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Intoxication of Cultured Human Lung Fibroblasts with *Clostridium difficile* Toxin

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The cytopathogenic effect of partially purified toxin from *Clostridium difficile* on cultured human lung fibroblasts was studied. Conditions for determination of 50% tissue culture dose were standardized. The cytopathogenic effect of the toxin was dependent on toxin concentration, exposure time, and density of the cells. Transfer of the cells to 0°C did not inhibit binding of toxin to the fibroblast surface, but prevented the development of the cytopathogenic effect. Both binding of toxin and some intracellular step(s) were prevented by 2,4-dinitrophenol. These preventative effects were reversible. Before and concomitantly with the appearance of the cytopathogenic effect, the cellular uptake of uridine and of amino acids was markedly stimulated. Protein synthesis was depressed when 100% of the cells showed the cytopathogenic effect, but the synthesis of nucleic acids was inhibited only several hours later. The primary cellular target for the toxin is still unknown.

The anaerobic bacterium *Clostridium difficile* was first isolated in 1935 from the intestinal flora of newborn infants (8). It was found to produce a soluble exotoxin which was lethal for several animals on subcutaneous injection (8, 17). However, neither the bacterium nor its toxin was considered to be of any pathogenic significance for humans until more than 40 years later. In 1977, it was found that the feces of patients with antibiotic-associated pseudomembraneous colitis were cytotoxic to cultured cells (9, 15). The responsible agent was identified as a toxin from *C. difficile* (3, 10).

The toxin, later purified and biochemically partially characterized (16, 18), was found to be toxic to a variety of cultured mammalian cells (3, 10, 19). In fibroblasts, it induces a typical morphological change described as actinomorphic (3), and in epithelium- and neuron-like cell lines it induces a rounding up of the cells (9, 19).

Chang et al. (4) reported on ultrastructural changes of the surfaces of toxin-treated human amnion cells. Thelestam and Brönnegård (19), using an indirect immunofluorescence technique, demonstrated that in human lung fibroblasts the toxin causes a disruption of the stress fibers (20) which are formed by actin microfilaments. The morphological effect, which appeared after a latency period of 3 to 5 h, was correlated in time to stress fiber breakdown, but the cells were irreversibly intoxicated already about 20 min after the initial contact with the toxin (19). In addition, the morphological and cytoskeletal changes resembled those induced by the fungal alkaloid cytochalasin B, although the latency period of the latter was shorter, and no competition between the two agents could be demonstrated. Furthermore, the toxin-induced effect was irreversible, whereas that produced by cytochalasins was completely reversed in less than 1 h after removal of the cytochalasin (13, 14, 19).

Thelestam and Brönnegård (19) speculated that the toxin might associate with some integral membrane protein directly or indirectly connected with submembraneous microfilaments, thereby producing a disordering in the putative link between the plasma membrane and the cytoskeleton (1). However, the rather long latency period before appearance of the morphological effect might indicate that the disruption of stress fibers occurs secondary to intracellular changes during the intoxication process. A similar breakdown of stress fibers has been described in connection with some viral infections of cultured cells, but the basis for this phenomenon is not understood (6, 12).

It is conceivable that the cytotoxic action of the *C. difficile* toxin constitutes the basis for its pathological effect on the intestine. Thus, an understanding of the mechanism of the cytotoxic action may improve the possibilities of preventing and treating the disease. In this first descrip-

tive paper we convey some basic features of the cellular response to treatment with the *C. difficile* toxin.

MATERIALS AND METHODS

Chemicals. Eagle minimal essential medium, newborn calf serum, and crude trypsin were obtained from Flow Laboratories, Ltd., Irvine, Scotland; Hanks balanced salt solution and prereduced, anaerobically sterilized chopped meat medium were from the National Bacteriological Laboratory, Stockholm, Sweden. [*methyl*-^{3}H]thymidine (specific activity, 20 Ci/mmol), [5-^{3}H]uridine (specific activity, 29 Ci/mmol), [α-*methyl*-^{3}H]aminoisobutyric acid (AIB; specific activity, 10 Ci/mmol) and Aquasol universal cocktail were from NEN Chemicals GmbH, Dreieich, Federal Republic of Germany, and *U*-^{14}C-labeled protein hydrolysate (specific activity, 59 mCi/matom of C) was from The Radiochemical Centre, Amersham, England. Triton X-100 (technical grade) was from Rohm and Haas Co., Philadelphia, Pa., and 2,4-dinitrophenol (DNP) was from Fluka AG, Chemische Fabrik, Buchs SG, Switzerland. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of toxin. *C. difficile* ATCC 9689 was cultivated in prereduced, anaerobically sterilized chopped meat medium for 72 h.

