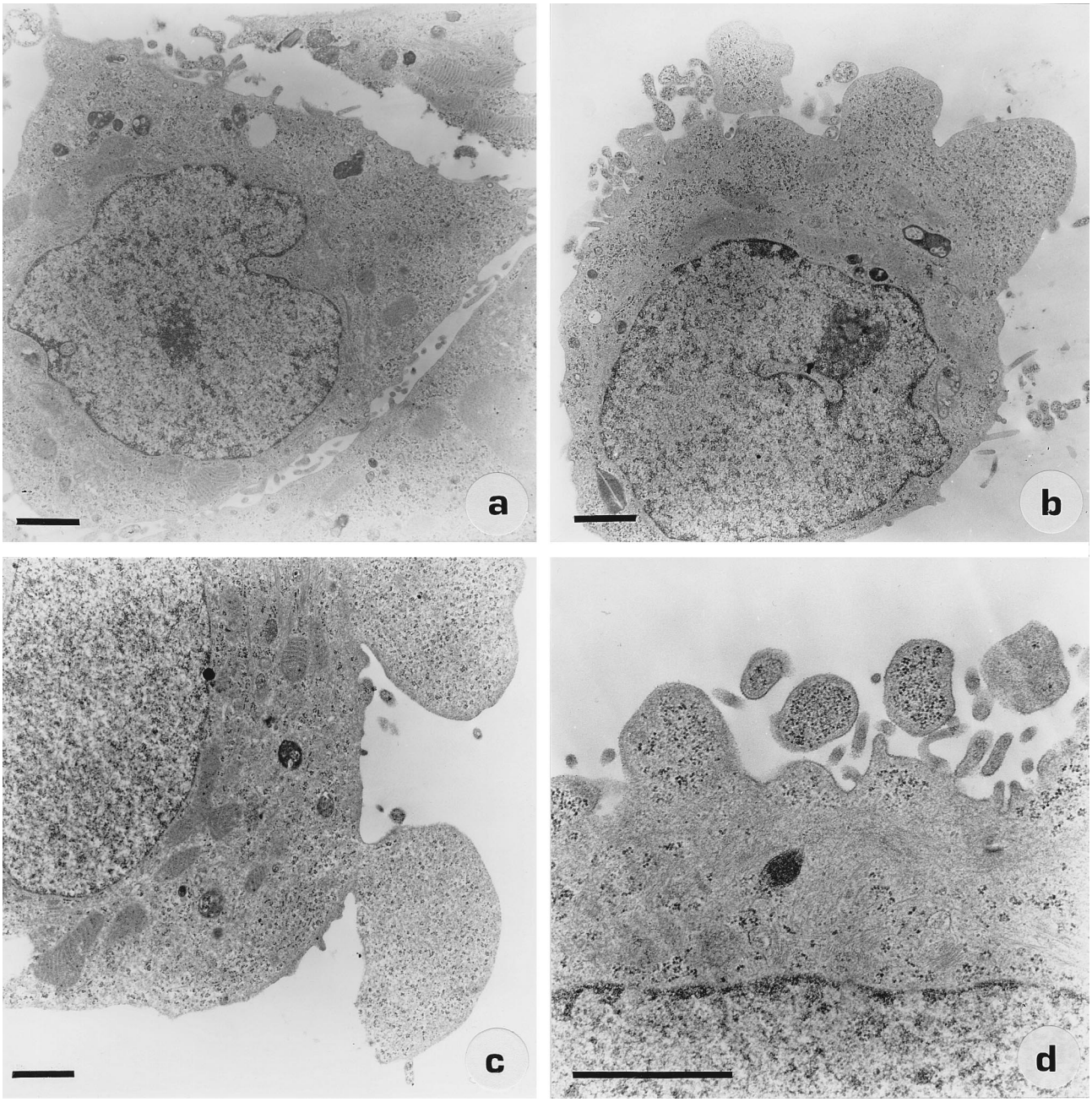

FIG. 2. *B. fragilis* ET induces potocytotic blebs in HT-29 cells. Transmission electron micrographs of control cells (a) and cells treated with ET for 3 h (b to d). The general morphology of cell organelles was not altered by exposure to ET. Bars, 1 μm.

