Interaction of *Clostridium difficile* Toxin A with Cultured Cells: Cytoskeletal Changes and Nuclear Polarization

C. Fiorentini, W. Malorni, S. Paradisi, M. Giuliano, P. Mastrantonio, and G. Donelli

Departments of Ultrastructures and Bacteriology and Medical Mycology, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy

Received 4 January 1990/Accepted 16 April 1990

Experiments done on in vitro-cultured cells exposed to toxin A from *C. difficile* showed a series of cytopathologic changes leading to cell retraction and rounding accompanied by the marginalization of the nucleus, which localized at one pole of the cell. Cytoskeleton appeared to be strongly involved in such modifications. In particular, the microfilament system seemed to be involved in cell retraction, while microtubule network integrity and function seemed to be necessary for the nuclear displacement. The carboxylic ionophore monensin completely blocked the cytopathic effect when added with the toxin. The serine protease inhibitor chymostatin appeared to be protective also upon addition long after the end of the binding step. The Ca⁰⁰²⁻-dependent cytosolic protease inhibitors antipain and leupeptin were uneffective in protecting cells. Thus, our results suggest the involvement of an acidic compartment and the action of a serine protease in toxin A-induced cytopathic effect.

Toxigenic strains of *Clostridium difficile* are recognized as the major cause of antibiotic-associated pseudomembranous colitis in humans (3). They produce at least two different toxins, designated toxins A and B, which are large, heat-labile cytotoxic proteins implicated in the pathogenesis of the disease (2). Toxin A elicits severe epithelial damage associated with hemorrhage and fluid secretion when injected into rabbit ileal loops (38). These effects are not accompanied by activation of adenylate cyclase (17), indicating that its mechanism of action differs from that of cholera toxin (12) and *Escherichia coli* heat-labile enterotoxin, which cause fluid secretion and stimulate intestinal adenylate cyclase without producing an inflammatory infiltrate (32). Conversely, toxin B appears to be devoid of enterotoxic activity, and its role in the pathogenesis of the disease is still unclear (19).

Both toxins A and B induce cytopathic effect (CPE) in cultured cells (7, 16, 33) and belong to the group of intracellularly acting proteins which have to be internalized and processed to exert their cytopathic activity (8, 11). In particular, toxin B appears to modify the cytoskeleton, disaggregating actin filaments (40) and rearranging some actin-binding proteins (23). On the other hand, toxin A has been shown to induce morphological changes in several cultured mammalian cells, mainly consisting of cell retraction and rounding (C. Fiorentini et al., Microsoc. Ther., in press). It has also been demonstrated to perturb the cytoskeleton (10) and to stimulate intracellular calcium release, directly altering epithelial cell permeability without producing cell death or disrupting the confluence of the monolayer (28).

To better clarify the subcellular mechanisms underlying *C. difficile* toxin cytotoxicity, we have planned an extensive study on in vitro-cultured cells different in origin and growth pattern. The present work focused on toxin A-induced CPE and was mainly concerned with an intracellular phenomenon named nuclear polarization. The mechanisms underlying such a morphological change were investigated by immunocytochemical and electron microscopic approaches.

**MATERIALS AND METHODS**

**Cell cultures.** Different human cell lines from cervix carcinoma (HeLa), larynx carcinoma (HEp-2), mammary breast carcinoma (CG5), epidermoid carcinoma (A431), and hepatoma (HEp-G2) as well as a murine myogenic cell line (L8) were cultured at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Flow Laboratories, Inc., McLean, Va.), 1% nonessential amino acids, 5 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

For light microscopy, both control and treated cells were grown on 13-mm-diameter glass cover slips in separated wells (5 × 10⁴ cells per well). For transmission electron microscopy, cells were subcultured in 25-cm² Falcon plastic flasks at a density of approximately 10⁴ cells per ml. Flasks and wells were placed in a 37°C incubator containing an atmosphere of 95% air and 5% CO₂.

