Actin-Specific ADP-Ribosyltransferase Produced by a *Clostridium difficile* Strain

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By screening possible ADP-ribosyltransferase activities in culture supernatants from various *Clostridium* species, we have found one *Clostridium difficile* strain (CD196) (isolated in our laboratory) that is able to produce, in addition to toxins A and B, a new ADP-ribosyltransferase that was shown to covalently modify cell actin as *Clostridium botulinum* C2 or *Clostridium perfringens* E iota toxins do. The molecular weight of the CD196 ADP-ribosyltransferase (CDT) was determined to be 43 kilodaltons, and its isoelectric point was 7.8. No cytotoxic activity on Vero cells or lethal activity upon injection in mice was associated with this enzyme. CDT was neither related to *C. difficile* A or B toxins nor to *C. botulinum* C2 toxin component I. However, Vero cells cultivated in the presence of *C. difficile* B toxin had a lower amount of actin able to be ADP-ribosylated by CDT or C2 toxin in vitro. Antibodies raised against CDT reacted by immunoblot analysis with a 43-kilodalton protein of *C. perfringens* type E culture supernatant producing the iota toxin.

Clostridium difficile induces its pathogenic effects by secreting two potent toxins named A and B according to their order of elution from anion-exchange columns (13). Toxin A is an enterotoxin that causes fluid accumulation in the isolated ileal loops of rabbits (34). The molecular weight of toxin A has been reported to be 470,000 (11). Although toxin A has cytotoxic activity, toxin B is much more potently cytotoxic. Toxin B, or cytotoxin, has no enterotoxic activity but induces a strong cytopathic effect on cultured cells (5, 34, 35). The molecular weight of toxin B is not precisely known and is currently the subject of controversy (15, 24, 38). Both toxins A and B from *C. difficile* are lethal to mice (35).

The molecular mechanisms for the enterotoxin and the cytotoxin are totally unknown. However, toxin B appears to modify the cytoskeleton (36). It has been recently reported that actin filaments are disaggregated with an increase of globular actin (G-actin) in cells treated with *C. difficile* B toxin (15, 24).

Recently, C2 toxin, which is lethal, enterotoxic, and cytotoxic and is secreted by *Clostridium botulinum* (C and D strains), has been shown to act on cellular G-actin (1, 22, 25). In addition, recent reports have demonstrated that the iota toxin secreted by *Clostridium perfringens* type E strains has a structure and activity similar to those of C2 toxin (31–33, 37).

C. botulinum C2 toxin is composed of two independent polypeptide chains called component I (or light chain) and component II (or heavy chain); these are not associated by either covalent or noncovalent bonds (10). The C2 and iota toxins belong to the family of the binary toxins (30), like leucocidin (18), staphylococcus gamma lysin (8), and anthrax toxins (12). The light chain of C2 toxin (55 kilodaltons (kDa) (22) has been shown to be an enzyme which covalently modifies, by ADP-ribosylation (29), cellular actin in its globular (G) form (1, 22). The heavy chain (100 kDa) (20) of *C. botulinum* C2 toxin recognizes a cell surface receptor to

which it binds. However, it is only after removal, by serine proteases such as trypsin, of a 12-kDa peptide from component II that this molecule is able to bind the toxin light chain, allowing this enzyme to penetrate and intoxicate target cells (20).

It appeared therefore that toxins A and B from *C. difficile* might share a common molecular mechanism with the binary pathogenic molecule C2. This possibility prompted us to examine whether *C. difficile* strains elaborate an ADP-ribosyltransferase that modifies cellular actin.

In the present paper, we show that one strain of *C. difficile* (CD196), isolated from a patient suffering from pseudomembranous colitis, was indeed able to produce a large amount of an actin ADP-ribosyltransferase, in addition to the enterotoxin and the cytotoxin. We have examined the relationship of this enzyme to the *C. botulinum* C2 and *C. difficile* A and B toxins.

MATERIALS AND METHODS

Bacterial strains. *C. difficile* 196 (CD196) was isolated from a 28-year-old woman who developed diarrhea following amoxycillin treatment. Pseudomembranous colitis was evident by endoscopic examination, and *C. difficile* cytotoxin was found in the stools by using the tissue culture assay with Vero cells (10^6 cytotoxic units per g of stools). The CD196 strain produced a good yield of both toxins A and B (23). CD196 had the typical microbiological characteristics of *C. difficile* (9). CD196 acidified glucose and fructose, hydrolyzed esculin, and produced gelatinase but not lecithinase or lipase. Its fermentation end products were acetic, isobutyric, butyric, isovaleric, isocaproic, pyruvic, lactic, succinic, and phenylacetic acids. The other *C. difficile* strains isolated in our laboratory and used in this study were CD126, CD303, CD1063, and CD660. *C. difficile* VPI 10463 was provided by the Virginia Polytechnic Institute and State University (Blacksburg, Va.), and the type strain ATCC 9689 was purchased from the American Type Culture Collection (Rockville, Md.). *C. perfringens* type E strain NCIB 10748, which produces iota toxin, was purchased from the Torry

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Research Station (Aberdeen, Scotland). *C. botulinum* type C strain 468 and type D strain 1873, both of which produce C2 toxin, were from the Prévot collection (Institut Pasteur, Paris, France).