(i) Extracellular toxin. Extracellular toxin was harvested by centrifugation (8,000 × *g*, 30 min, 4°C), and the culture supernatant was filtered (0.22-μm pore size; Millipore Corp.). The toxin was precipitated from the filtrate by addition of ammonium sulfate to 65% saturation. The precipitate was harvested and dissolved in tris(hydroxymethyl)aminomethane-buffered saline [TBS; i.e., 0.15 mM NaCl in 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.0] and fractionated on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Active fractions were pooled, concentrated and further purified by chromatography on a diethylaminoethyl-Sephadex A-25 column (Pharmacia).

(ii) Cell-bound toxin. Cell-bound toxin was prepared by repeated extraction of the centrifuged bacteria with small volumes of 1 M NaCl. These salt extracts were pooled, filtered, and concentrated. The specific cytotoxic activity obtained in salt extracts was considerably higher than that of concentrated, crude culture filtrates or of toxin partially purified as described in the preceding section (B. Aronsson and R. Möllby, manuscript in preparation). However, the cellular response was identical with all preparations.

Cultivation of cells. Human diploid embryonic lung fibroblasts (line MRC-5) were cultivated in Eagle minimal essential medium supplemented with 10% calf serum, 5 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), hereafter referred to as growth medium. The cultures were maintained in a humid atmosphere containing 5% CO_2. Cells to be used for cytotoxicity tests were grown in polystyrene microtiter plates (96 wells; area, 0.3 cm^2/well) (Falcon Plastics, Oxnard, Calif.) or in six-well plates (area, 9 cm^2/well) (Linbro Chemicals Co., New Haven, Conn.). The cells were free of mycoplasma infection as determined by cultivation and autoradiography.

Measurement of cytotoxic activity. The unit of toxin activity was the 50% tissue culture dose (TCD_{50}), i.e., the toxin dilution inducing a characteristic actinomorphic change in 50% of the exposed cells. For determination of the TCD_{50}, logarithmically growing cells in microtiter plates (5,000 to 10,000 cells per well) were exposed to serial twofold dilutions (200 μl) of the toxin in growth medium at 37°C for 20 to 22 h. The dilution of toxin causing an effect closest to 50% cytopathogenic effect (CPE) was established by counting the numbers of affected and unaffected cells. The cell counts were performed with an inverted microscope (Olympus Tokyo CK), and three randomly selected fields of view were counted per well.

In the experiments described in the present study the exposure time was usually shorter than 20 to 22 h; hence, larger amounts of toxin, i.e., 100 to 1,000 TCD_{50}, were used. Unless otherwise stated, the cells were rinsed with Hanks balanced salt solution after exposure to the toxin, fresh growth medium was then added, and the incubation was continued at 37°C until scoring the percent CPE. The values for percent CPE in the figures and tables are averages of duplicate or triplicate samples.

Measurement of uptake and incorporation of radiolabeled precursors into cellular macromolecules. These experiments were performed by the method of Crowe et al. (5). Toxin-treated cells in six-well plates were rinsed and incubated with radioactive thymidine (2 μCi/ml in growth medium), uridine (2 μCi/ml in TBS), or protein hydrolysate (0.5 μCi/ml in TBS) for 30 min at 37°C. Extracellular radioactivity was carefully removed by rinsing three times with ice-cold Hanks balanced salt solution. The cell material was transferred to test tubes in 1 ml of 0.1% sodium dodecyl sulfate. Bovine serum albumin (1 mg) was added to each tube as a carrier protein, and cellular macromolecules were precipitated by adding 100% trichloroacetic acid to a final concentration of 10%. The tubes were left in the cold for at least 30 min and then centrifuged (2,000 rpm, 10 min). The supernatant fractions were removed, and radioactivity was counted (0.5 ml in 10 ml Aquasol) in a Nuclear Chicago Scintillation Counter to measure uptake of the precursors into the acid-soluble pool. The radioactivity in acid-insoluble material was determined by suspending the pellets in 0.2 ml of 0.1 M NaOH and counting of incorporated radiolabel in 10 ml of Aquasol. Protein was determined by the method of Lowry et al. (11).