**Toxin purification.** The procedure adopted for toxin purification was that described by Sullivan et al. (37). *C. difficile* VPI strain 10463 was grown in 3 liters of brain heart infusion (dialysis flasks) for 72 h at 37°C. The culture supernatant was centrifuged at 8,000 × g for 10 min, filtered through a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.), and concentrated by ultrafiltration at 4°C with an XM-100 membrane filter (Amicon Corp., Lexington, Mass.). The concentrated supernatant was loaded onto a DEAE Trisacryl M column (2.5 by 40 cm) which had been equilibrated with 50 mM Tris hydrochloride (pH 7.5) and was eluted with a 600-ml linear NaCl gradient (0.05 to 0.5 M NaCl).

Fractions (5 ml) were collected and assayed for cytotoxicity with HEp-2 cells. For enterotoxicity, the rabbit ileal loop test was performed as previously described (19).

Cytotoxic fractions eluting at 0.1 M NaCl (toxin A) were further purified by acetic acid precipitation in acetate buffer (pH 5.5). When analyzed by nondenaturing gel electrophoresis, the toxin A preparation was homogeneous. The protein concentration was 118 μg/ml as determined by the method of Lowry et al. (18). The toxin was stored at −20°C in 50 mM Tris hydrochloride (pH 7.5), and dilutions were made in the same buffer.
Cell treatment. For light and electron microscopy, 24 h after seeding, cell cultures were treated with 4 μg of toxin A per ml. This toxin concentration caused the CPE in 100% of epithelial cells (HeLa, HEP-2, CG5, A431) in 1 h and 100% of the other cell lines (Hep-G2, L8) in 3 h. Treatments were performed at different temperatures (4 or 37°C).

Chemicals. Experiments with different chemical agents were done with the maximum dose producing no cytopathologic changes in the cells; all the experiments were performed in triplicate. The following chemicals were used: trifluoperazine (TFP) (4.8 μg/ml), which has been demonstrated to be capable of antagonizing the calcium-binding protein calmodulin (29); drugs which interact with the cytoskeletal components (31), such as cytochalasin B (0.48 μg/ml), phalloidin (7.88 μg/ml), and demecolcine (0.26 μg/ml); a Na+/H+-exchanging ionophore, monensin (340 μg/ml) (25); Ca2+-dependent protease inhibitors (1, 9) leupeptin (100 μg/ml) and antipain (100 μg/ml); and a serine protease inhibitor, chymostatin (400 μg/ml) (39). The stock solutions were made in dimethyl sulfoxide, and monensin was diluted in ethanol. For the experiments, final concentrations were obtained directly in growth medium. Parallel treatments with vehicles alone (dimethyl sulfoxide and ethanol) were used as controls. All the chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Fluorescence microscopy. For microtubule-associated proteins (MAPs), Tau, filamin, vinculin, tubulin, and vimentin detection, cells grown on cover slips were fixed in 3.7% formaldehyde in phosphate-buffered saline (pH 7.4) for 10 min at room temperature. After being washed in the same buffer, the cells were permeabilized with 0.5% Triton X-100 (Sigma) in phosphate buffer (pH 7.4) for 10 min at room temperature.

For vinculin and α-tubulin labeling, monoclonal antibodies directed against vinculin (Amersham Corp., Arlington Heights, Ill.) and α-tubulin (Amersham) were used. After 30 min at 37°C, cells were washed in phosphate-buffered saline and then incubated with a sheep anti-mouse immunoglobulin G fluorescein-linked whole antibody (Sigma) for 30 min at 37°C. For MAP and Tau detection, cells were incubated with rabbit anti-MAP and anti-Tau antibodies (Biolyeda), respectively, for 30 min at 37°C. After washing, an incubation with a goat anti-rabbit immunoglobulin G rhodamine-linked whole antibody (Sigma) was performed. For filamin and vimentin detection, cells were incubated with goat polyclonal antifilamin (Sigma) and antivimentin (Sigma) antibodies at 37°C for 30 min. After washing, an incubation with a rabbit anti-goat immunoglobulin G fluorescein-linked antibody for 30 min at 37°C was performed.