Bacterial growth and toxin production. CD196 was grown on tryptic glucose yeast extract (TGY) broth, as previously described (23). For toxin production, *C. difficile* organisms were cultivated inside dialysis bags with brain heart infusion (BHI) medium (Difco Laboratories, Detroit, Mich.) incubated at 35°C for 48 h (34). Kinetics were established by cultivating the bacteria in 1 liter of BHI medium and by removing 5-ml samples at various intervals. The 5-ml samples were used to estimate bacterial growth by measuring the optical density at 600 nm, the protein concentration was estimated by the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Federal Republic of Germany), and the number of spores was found by heating the preparation at 80°C for 10 min and by plating on solid meat yeast medium (23). Cytotoxic activity was estimated by inoculating serial dilutions to cell monolayers (Vero cells), and ADP-ribosyltransferase activity was estimated by the gel assay described below.

C. botulinum C2 components I and II were purified from *C. botulinum* type D strain 1873. The bacteria were cultivated for 3 days at 35°C in egg meat medium (Difco). C2 toxin components I and II were purified by a method derived from that of Ohishi et al. (21). This method is briefly described; culture supernatant was precipitated with ammonium sulfate (80% [wt/vol]). The resulting precipitate was suspended in a minimum of 50 mM Tris hydrochloride buffer (pH 7.5) (buffer A) and then chromatographed on an Ultrogel AcA34 (LKB, Paris, France) gel filtration column (100 by 2.6 cm) equilibrated with buffer A. Fractions showing cytotoxic activity on Vero cells (after trypsinization [21]) were pooled and loaded on a QAE Sephadex A50 (Pharmacia, Paris, France) column (20 by 1 cm) equilibrated with buffer A. The QAE column was washed first with 0.075 M NaCl in buffer A (until no protein was eluted), and then a 0.075 to 0.3 M NaCl gradient (in buffer A) was applied. ADP-ribosylating activity toward actin was then measured in each fraction. The enzymatic activity was pooled and applied to preparative isoelectric focusing for further purification of the C2 toxin component I (see paragraph on preparative isoelectric focusing). QAE column gradient flowthrough and 0.075 M NaCl wash were pooled and precipitated by ammonium sulfate (80% [wt/vol]). The resulting precipitate was suspended in a minimum volume of buffer A and chromatographed on an AcA34 Ultrogel column (100 by 2.6 cm) equilibrated with buffer A. Fraction showing cytotoxicity on Vero cells, after trypsinization and addition of the enzymatic component I, were pooled, dialyzed against distilled water, and submitted to preparative isoelectric focusing. Fractions of the isoelectric focusing preparative gel containing C2 toxin component II activity were pooled, and the purity of this protein was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see Fig. 5B).

Preparation of antisera. New Zealand White rabbits (2 to 3 kg) were injected with 6 to 10 μg of pure C2 toxin component I, II, or semipurified CD126 ADP-ribosyltransferase (CDT), in 2 ml of phosphate-buffered saline medium (PBS). An equal volume of complete Freund adjuvant was added for the primary injection. Booster injections with the same volume and protein concentration but with incomplete adjuvant were given 15, 30, and 45 days later. Only two injections were carried out with semipurified CDT. The

rabbits were bled 10 days after the last injection. Antisera were kept at 4°C with 0.2% sodium azide.

ADP-ribosyltransferase assays. Crude bacterial culture supernatants were first tested for ADP-ribosyltransferase activity by directly injecting the preparation into *Xenopus laevis* oocytes loaded with [^{32}P]NAD as previously described (7). Briefly, female adult *X. laevis* were obtained from W. de Rover (Hertenlaah, Dendolder, The Netherlands), ovarian lobes were surgically removed, washed with modified Barth saline medium (MBS-H) (14), and dissociated by overnight incubation at room temperature in MBS-H containing 1 mg of collagenase (Sigma Chemical Co., St. Louis, Mo.) per ml. Isolated oocytes were washed free of collagenase before use. Oocytes were first injected (20 nl) with 10-fold-concentrated [^{32}P]NAD (Dupont, NEN Research Products, Boston, Mass.) corresponding to 10^6 cpm. Preparations to be tested for ADP-ribosyltransferase were then microinjected (20 nl) into the oocytes. A total of five oocytes were used to assay each bacterial supernatant. The oocytes were incubated in MBS-H (100 μl) at room temperature for 1 h. They were then washed with MBS-H and homogenized in 100 μl of lysis buffer (150 mM NaCl, 50 mM Tris hydrochloride [pH 7.5], 0.1% SDS, 1% Triton X-100, 1 mM phenyl methylsulfonyl fluoride [PMSF]). Pigments were removed by a short (30-s) centrifugation in a microfuge. An aliquot (20 μl) of the supernatant was fractionated by SDS-PAGE. After being stained, destained, and dried, the gel was exposed to X-ray film with an intensifying screen at −80°C.

