Toxin B, a potent cytotoxin produced by *Clostridium difficile*, was purified to homogeneity from 6-day broth cultures of a toxigenic isolate. Cytotoxin was purified approximately 4000-fold by sequential ammonium sulfate precipitation, DEAE-Sepharose chromatography, and high performance liquid chromatography on a Mono Q anion-exchange column. The molecular weight of reduced purified toxin was 50,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, compared to 150,000 for unreduced toxin. Dose-response studies indicated that subpicogram concentrations of purified toxin caused rounding of approximately 20,000 IMR-90 fibroblasts. The phenomenon of cell rounding caused by toxin B was correlated with the ratio of globular to filamentous actin in fibroblasts as measured by two techniques. The toxin caused a significant increase in the ratio of globular to filamentous actin which was nearly completed prior to the onset of rounding. We conclude that cell rounding of fibroblasts exposed to toxin B is related to an increase in the ratio of globular to filamentous actin which is produced by small numbers of toxin molecules/cell.

*Clostridium difficile* has recently been recognized as the causative agent of antibiotic colitis in experimental animals (1, 2) and man (3, 4). This anaerobic pathogen colonizes the large bowel after alteration of the indigenous flora by antibiotics. Toxigenic strains of *C. difficile* then release several of globular to filamentous actin which is produced by exposed to toxin B is related to an increase in the ratio of rounding. We conclude that cell rounding of fibroblasts exposed to toxin B is related to an increase in the ratio of globular to filamentous actin which is produced by small numbers of toxin molecules/cell.

**Materials and Methods**

*C. difficile* strain 10463 and goat antitoxin B were obtained from the Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA. Human embryonic lung IMR-90 fibroblasts were from the Institute of Medical Research, Camden, NJ. Pre-reduced chopped meat carbohydrate broth was obtained from Gibco, brain-heart infusion broth from Becton, Dickinson and Co., and skim milk broth from Difco. DEAE-Sepharose CL-6B and the HPLC Mono Q column were purchased from Pharmacia. Nalgene filters (0.45 μm) were obtained from Nalgene, and the Amicon Diaflow Ultrafilter PM 10 from Amicon. PAGE molecular weight standards were purchased from Sigma.

Falcon 250-ml tissue culture plastic flasks and Falcon plastic Petri culture dishes (60 x 15 cm) were obtained from Becton, Dickinson and Co. Costar 24- and 96-well plastic tissue culture plates were from Costar. Complete medium for tissue culture consisted of the following reagents from Gibco: Dulbecco's minimal essential medium with d-glucose (1000 mg/liter) and L-glutamine (584 ml/liter) supplemented with sodium bicarbonate (3.7 g/liter), nonessential amino acids (10 mM), sodium pyruvate (100 mM), fetal bovine serum (10% v/v), penicillin (100 units/ml), and streptomycin (100 μg/ml). Salmon testis DNA was obtained from P-L Biochemicals. Deoxyribonuclease I from bovine pancreas and chicken gizzard actin were purchased from Sigma.

* Cultures of *C. difficile*—Stock cultures of a highly toxigenic strain of *C. difficile* (strain 10463) were stored in skim milk medium at -70 °C. Subcultures of *C. difficile* were incubated anaerobically in 5

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* The abbreviations used are: HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; G- and F-actin, globular and filamentous actin, respectively.
ml of chopped meat broth at 37 °C for 48 h. One ml of this subculture was then inoculated into a flask containing 4 liters of brain heart infusion broth which was incubated anaerothetically at 37 °C for 6 days. Preliminary studies revealed that toxin production peaked between days 5 and 6.

**Cytotoxicity Assay**—Toxin activity was monitored as described previously (10) on IMR-90 fibroblasts grown on Costar tissue culture plates (96 wells, 6.4-mm diameter each). Each well contained a confluent monolayer of 40,000 fibroblasts in 0.2 ml of medium. To each well was added 20 μl of serial 10-fold dilutions of toxin to obtain the highest dilution producing 50% rounding of cells after 24 h of incubation. The cytotoxic effect of the toxin preparations was completely inhibited by preincubation with equal volumes (20 μl) of C. difficile antitoxin for 30 min at 20 °C. The minimal toxic dose was determined as the amount of toxin which caused rounding of 50% of the fibroblast monolayer as described above. Protein concentration was determined by the method of Lowry et al. (17).