For the detection of the surface antigens epidermal growth factor receptor and β1-microglobulin, the light subunit of human leucocyte antigen, the cells were incubated with the polyclonal antibodies anti-epidermal growth factor receptor (Sigma) and anti-β1-microglobulin (Dako) at 4°C for 30 min. After being washed in phosphate buffer, cells were fixed in 3.7% formaldehyde in the same buffer for 30 min at 4°C and incubated with a goat anti-rabbit immunoglobulin G rhodamine-linked antibody (Sigma) at 37°C for 30 min. Surface glycoproteins recognized by the lectin from Triticum vulgaris (wheat germ agglutinin) are mainly represented by proteins carrying N-acetylglucosamine carbohydrates. Specimen preparation with fluorescein-linked wheat germ agglutinin (direct fluorescence) was performed as stated above.

Finally, after washings, cover slips were mounted with glycerol-phosphate-buffered saline (2:1) and analyzed with a Nikon Optiphot fluorescence microscope.

Transmission electron microscopy. Cells were fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer, postfixed with 1% OsO4 in the same buffer for 1 h, dehydrated through graded ethanol, and embedded in Agar 100 resin (Agar Aids). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 10 C electron microscope.

RESULTS

Morphological features of intoxicated cells. As a general rule, morphological effects of toxin A on the different cell lines considered here are represented by (i) cell retraction, (ii) cell rounding, and (iii) displacement of the nucleus toward one pole of the cell. In the present study, a dose of toxin of 4 μg/ml showed a maximal effect of rounding up and nuclear polarization after 1 h in HeLa, HEP-2, CG5, and A431 cells and after 3 h in L8 and Hep-G2 cells. Taking into account the strict overlapping among the results obtained with the different cell lines under different culture conditions, we report here only the data relative to HeLa cells, which can be assumed to be a representative model for cultured cell intoxication.

When an ultrastructural analysis was performed, control cells were characterized by a round or elongated nucleus with two or more nucleoli and several mitochondria with an electron-dense matrix (Fig. 1a). In toxin-treated cells undergoing the retraction process (data not shown), no modification in the morphology of cell organelles was detected. However, at the final stage of the intoxication process, the nucleus appeared to be kidney shaped and polarized to one pole of the cell (Fig. 1b). The endoplasmic reticulum was slightly diluted, while mitochondria were localized around the Golgi apparatus, opposite to the nucleus, and showed an unchanged general morphology but a rarefied matrix. The Golgi apparatus stalks appeared to be remarkably increased, numerous vesicles being visible; bundles of cytoskeletal filaments, probably microfilaments, were also observed (Fig. 1c). Moreover, when cells were maintained in suspension by continuous shaking, the exposure to the toxin induced nuclear polarization with the same ultrastructural features mentioned above.

Cytoskeletal changes induced by toxin treatment. The cytoskeletal components underwent a different behavior toward toxin A treatment. In the microfilament system, F-actin and α-actinin were localized only in the side opposite the nucleus in a patchily organized form, as previously described in HEP-2 cells (7). Filamin, normally localized together with α-actinin (Fig. 2a), displayed a diffuse positivity throughout the cytoplasm (Fig. 2b and c). Vinculin seemed to be unexpressed in intoxicated cells (Fig. 3b) compared with control cells (Fig. 3a).

The microtubule network, well visible in control cells (Fig. 4a), disappeared in the cytoplasm of treated cells. In fact, a diffuse positivity for α-tubulin was detectable in toxin-exposed cells (Fig. 4b), even though, by varying the focal plane of the microscope, a thin meshwork of microtubules was observed around the nucleus (Fig. 4c and d). Furthermore, the MAPs and Tau, normally scattered in the cytoplasm and mainly distributed near the nucleus, appeared to be diffused throughout the cytoplasm of the roundish intoxicated cells (data not shown).

Finally, the intermediate filament vimentin, organized in a complex network in untreated cells (Fig. 5a) was localized as
a positive, condensed meshwork at the side opposite the nucleus in treated, rounded cells (Fig. 5b).

**Surface antigen distribution after toxin treatment.** The effects of toxin A on the distribution of some surface antigens were also analyzed in Triton X-100-untreated cultured cells by immunocytochemical methods. Different surface markers were labeled: the receptor for epidermal growth factor, which is expressed by the A431 and CG5 epithelial cell lines; the light subunit of human leukocyte antigen (β2-microglobulin), which was evaluated in all human cell lines examined; and the glycoproteins of the N-acetyl-D-glucosamine group, labeled by the wheat germ agglutinin lectin. All these markers were uniformly distributed on the surfaces of control and treated cells (data not shown).