In vitro ADP-ribosylation assays were performed with actin isolated from *X. laevis* oocytes. A *X. laevis* homogenate was prepared with a Potter homogenizer with 1 part of packed oocytes and 4 parts of 0.32 M sucrose containing 1 mM PMSF. The homogenate was centrifuged first at 17,000 × *g* for 30 min, and the resulting supernatant was ultracentrifuged at 100,000 × *g* for 1 h. The 100,000 × *g* supernatant was used as a source of actin for the ADP-ribosyltransferase reactions. Assay mixture for the gel assay (total volume, 20 μl) contained 7 μg of oocyte actin preparation, 100 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.2), 10 mM thymidine, and 2 × 10^6 cpm of [^{32}P]NAD. Amounts between 1 and 10 μl of the fractions to be tested for enzymatic activity were added to the assay mixture. After incubation for 1 h at 37°C, sample buffer was added and the preparation was fractionated by SDS-PAGE and processed for autoradiography.

A mixture for a filter assay (total volume, 50 μl) contained 20 μg of *X. laevis* oocyte actin preparation, 100 mM HEPES buffer (pH 7.2), 10 mM thymidine, and 10^5 cpm [^{32}P]NAD, and 10 μl of the test sample. The mixture was incubated for 1 h at 37°C in a 96-well Falcon microplate (Becton Dickinson Labware, Oxnard, Calif.). The reaction was stopped by addition of 50 μl of 20% trichloroacetic acid. Samples were collected directly from the microplate on glass-fiber mats by using a cell harvester (Skatron, Oslo, Norway). The mat was dried, and each precut disk was detached and individually counted for radioactivity.

Immunoprecipitation of [^{32}P]ADP-ribosylated protein. Cytosolic extract of *Xenopus* oocytes was ADP-ribosylated by crude culture supernatant from CD196. The reaction mixture (total volume, 100 μl) contained 50 μg of *Xenopus* oocyte protein extract, 100 mM HEPES buffer (pH 7.2), 10 mM thymidine, 2 × 10^7 cpm of [^{32}P]NAD, and 10 μl of CD196 culture supernatant. After 1 h at 37°C, a 20-μl aliquot of the reaction was kept for direct SDS-PAGE analysis, the remainder was adjusted to 250 μl with H$_2$O, and SDS was brought to a final concentration of 2%. The mixture was

heated at 100°C for 5 min and then cooled down to room temperature before addition of 4 volumes of 50 mM Tris hydrochloride buffer (pH 7.4) containing 190 mM NaCl, 2.5% Triton X-100, and 1% aprotinin. After centrifugation for 2 min in a microfuge, 5 µl of anti-actin or nonimmune rabbit serum was added and the samples were processed as described previously (26).

Immunoblotting procedure. The method of Burnette et al. (3) was followed. Samples of proteins or crude culture supernatants were submitted to SDS-PAGE. The proteins were transferred electrophoretically to nitrocellulose (BA85; Schleicher & Schuell, Cera Labo, Paris, France). The nitrocellulose was first incubated for 1 h in PBS containing 5% milk and then was incubated overnight at room temperature with dilutions (in the same solution) of specific antibodies. Bound antibodies were detected with ^{125}I-labeled protein A and autoradiography.

Cell culture assays. Tests for ADP-ribosylation of actin were done either on African green monkey Vero cells or human fibroblasts (MRC5). Cells were cultivated in Dulbecco modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum. Cells were either plated into 96-well Falcon tissue culture plates or 6-well Falcon plate (5). For cytotoxic assay of *C. difficile* A, B, or C2 toxins, serial twofold dilutions of samples containing the toxins were prepared in DMEM and inoculated into cell monolayers. The cells were observed 24, 48, and 72 h after inoculation for morphological alterations. One cytotoxic unit was defined as the reciprocal of the highest dilution which produced a 100% cytopathogenic effect on cells. Activation by trypsin was carried out by incubating toxins or a combination of toxin components with trypsin at a final concentration of 200 µg/ ml for 30 min at 37°C. Proteolysis was stopped by the addition of soybean trypsin inhibitor (400 µg/ml).