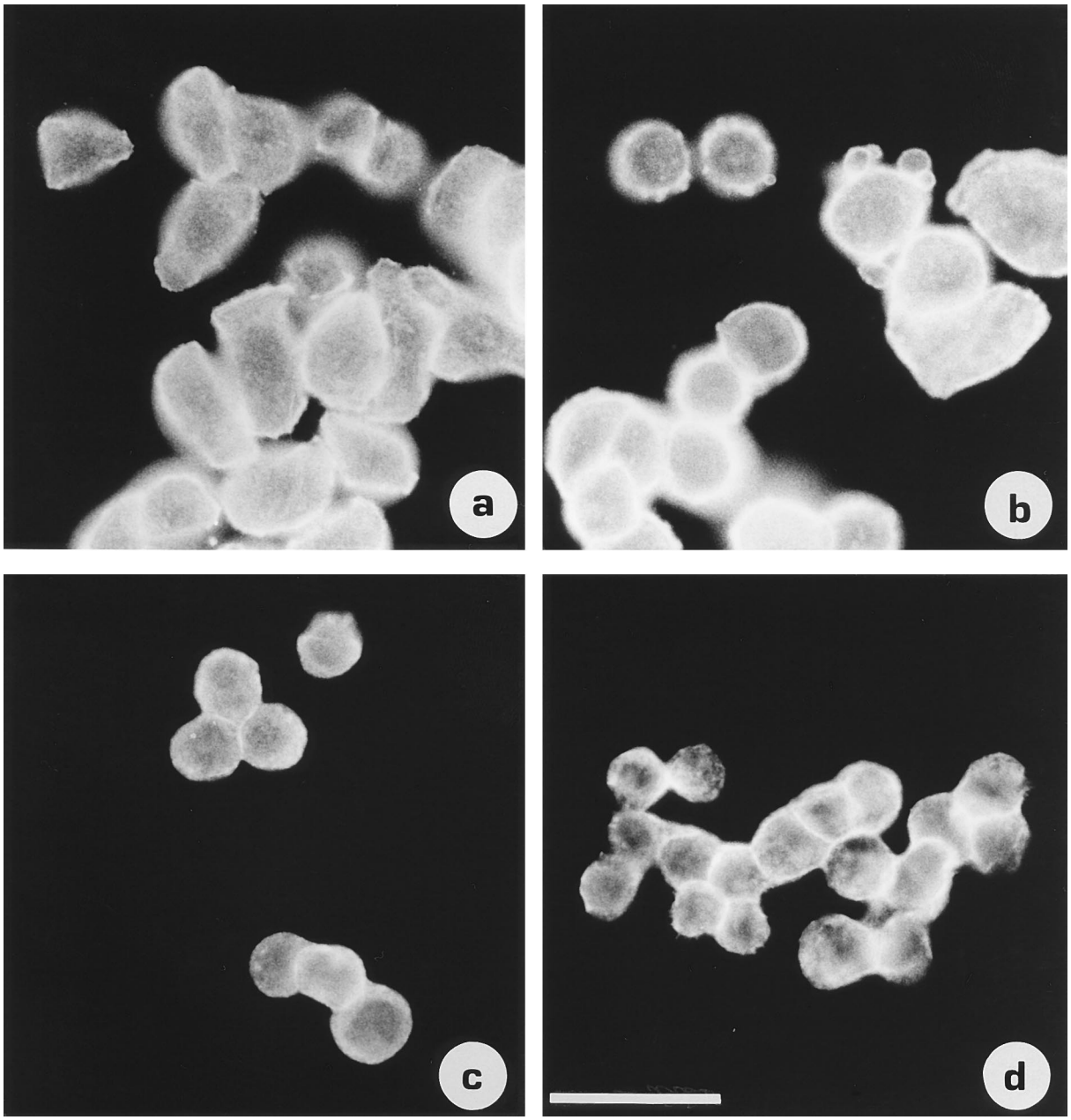

FIG. 3. Fluorescence micrographs of HT-29 cells stained with fluorescein phallacidin for F-actin detection. (a) Cells maintained in HEPES solution devoid of EDTA; (b) cells in HEPES solution devoid of EDTA exposed to ET for 40 min; (c) cells maintained in HEPES solution containing EDTA; (d) cells in HEPES solution containing EDTA treated with ET for 40 min. EDTA was able to inhibit the cytoskeleton-dependent blebbing induced by ET. Bar, 10 μm.

very similar to that of the control cells, i.e., they showed well-organized stress fibers with only a few blebs on the cell surface (Fig. 1h). The toxin effects were also dose dependent, the intensity of cell response increasing with the dose of ET (data not shown).

When an ultrastructural analysis was performed, control cells were characterized by a round or irregular nucleus with one or more nucleoli and several mitochondria with an electron-dense matrix. Vacuoles and electron-dense bodies were also detected (Fig. 2a). In toxin-treated cells, no modification in the morphology of cell organelles was observed, with the nucleus, mitochondria, and other structures appearing the same as those of the control cells. However, in accordance with the above-mentioned results, blebs rich in ribosomes were observed in cells exposed to the toxin for 3 h (Fig. 2b to d). Blebs of various sizes appeared as an enlargement of the cell body (Fig. 2b) or as protrusions attached to the cell by only a thin slice of cytoplasm (Fig. 2c). Two types of blebs, potocytotic and zeiotic blebs, have been described so far (3). Potocytotic blebs contain clear fluid and are devoid of cytosolic organelles, whereas zeiotic blebs originate as a herniation of the cytoplasm content. Blebs induced by ET belong to the potocytotic type, no organelles being present inside the bleb matrix. After 24 h of exposure to the toxin, the cells looked like controls without blebs (data not shown).

Metal chelators inhibit the morphological changes induced by ET. It has been reported that the *B. fragilis* ET is a zinc metalloprotease and that chelating agents inactivate the cell-