**Purification of Toxin B**—All purification steps were carried out at 4 °C. The 6-day broth culture was centrifuged at 3000 × g for 10 min to separate bacterial cells from the toxin-containing supernatant. The supernatant was filtered through a Nalgene filter (0.45 μm), and the pH was adjusted to 7.4 by addition of solid Tris base. After standing for 1 h, the precipitate was collected by centrifugation at 3000 × g for 30 min and resuspended in 100 ml of 50 mM Tris/HCl buffer, pH 7.4. This solution was then made 20% saturated by adding solid ammonium sulfate. After standing for 1 h, the supernatant was collected by centrifugation at 3000 × g for 30 min another and resuspended in 50 mM Tris/HCl buffer, pH 7.4. This solution of crude toxin was then dialyzed against 2 liters of 50 mM Tris/HCl buffer, pH 7.4, for 24 h.

Dialyzed crude toxin was loaded on a 9 × 2.5 cm column of DEAE-Sepharose Cl-6B and eluted with a NaCl gradient in 50 mM Tris/HCl buffer, pH 7.4, as described by Sullivan et al. (5) with minor modifications. Instead of a continuous salt gradient, the column was eluted with a step gradient consisting of 0.32, 0.34, 0.36, 0.38, and 0.4 M NaCl. Cytotoxicity of each fraction was monitored on IMR-90 fibroblasts as described above. Protein concentration was monitored by absorbance at 280 nm. The cytotoxin-containing fractions eluting with 0.34 M NaCl (approximately 100 ml) were pooled and concentrated 10-fold on an Amicon filter PM-10. The pooled concentrated partially purified toxin from the DEAE column was further purified by HPLC on a Mono Q anion-exchange column. One ml of partially purified concentrated toxin from the DEAE column was injected into the column, which was then eluted at 1 ml/min with a linear gradient of 0.1–1 M NaCl in 20 mM Tris/HCl buffer, pH 7.4. One-ml fractions were monitored for protein by absorbance at 230 nm and for cytotoxicity.

**Polyacrylamide Gel Electrophoresis of Toxin**—SDS gel electrophoresis was performed as described by Laemmli (18) on 7.5% acrylamide separating slab gels, 1.5 mm in thickness. Samples of toxin B were incubated in Laemmli sample buffer containing 1.25% mercaptoethanol with or without 5% 2-mercaptoethanol for 30 min. These samples were then heated at 100 °C for 2 min, and electrophoresis was performed at 25 mA for 4.5 h. Isoelectric focusing of toxin B was performed on slab gels according to the method of O’Farrell (19).

**Amino Acid Analysis**—For amino acid analysis, the fraction with the highest cytotoxicity from the HPLC column was used (see Fig. 2). This fraction (1 ml) was hydrolyzed in 6.0 N HCl by the addition of an equal volume of concentrated HCl under vacuum at 110 °C for 24 h. The hydrolysate was analyzed on a Beckman Model 110 CL amino acid analyzer. Results are expressed as residues/1000 amino acids and are not corrected for losses during hydrolysis.

**Estimation of Cytotoxicity and Cytoskeletal Actin**—Confluent fibroblast monolayers growing in 25-cm² flasks (2.5 × 10⁶ cells/flask) were extracted with Triton X-100 to isolate cytoskeletal proteins using a modification of the method of White et al. (14). After specific times in culture, the medium was removed and each cell layer was washed three times with phosphate-buffered saline, pH 7.4, at 4 °C. The thoroughly drained cell layer was carefully covered with 5 ml of extraction solution containing 10 μl of Triton X-100 (1 wt/vol), 10 ml of HEPES, 300 mM sucrose, 50 mM NaCl, and 3 mM MgCl₂, pH 7.4, containing 150 μg/ml phenylmethylsulfonyl fluoride. The flask was left undisturbed on ice for a period of 5 min. This extraction was repeated two more times. Actin solubilized by this procedure is referred to as cytosplastic actin. The insoluble cell layer containing cytoskeletal actin was then harvested with the aid of a rubber policeman in 5 ml of Laemmli sample buffer with 1.25% mercaptoethanol. Aliquots of the insoluble cell layer in sample buffer were heated at 100 °C for 5 min and then applied to the polyacrylamide gel. The proteins in the soluble fraction were precipitated by adding a 4-fold volume of cold acetone and allowing the mixture to stand at −20°C for 10 min prior to electrophoresis on 10% polyacrylamide gel with a 3% stacking gel. Protein bands were stained with Coomassie Brilliant Blue R-250, and quantitation was obtained by densitometric tracings at 450 nm. The major actin band in extracts was identified by its relative mobility compared to an authentic actin standard. Results are expressed as the ratio (percentage) of cytoplasmic actin to cytoskeletal actin.