Neutralization was performed by preincubating dilutions of toxin with antiserum for 2 h at room temperature prior to inoculation of the mixtures into the cell monolayers.

Estimation of globular actin in Vero cells treated by *C. difficile* toxins. Confluent monolayers of Vero cells (2 × 10^6 cells) were exposed to *C. difficile* toxin A (1 µg/ml), *C. difficile* toxin B (0.05 µg/ml), or *C. botulinum* toxin C2 (0.5 µg/ml) activated by trypsin as described in Table 1. After 5 h of incubation at 37°C, all of the toxin-treated cells displayed profound characteristic cytopathogenic effects. The monolayers were washed three times with 2 ml of DMEM and then carefully detached from the dishes with a rubber policeman. Each group of cells was suspended in 100 µl of 100 mM HEPES buffer (pH 7.2) containing 100 mM KCl and 5 mM MgCl$_2$. Cells were lysed by three cycles of freeze-thawing. An aliquot of 10 µl of each cell lysate was incubated with purified C2 component I (20 µg/ml), CDT (20 µg/ml), or diphtheria toxin fragment A (5 µg/ml) (ADP-ribosylation of elongation factor 2 by diphtheria toxin fragment A served as an internal control to check that the same amount of protein was used in each group of experiments), and 4 × 10^5 cpm of [^{32}P]NAD for 1 h at 37°C. The reaction products were analyzed by SDS-PAGE and autoradiography. The radioactive spots corresponding to actin (43 kDa) or elongation factor 2 (96 kDa) were cut out from the gel and counted.

Preparative isoelectric focusing. Preparative isoelectric focusing was performed in Ultrodex (LKB) on an LKB 2117 Multiphor system with a flat-bed isoelectric focusing kit (LKB) and 4% (wt/vol) gel slurry containing 5% LKB Ampholine (pH 6 to 9). A 2-ml sample of semipurified CDT was incorporated in the gel and focused at constant power (16 W) for 18 h at 10°C. The gel bed was fractionated, each

fraction was mixed with 10 ml of distilled water, and the solubilized proteins were then obtained by filtration. The enzymatic activity and gel electrophoresis pattern were determined for each fraction.

Immunoaffinity purification. Immunoglobulins from rabbit sera was purified by protein A-Sepharose affinity chromatography. The immunoglobulin G obtained were covalently linked to CNBr-activated Sepharose CL-4B beads (Pharmacia) according to the recommendations of the manufacturer. CDT, partially purified by AcA34 gel filtration of bacterial culture supernatant CD196, was applied to the affinity immunoglobulin G-Sepharose column. After the column was washed with PBS until no optical density at 280 nm could be recorded, 0.1 M glycine buffer (pH 3.0) was applied to the column and eluted proteins were collected (fraction of 1 ml) in tubes containing 20 µl of 4 M Tris hydrochloride buffer (pH 8.8) (which raised the final pH to 7.0). Fractions containing the enzymatic activity were pooled and extensively dialyzed against PBS.

RESULTS

An ADP-ribosyltransferase activity found in CD196 culture supernatant. By screening ADP-ribosyltransferase activity using our method of microinjection into *X. laevis* oocytes loaded with [^{32}P]NAD (7), we found that a protein of about 45 kDa was strongly labeled by the culture supernatant of one *C. difficile* strain (CD196) out of 15 tested. The radioactive protein migrated identically to the labeled band obtained by microinjecting purified C2 toxin component I (Fig. 1A). No incorporation of label from [^{32}P]NAD was catalyzed by supernatants of *Clostridium sordellii*, *C. perfringens* A, C, and D, or *Clostridium chauvoei* (Fig. 1A). In similar experiments, we found no [^{32}P] label incorporated into *X. laevis* oocyte proteins upon microinjection of culture supernatants from three strains of *Clostridium butyricum*, three strains of *Clostridium oedematiens* A, B, and D, and five strains of *Clostridium sporogenes* (data not shown). Culture supernatant of CD196 was also able to induce the transfer of ^{32}P from NAD to a protein of 45 kDa in *X. laevis* extract, again as C2 toxin light chain did (Fig. 1B). To demonstrate that the acceptor protein for the CD196 enzyme was actin, as is the case for C2 toxin (1, 22), we used antibodies directed against *X. laevis* oocyte actin to immunoprecipitate the ^{32}P-labeled molecule. The radioactive molecule was specifically immunoprecipitated by anti-actin antibodies (Fig. 2).

To confirm that the enzyme identified in the culture supernatant of CD196 was not due to a contamination by another *Clostridium* strain, 10 isolated colonies of CD196 on solid agar were picked randomly and grown individually in liquid medium. The supernatant of each of the 10 colonies was able to transfer ADP-ribose from NAD to cell actin and contained the classical enterotoxin and cytotoxin (data not shown).