**Effects of various compounds on toxin-induced CPE.** Experiments with several agents able to perturb cell structure and function were done on different cell lines. However, as previously explained, we report here only the results obtained with HeLa cells. Pretreatment with TFP, a compound capable of antagonizing the calcium-binding protein calmodulin, did not protect cells against intoxication. However, the characteristic retraction of the cell body and the changes in actin microfilaments were strongly delayed in the presence of this drug (Fig. 6a and b), even though after 3 h of treatment with the toxin, the CPE almost completely developed.

Drugs which interact with the cytoskeletal components were also tested (Table 1). Actin-depolymerizing or stabilizing compounds such as cytochalasin B and phallolidin were ineffective in protecting cells from toxin A. On the other hand, when cells were treated simultaneously with toxin A and the microtubule-depolymerizing agent demecolcine (Table 1), they normally retracted the cell body and became rounded, but the nucleus did not migrate (95%) toward one pole of the cell (Fig. 7a and b).

Monensin, which is able to raise the pH in intracellular acidic vesicles, was highly protective when added with the toxin at 37°C. In fact, only 5% of cells treated this way underwent rounding and nuclear polarization (Table 1). However, monensin was not protective when the cells were treated with a mixture of monensin-toxin at 4°C (to allow toxin binding but not entry) and then maintained in fresh medium at 37°C. In this case, 90% of cells showed the toxin-induced CPE. The time course for prevention of such a phenomenon by monensin indicated that this drug protected cells only when added immediately or shortly (within 10 min) after the binding step (Table 2).

Protease inhibitors were also used. Antipain and leupeptin, inhibitors of Ca2+-dependent proteases, were not protective at all (Table 1). On the other hand, chymostatin, a serine protease inhibitor, was highly protective when added with the toxin at 37°C (Table 1) as well as at 4°C with subsequent placement in fresh medium at 37°C. The cells remained well attached to the substratum without undergoing cell rounding and nuclear polarization. Moreover, the time course for prevention of toxin-induced CPE by chymo-
statin indicated that this drug appeared to be protective until 30 min after the binding step (Table 2).

**DISCUSSION**

The effects of toxin A from *C. difficile* on cultured cells seem to be mainly represented by a cascade of events leading to cell rounding, nuclear polarization, and cell death. In fact, three different phases can be detected in toxin A intoxication (Table 3). In the first phase, cell retraction, changes in the adhesion pattern and integrity of the cytoskeletal apparatus were observed; in the second phase, cell rounding, nuclear polarization and peculiar cytoskeletal changes were visible; finally, in the third phase, cells undergoing necrosis showed two or more kidney-shaped nuclei and a disrupted cytoskeleton.

It is well known that the cell shape and the polarity of the cell as well as the positioning of intracellular organelles are strongly dependent on the cytoskeletal components (31). Toxin A, like other bacterial toxins (6, 26, 27, 30, 34) and *C. difficile* toxin B (20, 23), has been described to perturb the cytoskeleton (Fiorentini et al., in press), causing changes in F-actin (7, 10) and α-actinin (7) distributions. Strikingly, experiments reported here with microfilament system-perturbing agents such as cytochalasin B and phalloidin, which, respectively, depolymerize or stabilize F-actin filaments (31), did not appear to be able to protect cells from both retraction and nuclear polarization. This could be due to an effect of the toxin on actin-binding proteins playing a role in the dynamic modifications of microfilament system rather than on F-actin itself. In fact, some plaque adhesion proteins like α-actinin, which acts as a cross-linking molecule, and vinculin, which connects stress fibers to the extracellular matrix, showed an altered pattern after toxin treatment. Moreover, cells seeded in toxin-containing medium failed to spread out but were still able to adhere to the substratum and displayed nuclear polarization. This can probably be related to the dependence of cell spreading on actin microfilaments and on the organization of stress fibers (4, 36). Furthermore, the organization of actin in a three-dimensional network is strongly dependent on the distribution of α-actinin and filamin, two gel-forming proteins (31). After exposure to toxin A, α-actinin appeared to be distributed together with actin, while filamin showed a diffuse pattern in the cytoplasm. These findings suggest an impairment of the relationships between actin and some actin-related proteins, which can also explain the homogeneous surface distribution of the surface protein epidermal growth factor receptor, known to be actin cytoskeleton related (41), in toxin-treated cells. In addition, the contraction-producing protein myosin, which is associated with actin filaments in