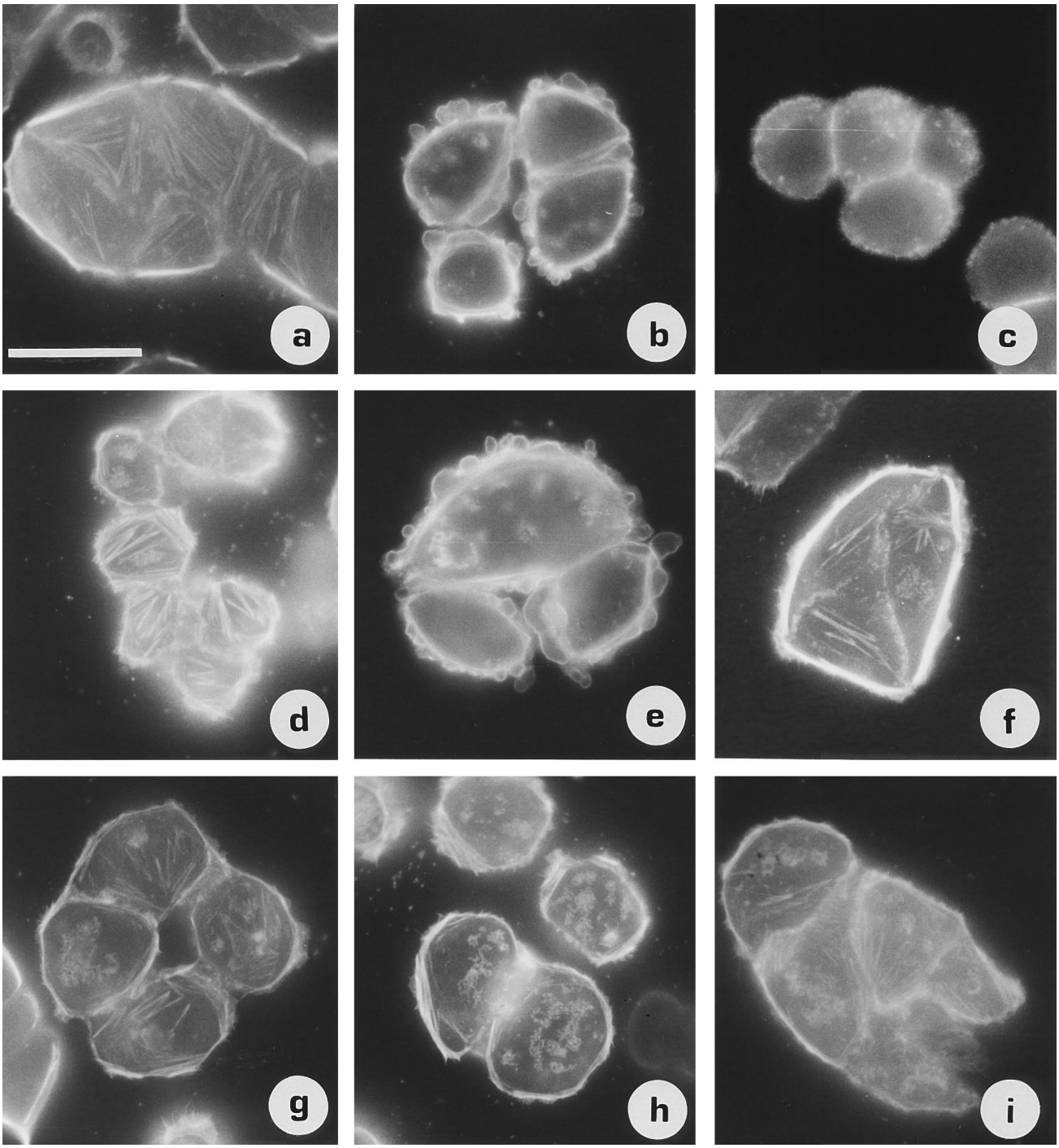

FIG. 4. Fluorescence micrographs of HT-29 cells stained with fluorescein phallacidin for F-actin detection. (a) Control cells; (b) cells treated with ET for 3 h; (c) cells treated with CdA for 3 h; (d) cells treated with NH_4Cl for 3 h; (e) cells pretreated with NH_4Cl for 30 min and then exposed to ET for 3 h; (f) cells pretreated with NH_4Cl for 30 min and then exposed to CdA for 3 h; (g) cells treated with monensin for 3 h; (h) cells pretreated with monensin for 30 min and then exposed to ET for 3 h; (i) cells pretreated with monensin for 30 min and then exposed to CdA for 3 h. Neither of the two inhibitors was able to prevent the disappearance of stress fibers caused by ET, whereas the blebbing was inhibited by monensin. Bar, 10 μm.

rounding activity (16). In order to check whether the cytoskeleton-dependent changes (i.e., disappearance of stress fibers and formation of surface blebs) can also be inhibited by metal chelators, we performed experiments with EDTA and 1,10-phenanthroline. As a marker for the cytoskeletal effects we used the surface blebbing and not the stress fibers, because the maintenance of the cells in HEPES medium was enough to cause the stress fibers to disappear, irrespective of the presence of the chelator (Fig. 3a and c [cells grown in HEPES medium without and with EDTA, respectively]). When ET was added to the solution devoid of EDTA, numerous surface blebs were detectable around the cells within 40 min (Fig. 3b). By con-