The secretion of CDT was studied as a function of bacterial growth and sporulation. ADP-ribosylation of cell actin was found to be catalyzed by CD196 culture supernatant after 2 h of growth (Fig. 3) and therefore was totally independent of bacterial sporulation. The enzyme could be found in the culture medium but also associated with the bacteria (data not shown).

Purification of CDT. Purification of CDT was achieved as follows. CD196 was grown in BHI medium in a dialysis bag for 48 h. Proteins in the culture supernatant were precipitated by ammonium sulfate (final concentration, 70% [wt/ vol]). The precipitate was redissolved in a minimum volume
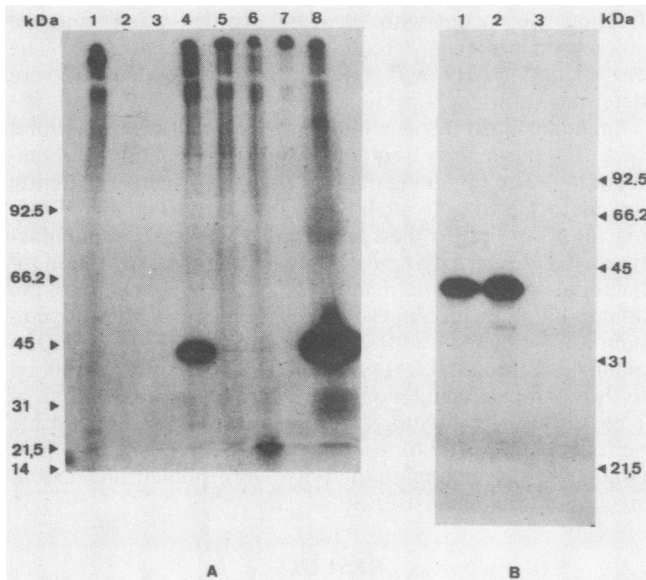
FIG. 1. (A) Effect of culture supernatants of various clostridia when injected into [^{32}P]NAD-loaded *X. laevis* oocytes. *X. laevis* oocytes (five per culture supernatant) loaded with [^{32}P]NAD (10^6 cpm) were injected with 20 nl (40 ng of protein) of bacterial culture supernatant. After microinjection, the oocytes were processed as described in Materials and Methods. Tracks: 1, Mock-injected oocytes; 2, *C. sordellii* (IP 82); 3, *C. chauvoei* (ATCC 10092); 4, *C. difficile* 196; 5, 6, and 7, *C. perfringens* A (NCTC 8798), C (CP93), and D (NCTC 2062), respectively; 8, purified *C. botulinum* C2 toxin component. (B) *C. difficile* 196 culture supernatant induces the transfer of ADP-ribose from NAD to a 45-kDa protein in *X. laevis* cytosolic extract. Crude bacterial supernatant from *C. difficile* 196 (10 μl) (track 1) or purified C2 toxin component I (0.1 μg) (track 2) was tested for ADP-ribosylation activity by the SDS-gel assay, as described in Materials and Methods. In track 3, no transferase was added to the assay reaction. Molecular weight standards are given in kilodaltons to the sides of the gels.

FIG. 2. Immunoprecipitation of the [^{32}P]ADP-ribosylated protein. Cytosolic extract of *Xenopus* oocytes was ADP-ribosylated by crude culture supernatant from CD196 (track A) and immunoprecipitated by anti-actin serum (track B) or nonimmune serum (track C).

of 50 mM Tris hydrochloride buffer (pH 7.5) and loaded on an Ultrogel AcA34 column equilibrated with the same buffer. Figure 4 shows the gel filtration elution pattern with the positions of the ADP-ribosyltransferase activity and the cytotoxic (A plus B toxin) activities. Enzymatically active fractions of the AcA34 column were pooled and applied to a QAE Sephadex column equilibrated with 50 mM Tris hydrochloride buffer (pH 7.5) and eluted in the same buffer. The CDT activity was totally recovered in the column flow-through. This material was further submitted to a preparative isoelectric focusing as described in Materials and Methods. Semipurified CDT was obtained by this procedure. A main 43-kDa protein band and minor peptide contaminants were observed by SDS-PAGE (data not shown). However, specific antibodies against CDT were obtained by immunizing a rabbit as described in Materials and Methods. Thereafter CDT antibodies were used to make an affinity column on which CDT was directly purified from the AcA34 column product (Fig. 5B).