---

**FIG. 2.** Immunofluorescence. Control (a) and toxin A-treated (b and c) HeLa cells. The actin-binding protein filamin appears to be diffused throughout the cytoplasm in treated cells (b and c [corresponding brightfield]). Bar, 5 μm.

**FIG. 3.** Immunofluorescence. Control (a) and toxin A-treated (b) HeLa cells after immunostaining with antivinculin antibodies. The antigen appears to be unexpressed in intoxicated cells (b). Bar, 5 μm.
stress fibers (15), has been shown to be modified by toxin A action at an early step of the intoxication process (Fiorentini et al., in press). This protein is known to be regulated by a calcium-calmodulin-dependent protein kinase (29, 31), and the perturbing effect of TFP on cell retraction can support the hypothesis of an involvement of myosin in this step of intoxication. Thus, our results seem to indicate that the relationships between the different components of the microfilament system are strongly involved in the early steps of the intoxication process.

The microtubular apparatus has been shown not to be involved in the early cell response to toxin treatment (7). At final stages, it showed a diffuse pattern in the cytoplasm, which is consistent with the homogeneous distribution of MAPs, proteins known to bind to microtubules and to cross-link them to other cell structures (31). On the other

FIG. 4. Immunofluorescence. Control (a) and toxin A-treated (b–d) HeLa cells after immunostaining with anti-α-tubulin antibodies. The antigen appears to be diffused in treated cells. However, a thin microtubular cage is observable in the perinuclear region (c and d [corresponding brightfield]). Bar, 5 μm.

FIG. 5. Immunofluorescence. Control (a) and toxin A-treated (b) HeLa cells after immunostaining with antivimentin antibodies. After treatment, vimentin filaments appear to be localized in the opposite side with respect to the nucleus. Bar, 5 μm.
hand, microtubules appeared to be still organized in a thin network around the polarized nucleus, where Tau protein, known to promote the assembly of microtubules and additionally to stabilize the resulting polymers (5), was mainly condensed. However, the inhibitory effect on nuclear displacement exerted by demecolcine, a microtubule-depolymerizing agent (42), suggested that integrity of the microtubular apparatus is required for the polarization of the nucleus.

The role of intermediate filaments in the intoxication process induced by toxin A remains to be defined. In fact, keratin did not show any change after toxin treatment and displayed a well-defined network in rounded polarized cells, probably because of its structural role (7), while vimentin appeared to be colocalized with actin and α-actinin at the side opposite the nucleus. Accordingly, it has recently been shown that intermediate filaments are packed near the nuclei of intoxicated CHO cells (14). Because of the known relationships between intermediate filaments and the nuclear envelope (13), this “crowding” of cytoskeletal components could be related to the observed modification in nuclear shape, with the nucleus becoming kidney shaped in almost all intoxicated cells. Furthermore, 11-nm filaments, probably related to the intermediate filament-type lamins, have been detected in the nucleoplasm of treated cells (14). The authors (14) suggested that the disorganization in lamins induced by toxin A could eventually affect cell mitosis and lead to cell death.

It has been demonstrated that an acidic compartment is needed in the toxin A activation pathway from the cell exterior to the cytosol (11). In fact, monensin, its major effects being to dissipate proton gradients (25) and to protect cells against a number of endocytosed toxins (22), seemed to inhibit the CPE induced by toxin A. The time course for

---

**TABLE 1. Effects of various compounds on CPE**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μg/ml)</th>
<th>% RC</th>
<th>% PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochalasin B</td>
<td>0.48</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>7.89</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Demecolcine</td>
<td>0.26</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>Monensin</td>
<td>340</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>400</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Antipain</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Compounds were added together with the toxin at 37°C for 3 h. The results in both columns are expressed as percentages of rounding cells (RC) and cells with a polarized nucleus (PN) compared with the same effects in control cultures treated with the toxin but without the tested compounds. In cultures treated with toxin A alone, the CPE was observed after 1 h.*

---

**FIG. 6.** Fluorescence microscopy. TFP-treated (a) and TFP- and toxin A-treated (b) Hep-G2 cells. Actin staining. No morphological modifications are observable after 1 h of incubation with the toxin. Bar, 5 μm.