The uptake and incorporation of radiolabel per microgram of protein were calculated, and the results are expressed as percentage of the corresponding values for control cultures in which the toxin treatment had been omitted. All experiments were performed in triplicate.

Measurement of uptake of AIB. Toxin-treated cells in six-well plates were rinsed and incubated for 10 min at 37°C with a prewarmed solution of AIB (2 μCi/ml in Hanks balanced salt solution buffered to pH 7.4 with 10 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid). The cells were quickly rinsed twice with ice-cold Hanks balanced salt solution. Accumulated AIB was released by incubation of cells with 0.1% Triton X-100 for 30 min at 37°C, and the radioactivity was determined in 0.1-ml portions of

the lysates. The AIB uptake per microgram of protein was calculated, and the values are expressed as percentage of the corresponding values for control cultures.

RESULTS

Standardization of conditions for determination of TCD_{50}. From a series of titrations under various conditions (Fig. 1) it was concluded that with longer exposure times less toxin was needed to obtain a 50% CPE; with higher cell densities more toxin was needed. More toxin was also needed to get a 50% CPE when the effect was recorded after 4 h than was needed when the effect was recorded after 20 h, indicating that the CPE is not fully developed after 4 h. Beyond 20 h, however, the CPE was not augmented.

Based on these findings, the TCD_{50} was defined as described above. Numerous subsequently repeated titrations with the same batch of toxin have indicated that the test is highly reproducible.

Dose-response and time course relationships. Table 1 demonstrates that toxin diluted in growth medium caused a significantly greater CPE than toxin diluted in TBS. Presumably the toxin was internalized or processed (or both) at the membrane level less effectively by cells deprived of serum and other components in the growth medium. Thus, in all subsequent experiments the toxin was diluted in growth medium.

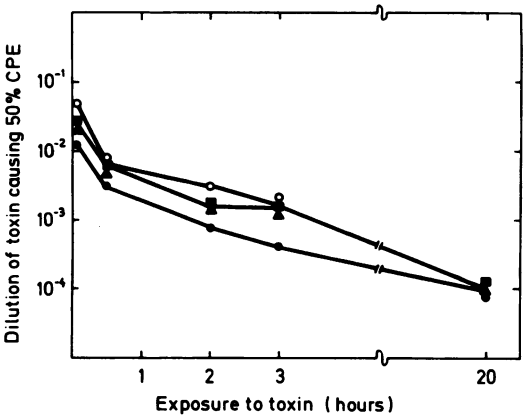

FIG. 1. *Determination of TCD_{50} as a function of toxin dose, cell density, and time of exposure of cells to toxin. Cells were treated at 37°C with serial twofold dilutions of toxin in TBS for the time periods indicated in the figure, whereupon the toxin solutions were replaced by growth medium. The TCD_{50} values were recorded 4 and 20 h after the beginning of the experiment. The experiment was repeated on 3 consecutive days with cultures containing approximately 5,000 (day 4), 10,000 (day 5), and 15,000 (day 6) cells per well. Symbols: ○, day 4, CPE recorded 4 h after the beginning of the experiment; ●, day 4, CPE recorded 20 h after the beginning of the experiment; ▲, day 5, CPE recorded 20 h after the beginning of the experiment; ■, day 6, CPE recorded 20 h after the beginning of the experiment.*

TABLE 1. *Influence of the incubation medium on cellular intoxication*[a]

Concentration of toxin (TCD_{50})	% CPE in	
	Growth medium	TBS
8	8	4
16	22	4
32	52	15
64	76	44
128	89	82

[a] Cells in microtiter plates were treated at 37°C for 30 min with increasing concentrations of toxin in growth medium or TBS. Percent CPE was scored after further incubation in growth medium for 20 h at 37°C.

Figure 2 shows a set of dose-response curves performed with different exposure times. Increasing concentrations of the toxin induced the cytopathogenic effect in an increasing proportion of the cells as scored 21 h after the first contact of the cells with the toxin. The final level of CPE was also dependent on the length of the exposure time to the toxin. However, the largest influence of time of exposure was seen during the first hour. The CPE was not increased after 6 h of exposure to the toxin.

Influence of temperature on cytopathogenicity. Previous experiments indicated that although binding of toxin to the cell surface was not blocked at low temperature, 0°C, the appearance of the cytotoxic effect was prevented by further incubation at 0°C (19). The present experiments confirmed that the CPE did not develop as long as the cells were held at 0°C (for up to 24 h). Upon elevation of the temperature to 37°C, however, the CPE appeared with the same length of latency period (i.e., 2 h) at 37°C, as in control cultures.