Molecular characteristics of CDT. Purified CDT had a molecular weight of 43,000 by SDS-PAGE (Fig. 5B). ADP-ribosyltransferase activity was tested on purified CDT directly from SDS-PAGE. After the electrophoresis run, the gel was cut out into 20 5-mm slices. Protein in each slice was eluted into 0.5 ml of 5 mM HEPES buffer (pH 8.0) containing 10 mg of bovine serum albumin per ml. Actin was found to be ADP-ribosylated by the 43-kDa protein. This enzymatic
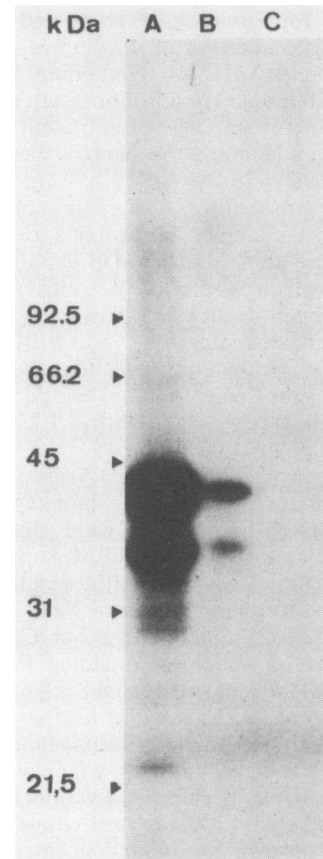
molecule is thus smaller than *C. botulinum* C2 component I, which in our hands was found at 45 kDa (Fig. 5B) (slightly different from the results of Ohishi et al. [22]). C2 toxin component II was found to have a M_r of 88 kDa (Fig. 5B), which corresponds to the trypsin-activated C2 component II (20).

The isoelectric point of CDT was determined by directly assaying the enzymatic activity of a partially purified enzyme (5 mg of protein) separated by preparative isoelectric focusing, as described in Materials and Methods. All the enzymatic activity was found in a broad peak with a maximum at pH 7.8 (data not shown). By the same technique, the isoelectric points of C2 toxin component I and II were found to be 4.3 and 7, respectively.

CDT does not induce a cytotoxic effect on cultured cells and is not lethal to mice. Purified CDT (trypsinized or not) gave no cytopathogenic (rounding up of cells) or cytotoxic effect on either Vero or MRC5 cells in culture (0.5 μg of protein per 10^5 cultured cells). It was not lethal upon intraperitoneal injection in mice (2 μg of protein per mouse). Crude CD196 culture supernatant (which contained both the enterotoxin and the cytotoxin activities) were trypsinized to activate a possible C2 toxin component II-like molecule (22). After trypsinization, crude 196 culture supernatant had lost all of its toxic effect for cells and mouse lethal activity (data not shown). We then tested the possibility that CDT could be toxic if it were complemented with activated C2 toxin
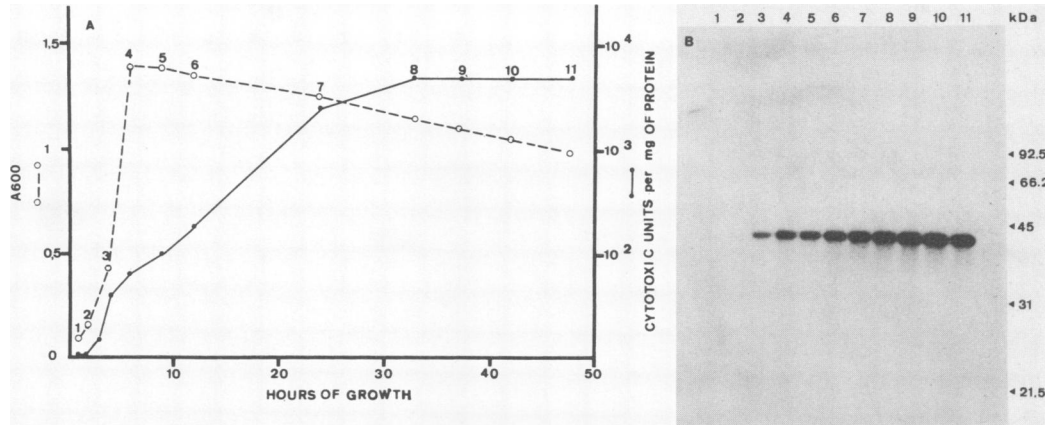
FIG. 3. Release of CDT in the culture medium as a function of time. (A) At each time indicated, 5-ml aliquots were removed from a 1-liter culture of CD196 in TGY medium. The A_{600} (O) and cytotoxicity (toxins A and B) to Vero cells (●) were measured. No spores could be detected in the culture before 50 h. (B) At the times indicated in panel A by the numbers (above the optical density curve), 10-μl aliquots of the CD196 culture supernatant were tested for ADP-ribosyltransferase activity by the gel assay described in Materials and Methods.
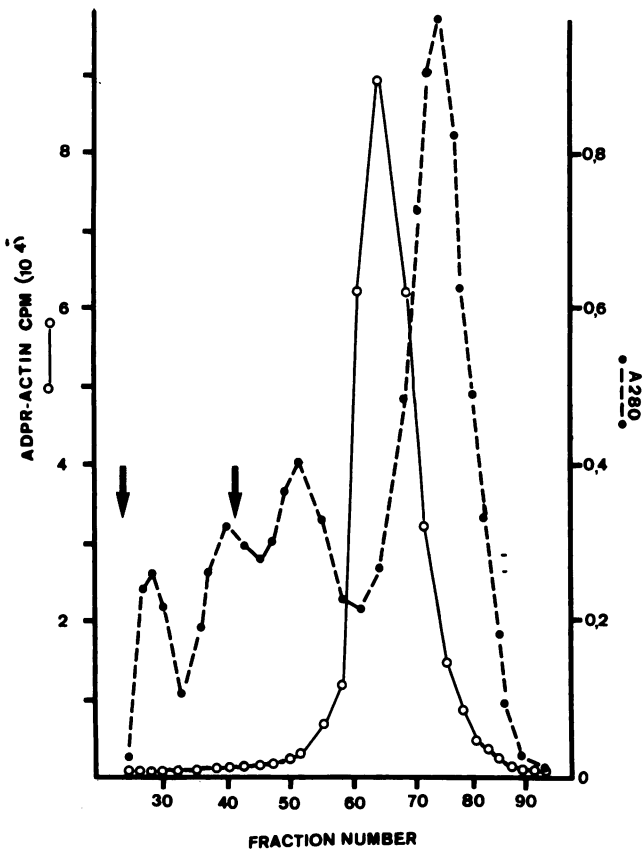

