INFECTION AND IMMUNITY, Sept. 1993, p. 3988–3993
0019-9567/93/093988-06$02.00/0

Vol. 61, No. 9

Clostridium difficile Toxin A Elicits Ca^{2+}-Independent Cytotoxic Effects in Cultured Normal Rat Intestinal Crypt Cells

CARLA FIORENTINI,[1]* GIANFRANCO DONELLI,[1] PIERLUIGI NICOTERA,[2] AND MONICA THELESTAM[3]

Department of Ultrastructures, Istituto Superiore di Sanità, Rome, Italy,[1] and Department of Toxicology[2] and Department of Bacteriology,[3] Karolinska Institutet, Stockholm, Sweden

Received 11 March 1993/Returned for modification 15 April 1993/Accepted 11 June 1993

In rat intestinal crypt cells, *Clostridium difficile* toxin A induces (i) early cytoskeletal alterations involving the whole population and (ii) late effects in 30 to 40% of the cells, consisting mainly of surface blebbing and nuclear fragmentation. All these effects were Ca^{2+} independent and were not abolished by protein synthesis inhibitors.

Clostridium difficile, the major etiological agent of antibiotic-associated pseudomembranous colitis (24), produces at least two high-molecular-weight protein exotoxins, toxins A and B, which are involved in the pathogenesis of the disease (17). Toxin A is an enterotoxin which upon injection into rabbit ileal loops elicits severe epithelial damage associated with hemorrhage and fluid secretion (17). It is also a cytotoxin which acts intracellularly (12) by modifying a cytosolic target that is probably involved in actin assembly (6–9). In this study, we used IEC-6 cells which originate from normal rat small intestinal tissue and display features of undifferentiated crypt cells (22). Thus, they have a diploid karyotype and a limited life span in vitro, show a strong density-dependent inhibition of growth, and in contrast to all epithelial cell lines used in toxin A studies so far (9) are not tumorigenic. It has been speculated that the cells in rat intestinal crypts may lack receptors for toxin A, since binding of the toxin in vivo was limited to the villi in both the small and the large intestines (26). However, the present study shows that at least cultured crypt cells from the small intestine do possess functional receptors for toxin A. The exclusion of the toxin from crypts in vivo probably depends on inhibited diffusion rather than a lack of receptors on crypt cells.

Rat intestinal IEC-6 cells (ATCC CRL 1592) were cultivated in Dulbecco modified Eagle's medium supplemented with 5% fetal calf serum. Toxin A, prepared from *C. difficile* VPI 10463 as previously described (8) and purified by the method of Sullivan and coworkers (25), was kindly provided by Paola Mastrantonio, Istituto Superiore di Sanità, Rome,

TABLE 1. Role of Ca^{2+} in toxin A-induced cellular effects[a]

Compound (reference)	Dose	Incubation (min)	% of cells showing CPE
None (control, toxin A only)			100
Ca^{2+} entry blockers			
Verapamil (13)	160 μM	30	100
$LaCl_3$ (1)	130 μM	30	100
Ca^{2+} ionophore A23187 (23)	25 μM	30	100
Intracellular Ca^{2+} chelator Quin-2 (19)	25 μM	15	100
Calmodulin antagonists			
Calmidazolium (20)	10 μM	30	100
Amitriptylin (3)	100 μM	30	100
Inhibitors of Ca^{2+}-activated catabolic enzymes			
Neomycin sulfate (4)	2.5 mM	60	100
Antipain (20)	500 μg/ml	30	100
Leupeptin (20)	500 μg/ml	30	100

[a] Confluent monolayers of IEC-6 cells in 96-well plates were pretreated at 37°C with each compound at the concentration and for the time indicated. The highest dose of each drug causing no morphological modification of the cells was used. Then, the cells were exposed to 1.5 μg of toxin A per ml for 3 h, and the effect of each compound on cellular intoxication was assessed microscopically and recorded as the percentage of cells showing the typical cytopathic effect (CPE). None of the tested compounds could counteract toxin A activity.