To determine the effect of toxin B on the ratio of cytoplasmic to cytoskeletal actin, partially purified toxin B (DEAE column) or purified toxin B (HPLC Mono Q column) was added to confluent monolayers of IMR-90 fibroblasts. Experiments were performed in which either the concentration of toxin or time of exposure to toxin was varied. Control flasks contained either no added toxin or heat-inactivated toxin (80 °C, 10 min). At the end of the incubation period, the medium was decanted, and the monolayers were extracted with Triton X-100 as described above.

**DNase I Inhibition Study for G-actin**—To estimate G-actin in toxin B-exposed fibroblasts, the DNase I inhibition assay was used according to the method of Malicka-Biszkiewicz and Roth (16). Confluent fibroblast monolayers (IMR-90) growing into 24-well Costar tissue culture plastic plates (10 cells/well) were incubated at 37 °C with partially purified toxin B or equal volumes of Tris buffer. After incubation was complete, cells were removed from plates with the aid of a rubber policeman and collected by centrifugation at 800 × g for 10 min. Cells were washed twice with 0.9% saline and resuspended in 1 ml of Buffer A (16). Cells were homogenized in a 2-ml glass homogenizer for 15 min at 4 °C and then centrifuged at 105,000 × g for 1 h. The 105,000 × g supernatants were immediately assayed for G-actin by DNase I inhibition assay exactly as described by Malicka-Biszkiewicz and Roth (16). In another set of experiments to determine if toxin B exerted a direct effect on the actin distribution of cell extracts, the 105,000 × g supernatant from untreated fibroblasts was incubated with toxin B or buffer for 30 min (37 °C). After a 30-min incubation, aliquots were assayed for G-actin.

**Quantitation of Cell Rounding**—The percentage of cell rounding was determined from 8 × 10⁻⁶-inch enlargements of phase micrographs of fibroblast monolayers. Percentages of rounded cells in Fig. 5 and Tables III and IV are the mean of 100 cells counted by three separate observers.

**RESULTS**

**Purification of Toxin B**

In preliminary experiments, Toxin B production by C. difficile strain 10463 was monitored by cytotoxicity assay. After

TABLE I

| Purification of C. difficile toxin B | Step | Volume | Total protein | Minimal toxic dose | Purification |
|---|---|---|---|---|---|
| DEAE-Sepharose-purified toxin | 10 | 1.6 | 3.2 fg | 6,625 |
| HPLC Mono Q | 1 | 0.765 | 5.1 fg | 3,920 |

*Protein was estimated by the method of Lowry et al. (17).

The minimal toxic dose, expressed here as the amount of protein/well, was estimated by calculating the highest dilution of toxin causing 50% rounding of IMR-90 fibroblasts exposed for 24 h. Each well contained a monolayer of 40,000 cells in 0.2 ml of medium.
5-6 days in anaerobic culture, 20 μl of culture supernatant diluted 10^2 in phosphate-buffered saline produced 50% rounding of a fibroblast monolayer. The toxin titer did not increase with further incubation; thus, 6-day cultures were routinely used as starting material for purification. As shown in Table 1, toxin B was purified approximately 4000-fold by a combination of ammonium sulfate precipitation and ion-exchange chromatography on DEAE-Sepharose. The major toxin-containing fraction was eluted from the DEAE column (Fig. 1) by 0.34 M NaCl eluting buffer. Minor toxin-containing fractions were noted to elute between 0.05 and 0.25 M NaCl and after elution with 0.3 M NaCl. These fractions have been reported by others (5) to represent toxin A, an enterotoxin which possesses weak cytotoxin activity. The toxin-containing fractions from the 0.34 M NaCl step were pooled and concentrated to 10 ml.

The cytotoxin in the pooled fractions from the DEAE-Sepharose column was further purified by HPLC on a Mono Q anion exchanger (Fig. 2). The toxin eluted as a sharp peak at 0.73 M NaCl. Several minor contaminating proteins eluted at lower molarity. Unfortunately, the toxin recovered from the HPLC column had lower specific activity in the cytotoxicity assay than the toxin from the DEAE column. This loss of activity may result from partial denaturation of toxin during passage through this column. The toxin-containing fractions from the DEAE and Mono Q columns were pooled and electrophoresed on 7.5% polyacrylamide gels containing 1.25% SDS (Fig. 3). It was observed that, in the presence of 5% 2-mercaptoethanol, toxin B had a molecular weight of 50,000. However, when toxin was incubated in sample buffer without 2-mercaptoethanol, a major band was found at 150,000 and only a faint band was observed at 50,000. Toxin activity could not be recovered from gel slices eluted with buffer for 24 h at 4 or 20 °C. Isoelectric focusing of the toxin revealed that the isoelectric point was at pH 4.3 (Fig. 4). The isoelectric focusing gel revealed additional minor bands in the DEAE column fraction (Fig. 4, lane 2) which were not present in the purified toxin from the HPLC column.