FIG. 4. Gel filtration of CD196 culture supernatant on Ultrogel ACA34. An Ultrogel AcA34 column (100 by 2.6 cm), equilibrated with 50 mM Tris hydrochloride buffer (pH 7.5), was loaded with CD196 culture supernatant precipitated with 70% (wt/vol) ammonium sulfate. Fractions (5 ml) were collected, and A_{280} (A280) was recorded (●). The ADP-ribosyltransferase (ADPR) activity of 10 μl of each fraction was determined by using the filter assay technique (O). Vero cell cytotoxicity (toxins A and B) was found between the two vertical arrows.

components. No cytotoxic effect or mouse lethal activity was observed when CDT was associated with C2 toxin component I or II at a 2:1 ratio in micrograms of protein. However, in similar experimental conditions, the combination of C2 toxin component I and II induced a cytotoxic effect of 1.2×10^2 CU in Vero cells and, upon intraperitoneal injection in mice, had a lethal effect of 3 mouse lethal doses (MLD) per μg of protein. Each C2 component individually was biologically inactive when tested at a minimal amount of 2 μg of protein per mouse or 0.5 μg of protein per 10^5 cultured cells.

Antibodies raised against CDT do not react with C2 toxin component I. Rabbit antibodies raised against the purified CDT potently neutralized the enzymatic activity of CDT (Fig. 5A) but had absolutely no effect on C2 toxin component I (either by enzymatic assay [Fig. 5A] or by Western immunoblotting [Fig. 5B]). Likewise, antibodies raised against C2 toxin component I did not neutralize CDT (Fig. 5A) or recognize CDT on a Western blot (Fig. 5B). Anti-CDT did not react with C2 toxin component II (Fig. 5B), and no hybridization with this antiserum was obtained with crude supernatant from VPI 10463, ATCC 9689, CD1063, CD201-83, CD660, and CD126 (Fig. 5C) or semipurified *C. difficile* toxin A or B (Fig. 5B). However, a clear-cut cross-reactivity with a 43-kDa protein was obtained with anti-CDT in the case of crude culture of CD196 and NCIB 10748 (*C. perfringens* E) (Fig. 5C).

We have also tested the possibility that antibodies raised against CDT might protect against the activity of either *C. difficile* enterotoxin or cytotoxin. However, all the experiments that we carried out to check this point gave negative results (data not shown).