* Corresponding author.

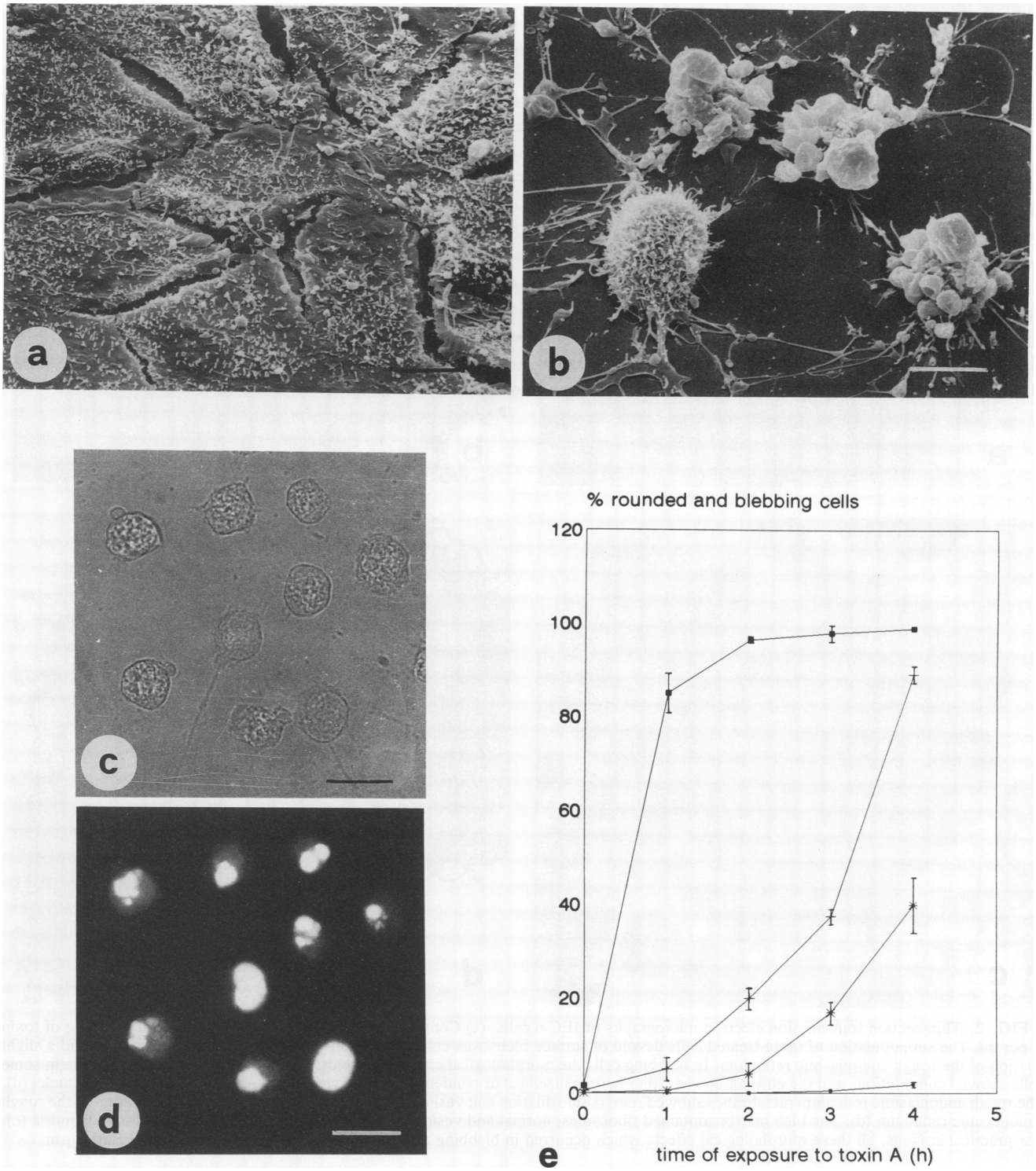

FIG. 1. Scanning electron micrographs of control IEC-6 cells (a) and IEC-6 cells treated with 1.5 μg of toxin A per ml for 3 h (b). All cells exposed to the toxin underwent cell retraction and rounding. Some cells, in addition, showed surface blebbing (b). All cells bearing surface blebs also showed nuclear fragmentation which was detected by light microscopy after the cells were stained with Hoechst 33258 (1 μg/ml in PBS for 5 min at 37°C). Bright-field (c) and fluorescence (d) micrographs of the same field are shown. Bars: a and b, 10 μm; c and d, 5 μm. Panel e shows that both cell rounding and blebbing increased with time and were also dependent on toxin concentration. The percentage of cells with fragmented nuclei was the same as that reported in panel e for blebbing cells. However, even by increasing the dose of the toxin to 20 μg/ml, we never observed more than 40 to 50% of treated cells bearing surface blebs (data not shown). Symbols: ■, blebbing cells, exposed to 0.15 μg/ml; +, rounded cells, exposed to 0.15 μg/ml; *, blebbing cells, exposed to 1.5 μg/ml; ■, rounded cells, exposed to 1.5 μg/ml.