The prevention of CPE at 0°C suggested that this might be due to abolition of a transmembrane signal or to inhibition of penetration of the cell membrane by the toxin because of a lowered fluidity of the membrane at 0°C. However, when the toxin was first allowed to act upon the cells at 37°C to allow for toxin penetration and these cells were then rinsed and placed on ice, the subsequent CPE was still blocked (Fig. 3). Furthermore, such prevention of the CPE was also obtained by lowering the temperature of intoxicated cells as late as immediately before the morphological change appeared in control cultures held at 37°C. Moreover, a partial effect already obtained at 37°C (e.g., 60% CPE, Fig. 3) was stopped at this level

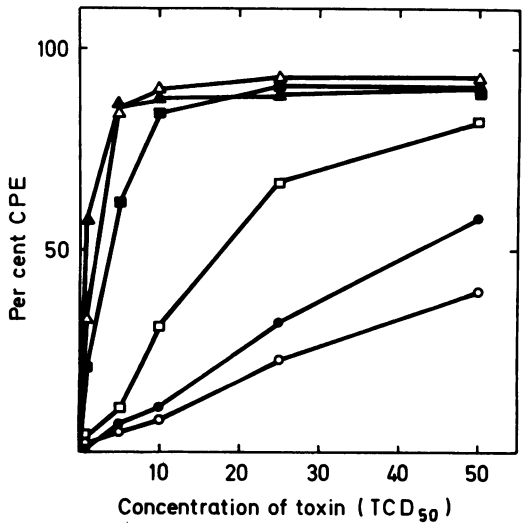

FIG. 2. *Dose-response curves. Cells were treated at 37°C for different times with the same series of increasing doses of toxin, whereupon the toxin solutions were replaced by growth medium. Percent CPE was scored 21 h after the beginning of the experiment. The CPE values in the figure are the averages of two separate experiments with duplicate samples in each. Times of incubation with toxin: ○, 5 min; ●, 10 min; □, 30 min; ■, 3 h; △, 6 h; ▲, 21 h.*

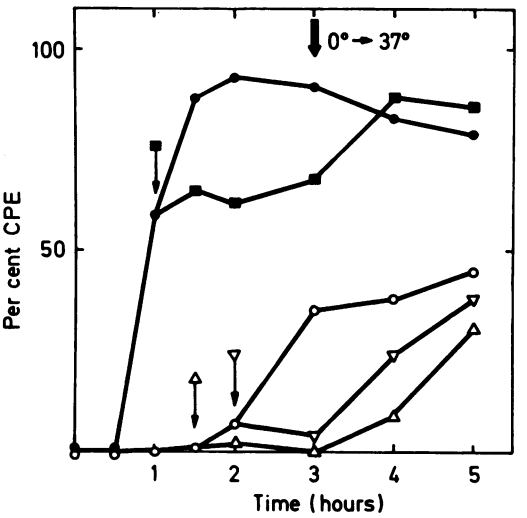

FIG. 3. *Time course for the effect of incubation temperature on toxin-induced CPE. Cells were treated at 37°C for 10 min with two different doses of toxin, whereupon the toxin solutions were replaced by growth medium. Control cultures were incubated at 37°C during the entire experiment. Other cultures were transferred to 0°C after various time periods (thin arrows). At 3 h after the beginning of the experiment all cultures in 0°C were transferred back to 37°C (thick arrow). Percent CPE was scored after the time periods indicated in the figure. Symbols: ●, 1,000 TCD_{50}, control; ■, 1,000 TCD_{50}, transfer to 0°C at 1 h; ○, 100 TCD_{50}, control; △, 100 TCD_{50}, transfer to 0°C at 1.5 h; ▽, 100 TCD_{50}, transfer at 0°C at 2 h.*

by transfer of the cells to 0°C. When the temperature was again elevated to 37°C, the cytotoxic process continued until the same CPE level as in the control cultures was reached.

Influence of DNP on cytopathogenicity. Previous experiments showed that 10 mM DNP added to the cells 30 min before the toxin exposure started prevented the appearance of the CPE whether or not the DNP was present during the latency period (19). The present experiments were designed to elucidate whether the preventative effect of DNP occurred only at the membrane level or at both the membrane level and intracellularly. Toxin-induced CPE was prevented by addition of DNP during the latency period (Fig. 4). Moreover, a partially developed CPE was stopped at that level by the addition of DNP (Fig. 4) as when using low temperature as the preventative agent. This indicates that some intracellular step(s) in the intoxication process was dependent on adenosine 5'-triphosphate.