**FIG. 7.** Light microscopy. Demecolcine-treated (a) and demecolcine- and toxin A-treated (b) cells. When intoxication followed demecolcine pretreatment, cell retraction occurred but without nuclear displacement. Bar, 5 μm.
TABLE 2. Time course for prevention of toxin-induced CPE by monensin and chymostatin*  

<table>
<thead>
<tr>
<th>Time interval (min)</th>
<th>Monensin</th>
<th>Chymostatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% RC</td>
<td>% PN</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>89</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>95</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>98</td>
<td>7</td>
</tr>
<tr>
<td>45</td>
<td>100</td>
<td>94</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>95</td>
</tr>
</tbody>
</table>

* Cells were treated with the toxin (4 μg/ml) for 30 min at 4°C. The toxin solution was then replaced by growth medium, and cells were incubated at 37°C. Monensin and chymostatin were added after the time intervals indicated above. The percentages of rounding cells (RC) and cells with a polarized nucleus (PN) were evaluated after 3 h of treatment. Cells treated with toxin A without the addition of chemicals displayed 100% RC and PN and were considered controls.

prevention of toxin-induced CPE by monensin is consistent with an involvement of Golgi apparatus in the internalization process (24) as previously hypothesized by Henriques et al. (11).

Furthermore, several findings indicate that at some stage of the intoxication process, a cytoplasmic protease is essential for the activity of toxin A. This toxin could possess, like Clostridium botulinum C2 (21) and Clostridium spiroforme (26, 35) toxins, a subunit which is required for its penetration in the cytosol and which must undergo limited proteolysis to be functionally active. Chymostatin, a serine protease inhibitor (39), seemed to block the action of toxin A. It appeared to be capable of inhibiting the binding of the toxin to the cell surface, and it was protective upon addition long after the end of the binding step. In the first case, chymostatin could inhibit the action of a membrane-bound protease which activates the toxin and allows it to penetrate into the cell. Henriques et al. (11) showed that membrane-bound toxin A can be transferred to the cytosol in active form directly across the membrane when exposed to a low pH. The low pH could alter the conformation of the toxin, allowing a membrane-bound protease to activate the toxin. On the other hand, the inhibitory effect induced by chymostatin after the binding step could be explained by an involvement of an enzyme which is present on the cell surface and in some subcellular compartment too. In fact, a number of membrane-bound proteases could be found in the Golgi complex, which seems to be involved in the toxin A internalization process (11). However, Henriques et al. (11) also found that chymostatin did not have a protective effect on toxin A-treated fibroblasts. This discrepancy with our results could be due to differences among the different cell types and in particular could depend on differences in inhibition of lysosomal and cytosolic proteases, on the different relationships that different cell lines establish with the substrate, as well as on differences in the organization of the cytoskeleton.

In conclusion, it appears that cell rounding and nuclear polarization induced by toxin A from C. difficile are separate phenomena which could depend on the microfilament system and on the microtubular apparatus, respectively. Furthermore, an acidic compartment and the action of a serine protease seem to be essential for the activity of toxin A. Presently, it is unknown how toxin A causes its irreversible effect leading to cell death. However, the cytotoxicity induced by the toxin seems not to involve a nonlysosomal proteolytic system in that the Ca2+-dependent cytosolic protease inhibitors, leupeptin and antipain, did not show any protective effect against the toxin. Eventually, toxin A could represent a useful tool for cell biology studies in the same manner as cholera and pertussis toxins. The understanding of its mode of action can give further information either on basic cytoskeletal functions or on the pathology of pseudomembranous colitis.

LITERATURE CITED