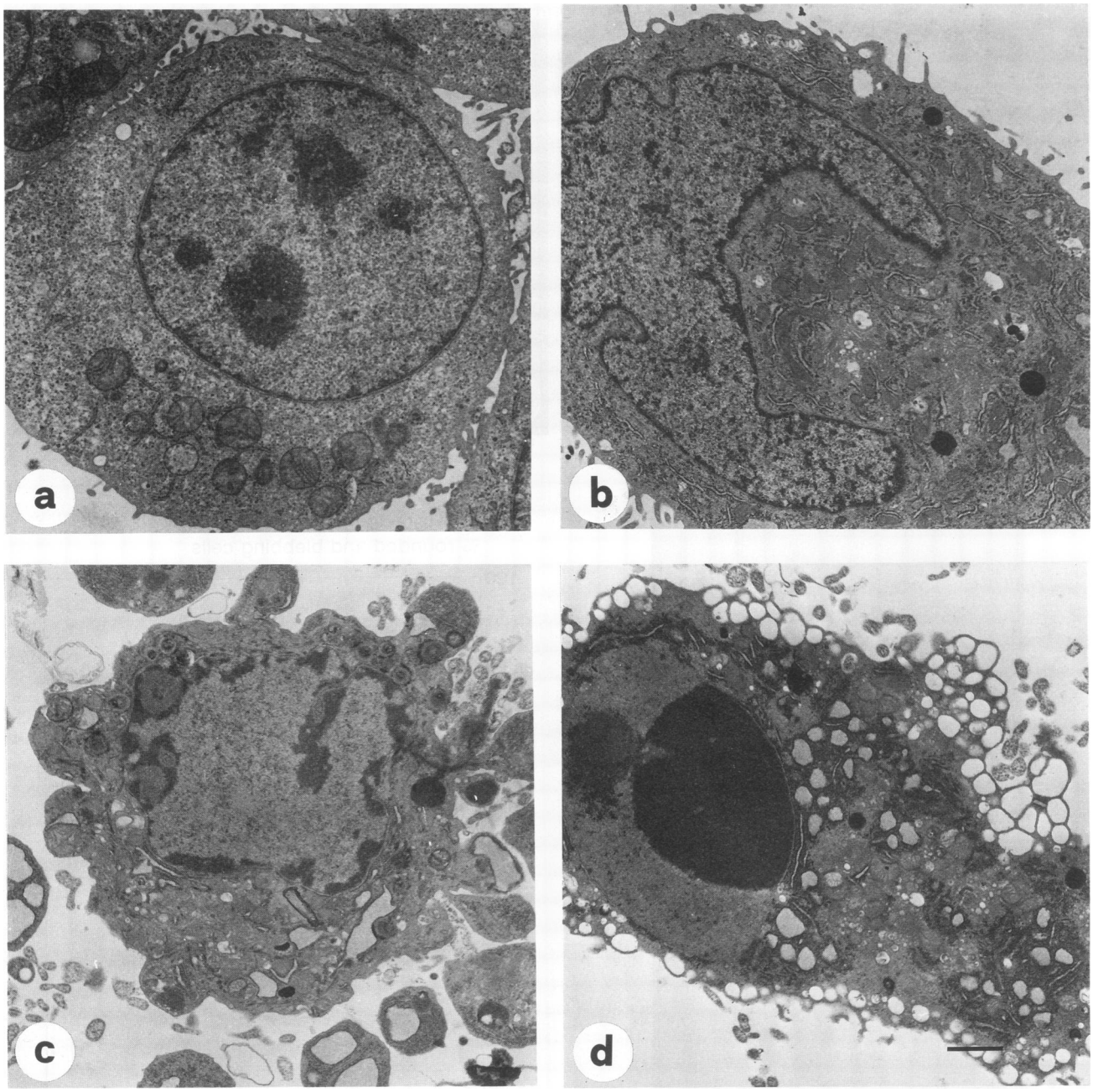

FIG. 2. Thin-section transmission electron micrographs of IEC-6 cells. (a) Control cells; (b to d) cells exposed for 3 h to 1.5 μg of toxin A per ml. The subpopulation of toxin-treated cells devoid of surface blebs was characterized by the polarization of the nucleus and a slight dilation of the rough endoplasmic reticulum. In blebbing cells the morphological changes were dramatic (b). The nuclear membrane in some cells showed convolutions and the chromatin was either marginalized (c) or condensed, with a clearly pyknotic appearance of the nuclei (d). The rough endoplasmic reticulum membranes showed remarkable dilation and vesiculation, probably produced by fragmentation of the rough endoplasmic reticulum (d). The bleb matrix contained ribosomes, normal and vesicular rough endoplasmic reticulum, and mitochondria (c). For practical reasons, all these morphological effects which occurred in blebbing cells are denoted collectively as BERN. Bar, 2 μm.

Italy. Amitriptylin was from H. Lundbeck & Co., Copenhagen, Denmark, and verapamil was from ACO, Solna, Sweden. [*methyl*-^{3}H]thymidine (specific activity, 20.0 Ci/mmol), [5-^{3}H]uridine (specific activity, 31.6 Ci/mmol), and Aquasol were from NEN. L-[4,5-^{3}H]leucine (specific activity, 75 Ci/mmol) and monoclonal antibodies against 5-bromo-2′-deoxyuridine (BrdU) were from Amersham (Buckinghamshire, United Kingdom). All