A reversibility similar to that found with low temperature as the preventative agent occurred with DNP, provided that the exposure to DNP was short enough (<90 min) not to cause irreversible cell damage by itself (Florin and Thelestam, unpublished data).

Effects of toxin on cellular macromolecular synthesis and transport functions. The incorporation of radiolabeled thymidine, uridine, and amino acids into deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein, respectively, gradually diminished in toxin-treated cells (Fig. 5). In the case of the nucleic acids, these effects developed slowly and after the appearance of the CPE in 90 to 100% of the cells. In the case of incorporation of uridine into RNA, there was first a stimulation simultaneous with the onset of the CPE, followed by a depression to the level of the controls. After overnight incubation with the toxin, a slight inhibition of the RNA synthesis was evident, and the DNA synthesis then also diminished but to a much lower level (Fig. 5A and B). Protein synthesis (after an early stimulation) was depressed to about 50% already after 4 h of toxin exposure. At this stage, the intoxication in terms of CPE had already reached its maximum.

Mitomycin C, actinomycin D, and puromycin were used as positive controls for inhibition of the synthesis of DNA, RNA, and protein (2). As

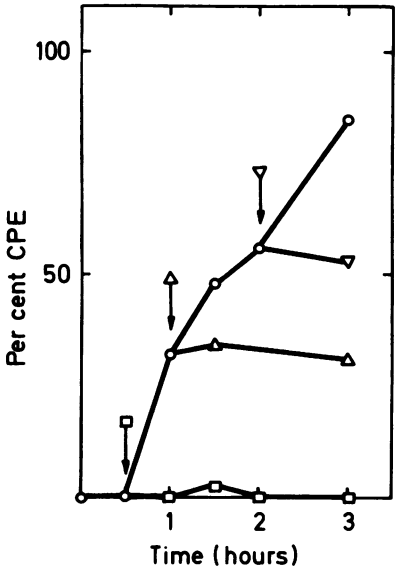

FIG. 4. *Time course for the effect of 2,4-dinitrophenol on toxin-induced CPE. Cells were treated at 37°C for 10 min with toxin (1,000 TCD_{50}), whereupon the toxin solutions were replaced by growth medium; 10 mM DNP (in growth medium) was added to the cultures after various times after treatment with toxin (arrows). Percent CPE was scored after the time periods indicated in the figure. Symbols: ○, control without DNP; □, DNP added at 0.5 h; △, DNP added at 1 h; ▽, DNP added at 2 h.*

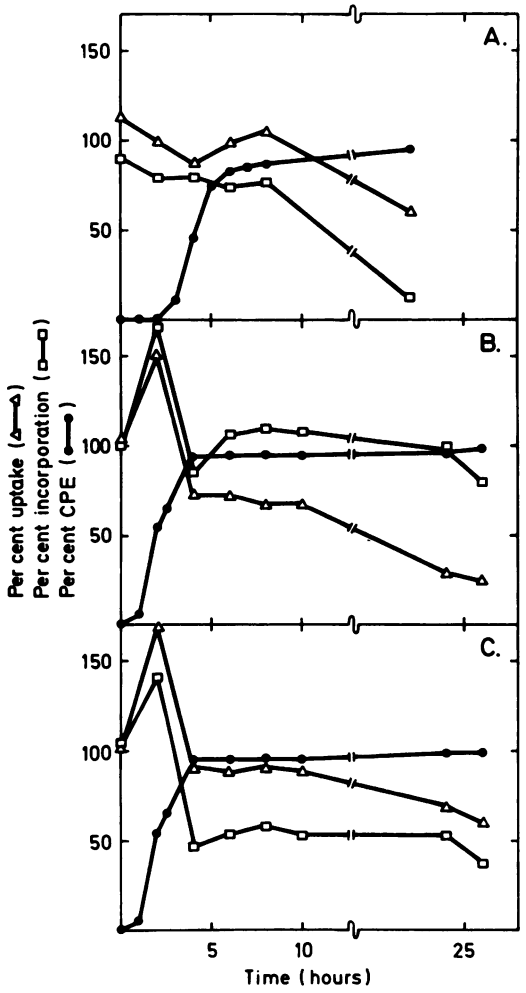

FIG. 5. *Effect of toxin on macromolecular synthesis and precursor uptake. Uptake (△) and incorporation (□) of precursors into cellular macromolecules were measured in toxin-treated (200 TCD_{50}) cells after the time periods indicated in the figure (see text). Percent CPE (●) was scored after the time periods indicated in the figure. Panels: A, uptake of thymidine and incorporation into DNA; B, uptake of uridine and incorporation into RNA; C, uptake of protein hydrolysate and incorporation into protein.*

expected, these substances inhibited incorporation of the respective precursors into acid-insoluble material in the cells (Table 2).