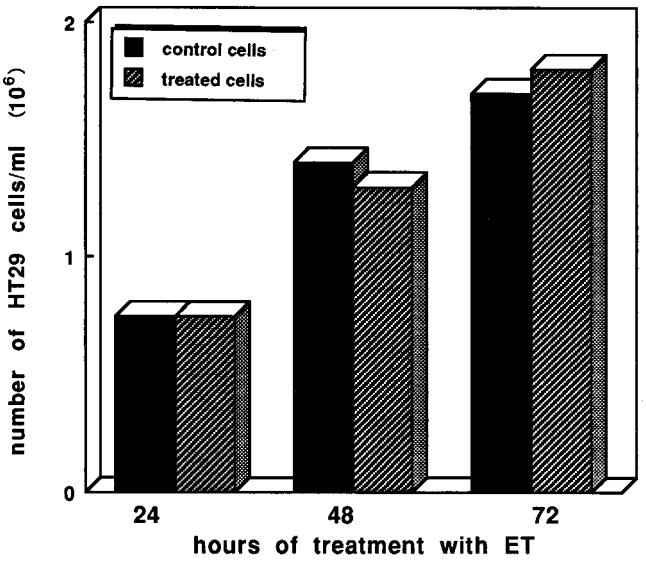

FIG. 5. Graph showing the number of HT-29 cells per ml at different times of treatment with ET. Cells exposed to ET continue to grow at the same rate as that of untreated cells.

trast, pretreatment with EDTA totally prevented cells from undergoing surface blebbing when exposed to ET (Fig. 3d). The same experimental protocol was followed by using the zinc-specific metal chelator 1,10-phenanthroline instead of EDTA, and similar results were obtained (data not shown).

Does ET behave as a classical toxin which needs an acidic compartment in order to act? Most bacterial toxins acting intracellularly need an acidic compartment in order to exert their cytotoxic activity (20). A classical method for studying this aspect is the use of compounds (such as NH_4Cl and monensin) which are able to increase the pH in those cytosolic compartments necessary for the activation of the toxins (22, 23). In Fig. 4, untreated cells, cells exposed to ET, and cells exposed to *Clostridium difficile* toxin A (CdA) (panels a through c, respectively) are shown. CdA was used as a positive control, since its characteristic effect, i.e., the disruption of the microfilament system, is known to be totally inhibited by NH_4Cl and monensin (8).

When cells were pretreated with 1 mg of NH_4Cl per ml, ET was still fully active. In fact, while HT-29 cells exposed to NH_4Cl alone presented well-organized stress fibers (Fig. 4d), cells pretreated with NH_4Cl for 30 min and then exposed to ET for 3 h (Fig. 4e) showed the phenotype typical of ET-treated cells. In the positive control, represented by cells exposed to NH_4Cl followed by CdA (Fig. 4f), the F-actin was still well organized in stress fibers.

Pretreatment with 150 μg of monensin per ml did not protect cells from the actin-disrupting effect of ET, although it can induce full protection from the blebbing (Fig. 4h). By contrast, the CdA-induced cytoskeletal effect was totally prevented by monensin (Fig. 4i).

ET does not interfere with cell growth and the cell cycle. By counting the cells, after exposure to 15 ng of ET per ml for 24, 48, and 72 h, it was evident that ET did not interfere with cell proliferation (Fig. 5). In fact, cells exposed to ET continued to grow at a rate equivalent to that of the untreated cells.

Although the modifications induced by the toxin are reversible within 24 h, ET might have modified the cell cycle. In order to explore this possibility, we checked the cell cycle by fluorescence-activated cell sorter analysis. Our results showed that the percentages of cells in G_0, G_1, S, and G_2 phases are the same before and after treatment with ET for 24, 48, and 72 h (data not shown).

DISCUSSION

It has been suggested that the cytotoxicity (16) and enterotoxicity (19) of ET are due to its proteolytic activity. Accordingly, we have shown that the formation of surface blebs, one of the hallmarks of cytotoxicity of ET in HT-29 cells, was totally inhibited by pretreatment of the toxin with either the metal chelator EDTA or the specific Zn^{2+} chelator 1,10-phenanthroline. Our in vitro findings strongly support the hypothesis that the activity on the cytoskeleton might also be dependent on the proteolytic activity of the toxin.