other compounds were from Sigma Chemical Co., St. Louis, Mo. For scanning and transmission electron microscopy, control and treated cells were prepared as previously described (18). For detecting cells in S phase, cells were loaded with BrdU for 5 h, fixed with methanol (95%) plus acetic acid (5%) for 20 min in the dark, and air dried. Then the cells were treated with HCl (1.5

TABLE 2. Percentage of cells in S phase after toxin A treatment[a]

BrdU/toxin A treatment	Total toxin exposure time (h)	% of cells in S phase (mean ± SD)[b]
Control (BrdU only)	0	60.0 ± 3.2
BrdU for 2 h before toxin A addition	3	60.0 ± 5.8
BrdU and toxin A at the same time	5	58.0 ± 4.7
Toxin A for 1 h before BrdU addition	6	45.5 ± 2.5
Toxin A for 2 h before BrdU addition	7	35.7 ± 2.0
Toxin A for 3 h before BrdU addition	8	21.4 ± 1.1

[a] IEC-6 cells in 24-well plates were exposed to toxin A (1.5 μg/ml) for the times indicated. The cells were loaded with BrdU and processed as described in the text. After toxin A exposure for up to 5 h, the percent cells in S phase was identical in control and treated cells, whether the BrdU was added before or with the toxin. After longer toxin treatments, a decrease in the percent cells in S phase was detected. However, the blebbing cells were either positive or negative for BrdU, i.e., there was no correspondence (positive or negative) between the blebbing phenomenon and the S phase of the cell cycle.