The cellular uptake of these precursors was also gradually lowered in toxin-treated cells. The inhibition of incorporation of thymidine into DNA parallelled the lowered uptake of thymidine into the acid-soluble pool (Fig. 5A). The eventual inhibition of RNA synthesis, however, seemed to depend on the lowered uptake of uridine (Fig. 5B). Protein synthesis was significantly depressed for at least 6 h before the uptake of amino acids was depressed (Fig. 5C). Thus, the inhibition of protein synthesis did not seem to depend on a lowered uptake of amino acids.

An initial elevation in the acid-soluble pool was observed for amino acids (Fig. 5C). To test whether this was really due to stimulation of membrane transport, the uptake of non-metabolizable AIB was measured in toxin-treated cells (Fig. 6). Indeed, the transport of AIB was stimulated in toxin-treated cells before the onset of any morphological change. However, the AIB uptake was gradually inhibited in cells which had undergone the morphological change, as was the uptake of other amino acids.

DISCUSSION

The toxin from *C. difficile* is cytopathogenic for all mammalian cell lines tested so far (7, 19). As an in vitro model for studies of the potential in vivo pathogenicity of the toxin, diploid intestinal epithelial cells would probably be most

TABLE 2. *Effects of metabolic inhibitors on macromolecular synthesis*[a]

Inhibitor	Exposure time (h)	Macromolecular synthesis in % of control cultures
Mitomycin C (50 μg/ml in growth medium)	0	86
	1	39
	6	26
Actinomycin D (5 μg/ml in growth medium)	0	75
	1	43
	3	36
Puromycin (500 μg/ml in TBS)	0	113
	0.25	32
	0.50	37

[a] Macromolecular incorporation of radiolabeled thymidine, uridine, and protein hydrolysate were measured after exposure to mitomycin C, actinomycin D, and puromycin, respectively, as described in the text.

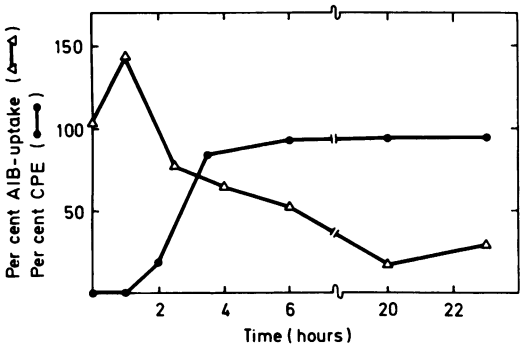

FIG. 6. *Transport of AIB into toxin-treated cells. Uptake of AIB* (△) *was measured in toxin-treated (200 TCD_{50}) cells after the time periods indicated in the figure (see text). Each point is the average of triplicate samples. Percent CPE* (●) *was scored after the time periods indicated in the figure.*

relevant, but cultures of such cells have so far not been possible to propagate serially. However, the basic mechanism for cellular intoxication should be independent of the cell type used. It was found earlier that diploid fibroblasts (lung and intestine) were more sensitive to the toxin than were various epithelium-like established cell lines (19). Therefore, fibroblasts were used as test cells in the present study. The lung fibroblast was chosen instead of the intestinal fibroblast because of the slow growth of the latter cell.

Our findings indicate that the cytotoxic effect of the *C. difficile* toxin, besides being related to the toxin concentration and time of exposure (Fig. 1 and 2), is also dependent on the density of the cells and the time for scoring the effect (Fig. 1) as well as the composition of the incubation medium during exposure to toxin (Table 1). Thus, the cytotoxicity test, when applied for diagnostic purposes, should be carefully standardized to permit comparison of findings within the same and between different laboratories.

In Table 3, the observations up to now regarding the sequence of events during cellular intoxication with the *C. difficile* toxin have been summarized and combined with questions and speculations relevant to each observation. The discussion below will follow this hypothetical scheme.