Amino acid analysis (Table II) of the purified toxin revealed a large proportion of Asx, Gly, Ala, Val, and Leu. No cysteine was detected in three separate amino acid analyses.

**Effects of Toxin B on Cellular Actin**

DNase I Inhibition Assay.—The effect of purified toxin B on the G-actin content of fibroblasts, as measured by DNase I inhibition, is shown in Fig. 5. Toxin B caused a sharp increase in DNase I inhibition within 30 min, with a somewhat slower rate of increase between 30 and 120 min. In contrast, cell rounding did not begin until 60 min and increased steadily up to 240 min (not shown) when all cells were rounded. In separate experiments, neither the mean number of cells/well nor the protein content of the cell monolayer was altered by toxin exposure up to 120 min.

In order to determine whether toxin B acted directly on actin in cell extracts, the following experiment was performed. Cell extracts were prepared from control fibroblasts and then toxin B (2.4 μg/ml) was added and the mixtures were incubated for 30 min at 37 °C. Toxin B had no effect on G-actin in cell extracts as measured by DNase I inhibition, when compared with control extracts incubated without toxin.

**Triton Extraction Method**—A typical Triton X-100 extraction of a confluent monolayer of IMR-90 fibroblasts is shown in Fig. 6. When the monolayers were extracted five times (5 min each time), only negligible amounts of protein were detectable by SDS-PAGE in the last two extractions. Therefore, the cells were routinely extracted sequentially three
times with the Triton X-100 solution. Densitometric analysis of the actin band (Mr 42,000) revealed that 50-60% of actin in IMR-90 fibroblast cultures was soluble in Triton X-100, whereas the remainder was insoluble. Triton-soluble actin will be referred to as cytoplasmic actin and Triton-insoluble actin as cytoskeletal actin as suggested by White et al. (14).

The effects of toxin B concentration and duration of exposure to toxin on cytoplasmic or cytoskeletal actin and cellular rounding are shown in Tables III and IV. Toxin B at a concentration of 2.5 μg/ml caused an increase in cytoplasmic actin from 50% in control cells to 76% at 1 h, when only 4% of cells were rounded (Table III, part A). No further increase in cytoplasmic actin was observed at 2 and 5 h. Similar results were obtained with HPLC-purified toxin (Table III, part B). As shown in Table IV, the effect of toxin B on cytoplasmic actin was somewhat dose-dependent. Significant increases in cytoplasmic actin and cell rounding were observed at toxin concentrations above 2.5 ng/ml. It should be noted that, while the absolute values for actin varied slightly from experiment to experiment, the trend toward increased cytoplasmic actin was consistent in all experiments.

The fungal metabolite cytochalasin B causes rounding of fibroblasts presumably by disaggregation of actin-containing filaments. We, therefore, treated fibroblasts with cytochalasin P (20 μg/ml) which caused rounding of one-third to one-half of the cells after 90 min. After a 1-h exposure to cytochalasin B, cytoplasmic actin was increased by 53% above untreated controls and by 60% after a 24-h exposure. In order to ascertain that cell rounding by itself did not cause an increase in the percentage of soluble actin, we exposed fibroblast monolayers to medium containing 0.025% trypsin and 0.01% EDTA for 10 and 20 min. After a 10-min exposure, the cells were rounded but still attached to the plate, whereas after 20 min, the cells were completely rounded and floating. In both cases, the percentage of cytosolic actin was within 2% of that observed in adherent control fibroblasts.