[b] Percentage of cells which incorporated BrdU after toxin A treatment.

N) for 10 min and washed with Triton X-100 (0.5% in phosphate-buffered saline [PBS]). After incubation with a monoclonal anti-BrdU antibody for 1 h at room temperature and then with tetramethyl rhodamine isothiocyanate–anti-mouse conjugate (diluted 1:20) for 1 h at 37°C, the cells were washed in PBS, and the coverslips were mounted on glass slides with Permount. The cytosolic free calcium concentration was assayed as previously described (19) with the fluorescent indicator Fura-2/AM. The assays of precursor incorporation in DNA, RNA, and protein were performed as previously described (10).

The IEC-6 cells exposed to toxin A showed a cytoskeletal response similar to that described for other cell types (6, 9). Cytoskeletal changes can be caused by fluctuations in cytosolic Ca^{2+} or alterations of Ca^{2+}-binding proteins, including calmodulin (20). *C. difficile* toxin B was reported to require active calmodulin and uptake of extracellular Ca^{2+} in order to intoxicate human lung fibroblasts (3). By contrast, neither Ca^{2+} nor Ca^{2+}-binding proteins or enzymes appeared to be involved in the cellular response to toxin A (Table 1). Accordingly, Lima and coworkers (16) observed that the toxin A-induced cytopathic effect in CHO cells was not affected by the Ca^{2+} channel blocker diltiazem or by the calmodulin inhibitor trifluoperazine. Furthermore, the primary cytoskeletal alterations induced by toxin A in cultured intestinal cells were not due to changes in the cytosolic Ca^{2+} level since, when measured by fura-2 (11), the cytosolic Ca^{2+} concentration was unmodified in IEC-6 cells within 1 h of exposure to the toxin (data not shown). Parallel experiments with Y1 cells indicated that the lack of effect on cytosolic Ca^{2+} was not restricted to IEC-6 cells. In contrast to these findings, toxin A was reported to stimulate intracellular Ca^{2+} release in human granulocytes within 3 s (21). This rapid effect is suggestive of a transmembrane activation immediately upon contact with toxin A. It can be dependent on the peculiar characteristics of granulocytes and not related to the cytoskeletal alterations which require toxin internalization.

The blebbing phenomenon induced by *C. difficile* toxin B in various types of cultured cells (18), including IEC-6 cells, has been observed previously (unpublished observations). By contrast, toxin A has so far been described as causing blebbing only in IEC-6 (Fig. 1). Although the general modes of action of the two toxins are very similar (4), they act differently on the associations between actin and actin-binding proteins (6, 8, 18) and with respect to Ca^{2+} involvement (3). Speculatively, these last findings could explain why the toxin B-induced blebbing occurred as a more general response in different cell types. Since the blebbing, endoplasmic reticulum, and nuclear change (BERN) effects caused by toxin A (Fig. 2) are present only in a fraction of treated cells, they cannot be merely a consequence of the general cytoskeletal breakdown. Moreover, the BERN response was linked neither to the cell cycle (Table 2) nor to the age of the cells in vitro (the number of passages), and the response was similar whether the cells were sparsely growing or the cultures were confluent (data not shown). Thus, the reason why only a cell fraction shows surface blebs remains to be defined. Since IEC-6 cells have the potential to differentiate in vitro into adsorptive and secretive cells, respectively (14), the different responses may be due to the concomitant presence in the culture of cells in different stages of spontaneous differentiation. Another reason may be a different stage of cell aging, mainly in terms of an unbalance in the enzyme machinery, which is known to