The toxin binds to the cell surface at low

TABLE 3. *Sequence of events during cellular intoxication with C. difficile toxin; observations, questions, and speculations*

Step[a]	Observations	Questions/speculations
1	Toxin binds to cell surface. Inhibited by DNP, but not by low temperature.	Cell surface receptor?
2	Toxin processed; irreversible intoxication in a few minutes. Inhibited by DNP and by low temperature.	Transmembrane signal or internalization of active fragment?
3	Effects observed before the CPE:	
	Ultrastructural surface changes (4)	Aggregation of surface-bound toxin-receptor complex?
	Stimulation of transport activities (uptake of uridine, amino acids).	Due to toxin internalization?
4	CPE observed in the light microscope. Inhibited by DNP and by low temperature.	
	Concomitant effect: stress fibers disintegrate (19).	Cytoskeletal hypercontraction due to effect on unknown regulator of cytoskeletal structure/function?
5	Effects observed secondary to the CPE:	
	Inhibition of membrane transport activities.	Due to loss of putative link between cytoskeleton and cell membrane?
	Inhibition of protein synthesis.	
	Gradual inhibition of DNA and RNA synthesis.	
6	Cells deteriorate after 3 to 4 days.	

[a] Steps 1, 2, and 3 occur during the latency period.

temperature and remains bound for at least 24 h in the cold. This indicates that binding of the toxin to the cell surface is not dependent on any active metabolic process or on the fluidity of the cell membrane. However, this contrasts with the observation that cells pretreated with DNP did not bind the toxin (19). The reason for this discrepancy is not clear at present. The cell surface receptor for *C. difficile* toxin has not yet been identified, but should be of a ubiquitous nature since the toxin is active upon all cultured mammalian cells investigated so far.

The assumption that irreversible intoxication occurs already after a few minutes is based on the following observations: (i) trypsinization of toxin-treated cells, a procedure which inactivates external toxin, does not prevent intoxication (19; Florin and Thelestam, in preparation), and (ii) failure of addition of anti-toxin to toxin-treated cells to neutralize intoxication (Florin and Thelestam, in preparation). Whether the toxin (or a part of it) is internalized or acts directly by giving an irreversible transmembrane signal is not clear at this stage. In any case, this step is energy requiring.

Chang et al. (4) reported ultrastructural surface changes in toxin-treated human amnion cells before they underwent the characteristic actinomorphic change. It might be speculated that the rearrangement of microvilli observed by these workers accompanies an aggregation of the surface-bound toxin-receptor complex. Other early effects at the membrane level are the stimulation of uptake activities just before and concomitant with the beginning of the CPE (Fig. 5 and 6). Conceivably, such stimulation phenomena may accompany internalization of toxin or an active part of it.

The most conspicuous manifestation of intoxication, i.e., the CPE visible in the light microscope, appears after a latency period of 1 to 3 h or even longer, depending on the dose of the toxin. The studies presented herein showed that this change in cellular morphology is inhibited at low temperature or in the presence of DNP (Fig. 3 and 4). Evidently, intoxicated cells require an adequate supply of adenosine 5'-triphosphate to express the toxic effect in the form of an actinomorphic change. This typical CPE is correlated to an irreversible conversion of the straight microfilament stress fibers to globular actin (as seen with indirect immunofluorescence) (19). Such a stress fiber modification may be due to a cytoskeletal hypercontraction, as has been suggested for the action of cytochalasins (14). These are known to cause similar but reversible actinomorphic changes by an energy-dependent process (13, 19). Hypercontraction may arise by an interaction of the toxin with some key regulator of the function or supramolecular organization of the cytoskeleton. The irreversibility of the CPE need not imply a continued hypercontraction. At later stages, the cells may be released from the contraction but be unable to regenerate their cytoskeletons and, therefore, continuously show the rounded morphology.

Since depression of membrane transport functions and inhibition of the synthesis of protein and nucleic acids occur after the toxin-induced CPE, these metabolic effects appear not to be the primary cause of the CPE. The intoxicated cells are unable to proliferate since DNA synthesis is shut down. However, after 22 h with manifest symptoms (95 to 100% CPE) the intoxicated cells still show residual metabolic function in the form of RNA and protein synthesis. Moreover, they are not "dead" according to classical viability tests based on vital staining with trypan blue (19). Neither do they release significant amounts of radiolabeled cytoplasmic components after up to 24 h of incubation with the toxin (19), or show any tendency to lyse, as observed microscopically during the following three days. Indeed, intoxicated cultures give the impression of being in a quiescent state rather than dead. It is not possible, however, to reverse the intoxication by growth stimulatory agents, such as serum or insulin (Florin and Thelestam, unpublished data). After about 3 to 4 days intoxicated cultures finally begin to deteriorate.