**DISCUSSION**

We report here the purification of toxin B from broth cultures of *C. difficile* using ion-exchange chromatography and HPLC. As shown in Table I, the cytotoxin causes rounding of 50-75% of 40,000 fibroblasts at subpicogram concentrations. However, since the cytotoxicity assay is accurate only to plus or minus one 10-fold dilution, there is a potential variability of up to 100-fold. This makes it difficult to accurately calculate the minimal number of toxin molecules/cell required for toxicity. It should be noted here that rounded

**TABLE II**

| Amino acid | Residues/1000 amino acids |
|------------|--------------------------|
| Aspartic acid | 111                      |
| Threonine | 49                       |
| Serine | 51                       |
| Glutamic acid | 106                     |
| Proline | 29                       |
| Glycine | 109                      |
| Alanine | 97                       |
| Valine | 97                       |
| Methionine | 23                      |
| Isoleucine | 62                      |
| Leucine | 97                       |
| Tyrosine | 26                       |
| Phenylalanine | 27                     |
| Lysine | 67                       |
| Histidine | 11                      |
| Arginine | 35                       |
fibroblasts exposed to toxin B are able to continue synthesizing protein and DNA (20) but are unable to divide and cannot be subcultured (9).

We estimated the molecular weight of reduced toxin in SDS to be 50,000, which is similar to that reported by Rolfe and Finegold (7). However, these authors also reported a molecular weight of 530,000 by gel filtration in aqueous buffers. Similar results were reported by Sullivan et al. (5) who found an average molecular weight of 450,000–500,000 by gel filtration and 360,000 by gradient PAGE and by Banno et al. (8) who reported a molecular weight of 450,000–500,000 by gel filtration. Our results showed a molecular weight of 150,000 for unreduced toxin in SDS-PAGE and 50,000 after exposure to 2-mercaptoethanol. This effect of reducing agents suggests the presence of disulfide bonds between subunits. Although we were unable to detect cysteine or cysteic acid by amino acid analysis, we cannot exclude the presence of very small numbers of cysteine residues/molecule of toxin. Interestingly, we previously reported that exposure of partially purified toxin to mercaptoethanol increased the cytotoxic activity of partially purified toxin (21). Taken together, these observations suggest that toxin B forms aggregates of 8–10 monomers ($M_r$, 400,000–500,000) in aqueous buffers. The $M_r$, 50,000 monomer form is obtained only after exposure to reducing agents and SDS. Unfortunately, efforts to recover active cytotoxin from polyacrylamide gel slices have thus far been unsuccessful. Therefore, the molecular configuration of active toxin cannot be determined on the available data.

We report here for the first time that purified toxin B causes an increase in the ratio of G- to F-actin in tissue culture cells. Whereas other clostridial toxins have been reported to have phospholipase or hemolytic activity (22, 23), C. difficile toxin B appears to exert its effect on cell shape via disaggregation of actin. In this regard, toxin B more closely resembles cytochalasin B. The results reported here are consistent with our previous morphologic observations (10) and in addition allow quantitation of the effect of purified toxin on the ratio of globular to filamentous actin. Toxin B caused

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**TABLE III**

| Time  | Cytoplasmic actin | Cell rounding |
|-------|------------------|---------------|
| min   | %                | %             |
| A.    |                  |               |
| DEAE-Sepharose toxin (2.5 μg/ml) | 0 | 50* | <1 |
|       | 15              | 55            |
|       | 30              | 76            |
|       | 60              | 76            |
|       | 120             | 75            |
|       | 300             | 74            |
| B.    |                  |               |
| HPLC toxin (1.6 μg/ml) | 0 | 56 | <1 |
|       | 30              | 67            |
|       | 60              | 73            |
|       | 240             | 84            |

*Each result represents the mean of three flasks of cells extracted and electrophoresed separately. Cytoplasmic actin was determined by Triton extraction followed by SDS-PAGE.
TABLE IV
Effect of toxin B concentration on cytoplasmic actin in IMR-90 fibroblasts

| Toxic B concentration | Cytoplasmic actin | Cell rounding |
|-----------------------|-------------------|--------------|
| 0                     | 62%               | <1           |
| 25 μg/ml              | 84                | 52           |
| 0.25 μg/ml            | 79                | 18           |
| 2.5 ng/ml             | 56                | <1           |

*Monolayers were exposed to toxin for 4 h prior to extraction. Results are means of three separate flasks of fibroblasts extracted and electrophoresed separately.

In summary, purified C. difficile toxin B at very low concentrations produces a significant increase on cytoplasmic G-actin of fibroblast monolayers. This effect probably causes or contributes to the cell rounding which occurs after the change in G-actin. This effect on actin appears to be indirect, perhaps mediated by an enzymatic action of toxin on proteins which modulate actin polymerization or which attach actin filaments to other cell organelles. Further studies of toxin B action may provide useful insights into the relationship of actin to cell shape.

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