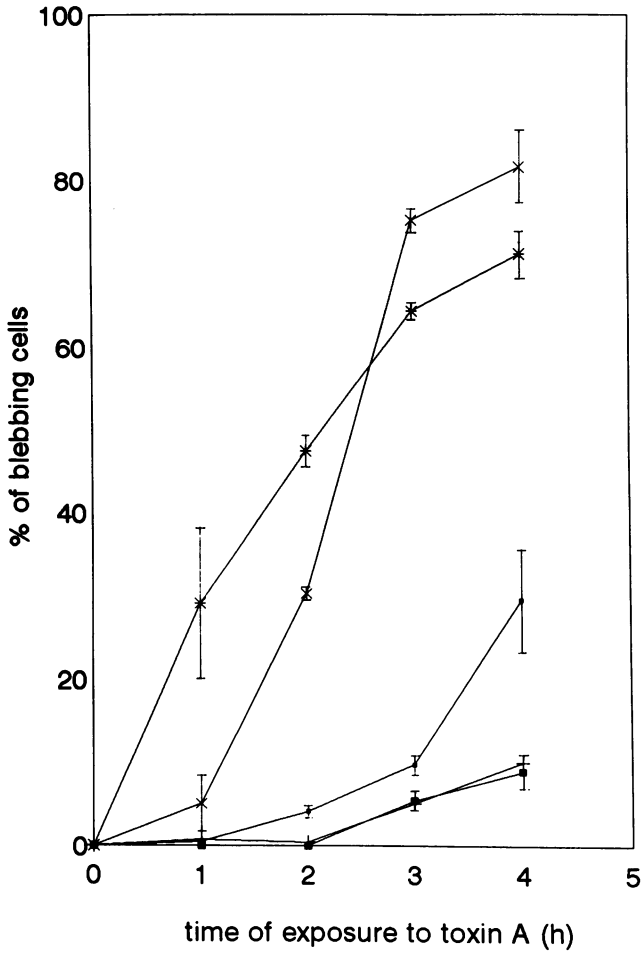

FIG. 3. Influence of cycloheximide (CHX) and actinomycin D (AcD) on toxin A-induced blebbing in IEC-6 cells. The cells were treated either with 50 μg of CHX or with 10 μg of AcD per ml for 30 min before the addition of 1.5 μg of toxin A per ml. The mean percent blebbing cells ± standard deviation was scored at the times indicated. In toxin A-treated cells, both drugs strongly increased the proportion of blebbing cells. Symbols: ■, toxin A alone; +, CHX alone; *, CHX plus toxin A; ■, AcD alone; ×, AcD plus toxin A.

TABLE 3. Effect of toxin A on macromolecular synthesis[a]

Time (h) of toxin exposure	% of cells showing CPE[b]	% Precursor incorporation in[c]:		
		DNA	RNA	Protein
0	0	100	100	100
1	0	111.5 ± 15.3	102.8 ± 9.06	97.1 ± 8.50
3	100	95.2 ± 9.30	80.4 ± 5.22	102 ± 10.5
6	100	51.8 ± 3.88	56.7 ± 5.97	80.6 ± 19.1

[a] Confluent monolayers of IEC-6 cells in 24-well plates were exposed for the times indicated to toxin A diluted in growth medium to 1.5 μg/ml at 37°C. Each experiment included six control cultures and triplicate toxin-treated cultures for each time point. Each experiment was performed at least three times. Toxin A in itself did not inhibit protein synthesis within the first 3 h of exposure, and RNA synthesis was only slightly depressed as measured by the incorporation of radiolabelled leucine and uridine, respectively. In addition, a decrease in thymidine incorporation in DNA was seen only after 6 h, when the rounding up had developed in 100% of the cells, and the inhibition was complete after 24 h (data not shown).