In summary, the present investigation describes some basic parameters relevant to cellular intoxication with the cytopathogenic toxin from *C. difficile*. However, the primary cellular target is still unknown.

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LITERATURE CITED

1. **Bourguignon, L. Y. W., and S. J. Singer.** 1977. Transmembrane interactions and the mechanism of capping of surface receptors by their specific ligands. Proc. Natl. Acad. Sci. U.S.A. **74**:5031-5035.
2. **Caspersson, T., S. Farber, G. E. Foley, D. Killander, and A. Zetterberg.** 1965. Cytochemical evaluation of metabolic inhibitors in cell culture. Exp. Cell Res. **39**: 365-385.
3. **Chang, T.-W., J. G. Bartlett, S. L. Gorbach, and A. B. Onderdonk.** 1978. Clindamycin-induced enterocolitis in hamsters as a model of pseudomembraneous colitis in patients. Infect. Immun. **20**:526-529.
4. **Chang, T.-W., P.-S. Lin, S. L. Gorbach, and J. G. Bartlett.** 1979. Ultrastructural changes of cultured hu-

man amnion cells by *Clostridium difficile* toxin. Infect. Immun. **23**:795–798.

5. **Crowe, R., H. Ozer, and D. Rifkin.** 1978. Experiments with normal and transformed cells. A laboratory manual for working with cells in culture, p. 153. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Fagraeus, A., D. L. J. Tyrrell, R. Norberg, and E. Norrby.** 1978. Actin filaments in paramyxovirus-infected human fibroblasts studied by indirect immunofluorescence. Arch. Virol. **57**:291–296.
7. **George, W. L., R. D. Rolfe, M. E. Mulligan, and S. M. Finegold.** 1979. Infectious diseases 1979—antimicrobial agent-induced colitis: an update. J. Infect. Dis. **140**: 266–268.
8. **Hall, I. C., and E. O'Toole.** 1935. Intestinal flora in newborn infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*. Am. J. Dis. Child. **49**:390–402.
9. **Larson, H. E., and A. B. Price.** 1977. Pseudomembraneous colitis: presence of clostridial toxin. Lancet **ii**: 1312–1314.
10. **Larson, H.E., A. B. Price, P. Honour, and S. P. Borriello.** 1978. *Clostridium difficile* and the aetiology of pseudomembraneous colitis. Lancet **i**:1063–1066.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **McClain, D. A., P. F. Maness, and G. M. Edelman.** 1978. Assay for early cytoplasmic effects of the src gene product of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **75**:2750–2754.
13. **Miranda, A. F., G. C. Godman, A. D. Deitch, and S. W. Tanenbaum.** 1974. Action of cytochalasin D on cells of established lines. I. Early events. J. Cell Biol. **61**:481–500.
14. **Miranda, A. F., G. C. Godman, and S. W. Tanenbaum.** 1974. Action of cytochalasin D on cells of established lines. II. Cortex and microfilaments. J. Cell Biol. **62**: 406–423.
15. **Rifkin, G. D., F. R. Fekety, J. Silva, Jr., and R. B. Sack.** 1977. Antibiotic-induced colitis. Implication of a toxin neutralized by *Clostridium sordellii* antitoxin. Lancet **ii**:1103–1106.
16. **Rolfe, R. D., and S. M. Finegold.** 1979. Purification and characterization of *Clostridium difficile* toxin. Infect. Immun. **25**:191–201.
17. **Snyder, M. L.** 1937. Further studies on *Bacillus difficilis* (Hall and O'Toole). J. Infect. Dis. **60**:223–231.
18. **Taylor, N. S., and J. G. Bartlett.** 1979. Partial purification and characterization of a cytotoxin from *Clostridium difficile*. Rev. Infect. Dis. **1**:379–385.
19. **Thelestam, M., and M. Brönnegård.** 1980. Interaction of cytopathogenic toxin from *Clostridium difficile* with cells in tissue culture. Scand. J. Inf. Dis. Suppl. **22**:16–29.
20. **Weber, K.** 1976. Biochemical anatomy of microfilaments in cells in tissue culture using immunofluorescence microscopy, p. 51–66. *In* S. V. Perry et al. (ed.), Contractile systems in non-muscle tissues. Elsevier/North-Holland Biomedical Press, Amsterdam.