In view of the data herein presented, *B. fragilis* ET may be considered a new toxin belonging to the group of protein toxins acting on the cell cytoskeleton. In fact, when HT-29 cells, a cell line very sensitive to ET, were used, the F-actin organization was clearly modified. The effect on actin appeared to be specific, since neither tubulin nor the intermediate filament-type vimentin were remarkably modified in HT-29 cells exposed to ET (data not shown). A number of bacterial protein toxins are known to exert their cytotoxic activity via a modification of cytoskeletal components (for a review, see reference 4). Some toxins, such as *Clostridium botulinum* C2, *Clostridium perfringens* iota-toxin, *Clostridium spiroforme* iota-like toxin, and *C. difficile* ADP-ribosyltransferase, directly induce the ADP-ribosylation of actin. Others, such as *C. difficile* toxin B and *Escherichia coli* CNF1 and CNF2, interact with the actin cytoskeleton via the small GTP-binding protein Rho by either inhibiting (11) or activating (6, 21) this molecule. Although most toxins which modify the actin cytoskeleton, including *C. difficile* toxins A and B (8, 9, 11), exert their action in an irreversible mode, the F-actin-disrupting effect induced by ET appeared to be reversible. The reversibility of the effect, the activity on the actin cytoskeleton, and the low molecular weight are characteristics that ET shares with *Vibrio cholerae* Zonula Occludens Toxin, which is also both cytotoxic and enterotoxic (5).

Another feature of cells exposed to ET was the formation of blebs on the cell surface, a phenomenon which has often been reported as a consequence of several toxic injuries (14). It has been hypothesized that bleb formation is related to a perturbation of the cytoskeleton, as also suggested by the finding that two well-known toxins, cytochalasin B and phalloidin, can induce deep cytoplasmic clefts and bulbous protuberances recognized as blister-like, zeiotic blebs (2, 15). The blebbing phenomenon can also be provoked by some bacterial protein toxins acting on the microfilament system, such as *C. difficile* toxins A and B, whose effects have been reported as irreversible (7, 13). However, the blebbing caused by ET was reversible in the same manner as that which occurs spontaneously in cultured cell lines which can display a permanently blebbed surface (1) or may undergo blebbing in a particular phase of the cell cycle (24). Since ET induced a blebbing phenomenon which appeared early and disappeared by prolonging incubation, we investigated whether this toxin could interfere with cell growth and whether its activity might be dependent on the cell cycle. Our results showed that the blebbing phenomenon was not cell cycle dependent and probably represented only a transient cellular response to cytoskeletal alterations.

Since most bacterial toxins acting intracellularly need an acidic compartment in order to act (20), we investigated whether ET was able to exert its activity in cells pretreated with NH_4Cl and monensin, both of which increase the pH in endo-

cellular compartments (22, 23). Interestingly, while NH_4Cl did not protect cells from both the F-actin-disrupting effect and the blebbing phenomenon, monensin was able to protect HT-29 cells from the blebbing but not from the disruption of the F-actin. Thus, the formation of blebs seems to follow the alteration in F-actin caused by the toxin. These results indicate that ET does not need an acidic compartment with a very low pH in order to exert its cytotoxic activity, suggesting that it may possibly exit from an early endosome. However, we cannot rule out the possibility that ET acts from outside, sending a signal across the cell which may account for at least some of the reported effects.

In conclusion, *B. fragilis* ET appears to act on cells by reversibly modifying the actin cytoskeleton, an effect probably dependent on the proteolytic activity of the toxin. Actin is involved in a lot of cellular functions, and in particular, it plays a key role in controlling the permeability of the intestinal barrier. Thus, it is not surprising that this cytoskeletal component represents a very sensitive target for an increasing number of enterotoxins, including the *B. fragilis* ET.

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