[b] CPE, cytopathic effect.

[c] Results were calculated as counts per minute per microgram of protein and expressed as percents (means ± standard deviations) of similarly treated controls in which toxin treatment was omitted.

counteract several degenerative processes including blebbing (for a review, see reference 5). The BERN effects closely resemble those seen in apoptosis induced by various agents (27) such as the phosphatase inhibitor okadaic acid (2), which is also a diarrheogenic toxin. Apoptosis has traditionally been associated with programmed cell death, in which dependence on active synthesis of mRNA and protein has been considered a cardinal feature. However, protein synthesis does not seem to be required in all instances (15). Toxin A-treated cells were actually sensitized to cycloheximide and actinomycin D, which inhibit synthesis of protein and mRNA, respectively (Fig. 3). These metabolic inhibitors might confer an additional stress on the exposed cells, which results in blebbing of a larger cell population. However, as shown in Table 3, toxin A in itself did not inhibit protein or DNA synthesis before the development of the cytoplasmic effect, and RNA synthesis was only slightly depressed. Thus, it seems that the toxin A-induced depression of macromolecular synthesis can be a consequence but not the cause of the cytoplasmic effect and/or the BERN response.

In conclusion, our data strongly suggest that the cytoskeletal changes induced by toxin A are achieved without any measurable effects on the cellular Ca^{2+} homeostasis. The reason why IEC-6 cells exposed to toxin A undergo cell death through two morphologically separated pathways is now under investigation. Taking into account the characteristics of these crypt cells, we propose IEC-6 cells as a useful cell model for studies on the cellular mode of action of enterotoxins.

We are grateful to Paola Mastrantonio for her continuing cooperation and for providing purified toxin A. We also thank Ove Lundgren for providing IEC-6 cells.

This study was supported in part by the National Research Council (CNR)-targeted project Prevention and Control of Disease Factors grant 93 00769 PF41 and by the Swedish Medical Research Council grant 16X-05969.

REFERENCES

1. **Andersson, K.-E., and E. D. Hogestatt.** 1984. On the mechanism of action of calcium antagonists. Acta Med. Scand. **681**(Suppl.): 11–24.
2. **Boe, R., B. T. Gjertsen, O. K. Vintermyr, G. Houge, M. Lanotte, and S. O. Doskeland.** 1991. The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. Exp. Cell Res. **195:**237–246.
3. **Caspar, M., I. Florin, and M. Thelestam.** 1987. Calcium and calmodulin in cellular intoxication with *Clostridium difficile* toxin B. J. Cell. Physiol. **132:**168–172.
4. **Caspar Shoshan, M., C. Fiorentini, and M. Thelestam.** 1993. Signal transduction pathways and cellular intoxication with *Clostridium difficile* toxins. J. Cell. Biochem. **52:**1–9.
5. **Cutler, R. G.** 1991. Human longevity and aging: possible role of reactive oxygen species. Part I. Basic aging processes. Ann. N.Y. Acad. Sci. **621:**1–28.
6. **Fiorentini, C., G. Arancia, S. Paradisi, G. Donelli, M. Giuliano, F. Piemonte, and P. Mastrantonio.** 1989. Effects of *Clostridium difficile* toxins A and B on cytoskeleton organization in HEp-2 cells: a comparative morphological study. Toxicon **27:**1209–1218.
7. **Fiorentini, C., S. C. Chow, P. Mastrantonio, M. Jeddi-Tehrani, and M. Thelestam.** 1992. *Clostridium difficile* toxin A induces multinucleation in the human leukemic T cell line JURKAT. Eur. J. Cell Biol. **57:**292–297.
8. **Fiorentini, C., W. Malorni, S. Paradisi, M. Giuliano, P. Mastrantonio, and G. Donelli.** 1990. Interaction of *Clostridium difficile* toxin A with cultured cells: cytoskeletal changes and nuclear polarization. Infect. Immun. **58:**2329–2336.
9. **Fiorentini, C., and M. Thelestam.** 1991. *Clostridium difficile* toxin A and its effects on cells. Toxicon **29:**543–567.
10. **Florin, I., and M. Thelestam.** 1981. Intoxication of cultured human lung fibroblasts with *Clostridium difficile* toxin. Infect. Immun. **33:**67–74.
11. **Grynkiewicz, G., M. Poenie, and R. Y. Tsien.** 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260:**3440–3450.
12. **Henriques, B., I. Florin, and M. Thelestam.** 1987. Cellular internalisation of *Clostridium difficile* toxin A. Microb. Pathog. **2:**455–463.
13. **Hosey, M. M., and M. Lazdunski.** 1988. Calcium channels. Molecular pharmacology, structure and regulation. J. Membr. Biol. **104:**81–105.
14. **Kurokawa, M., K. Lynch, and D. K. Podolsky.** 1987. Effects of growth factors on an intestinal epithelial cell line: transforming growth factor beta inhibits proliferation and stimulates differentiation. Biochem. Biophys. Res. Commun. **142:**775–782.
15. **Larrick, J. W., and S. C. Wright.** 1990. Cytotoxic mechanism of tumor necrosis factor-alpha. FASEB J. **4:**3215–3223.
16. **Lima, A. A. M., D. M. Lyerly, T. D. Wilkins, D. J. Innes, and R. L. Guerrant.** 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine in vivo and on cultured cells in vitro. Infect. Immun. **56:**582–588.
17. **Lyerly, D. M., H. C. Krivan, and T. D. Wilkins.** 1988. *Clostridium difficile*: its disease and toxins. Clin. Microbiol. Rev. **1:**1–18.
18. **Malorni, W., C. Fiorentini, S. Paradisi, M. Giuliano, P. Mastrantonio, and G. Donelli.** 1990. Surface blebbing and cytoskeletal changes induced in vitro by toxin B from *Clostridium difficile*: an immunochemical and ultrastructural study. Exp. Mol. Pathol. **52:**340–356.
19. **Nicotera, P., H. Thor, and S. Orrenius.** 1989. Cytosolic-free Ca^{2+} and cell killing in hepatoma lclc7 cells exposed to chemical anoxia. FASEB J. **3:**59–64.
20. **Orrenius, S., and P. Nicotera.** 1987. On the role of calcium in chemical toxicity. Arch. Toxicol. Suppl. **11:**11–19.
21. **Pothoulakis, C., R. Sullivan, D. A. Melnick, G. Triadafilopoulos, A. S. Gadenne, T. Meshulam, and J. T. La Mont.** 1988. *Clostridium difficile* toxin A stimulates intracellular calcium release and chemotactic response in human granulocytes. J. Clin. Invest. **81:**1741–1745.
22. **Quaroni, A., J. Wands, R. L. Trelstad, and K. J. Isselbacher.** 1979. Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. J. Cell Biol. **80:**248–265.
23. **Reed, P. W., and H. A. Lardy.** 1972. A23187: a potentiation ionophore. J. Biol. Chem. **247:**6970–6977.

24. **Rolfe, R. D., and S. M. Finegold (ed.).** 1988. *Clostridium difficile*: its role in intestinal disease. Academic Press, Inc., New York.
25. **Sullivan, N. M., S. Pellet, and T. D. Wilkins.** 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1032–1040.
26. **Torres, J., E. Jennische, S. Lange, and I. Lonnroth.** 1990. Enterotoxins from *Clostridium difficile*: diarrhoeogenic potency and morphological effects in the rat intestine. Gut **31**:781–785.
27. **Trump, B. F., and I. K. Berezesky.** 1992. The role of cytosolic Ca^{2+} in cell injury, necrosis and apoptosis. Curr. Opin. Cell Biol. **4**:227–232.