Relationships of CDT with *C. difficile* toxins A and B. Pothoulakis et al. (24) and more recently Mitchell et al. (15) have concluded that cells treated by *C. difficile* toxin B (cytotoxin) have a higher content of G-actin than control cells do. Since it has been established that C2 toxin component I selectively ADP-ribosylates G-actin (1), this toxin could be used to estimate the amount of globular actin present in cells. We therefore incubated Vero cells with either *C. difficile* toxins A, B, and C2 until a strong characteristic (rounding up of cells) cytopathogenic effect was obtained. The cells were then detached from the plastic dishes and washed, and cytosolic extracts were made. These
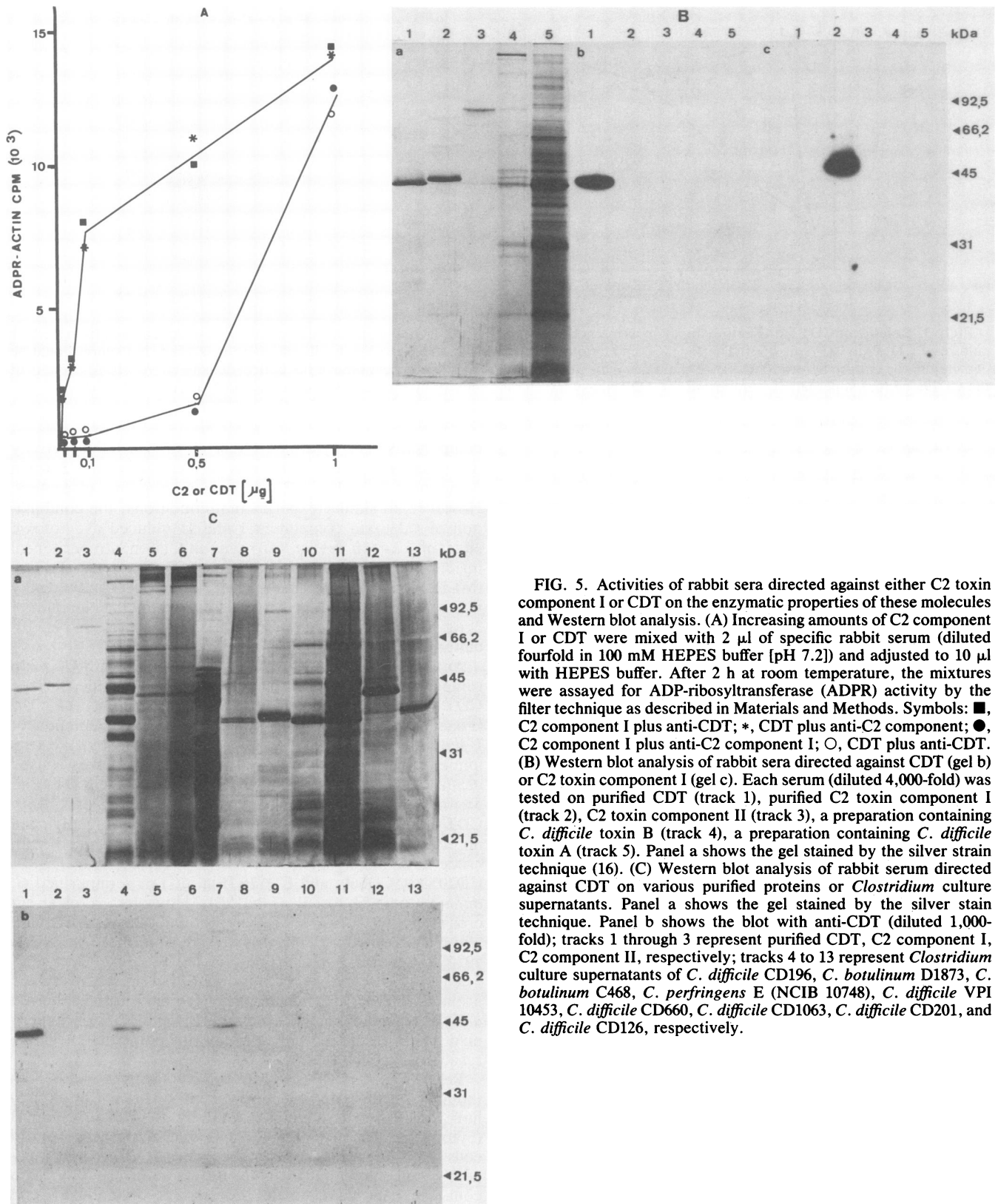
FIG. 5. Activities of rabbit sera directed against either C2 toxin component I or CDT on the enzymatic properties of these molecules and Western blot analysis. (A) Increasing amounts of C2 component I or CDT were mixed with 2 μl of specific rabbit serum (diluted fourfold in 100 mM HEPES buffer [pH 7.2]) and adjusted to 10 μl with HEPES buffer. After 2 h at room temperature, the mixtures were assayed for ADP-ribosyltransferase (ADPR) activity by the filter technique as described in Materials and Methods. Symbols: ■, C2 component I plus anti-CDT; *, CDT plus anti-C2 component; ●, C2 component I plus anti-C2 component I; ○, CDT plus anti-CDT. (B) Western blot analysis of rabbit sera directed against CDT (gel b) or C2 toxin component I (gel c). Each serum (diluted 4,000-fold) was tested on purified CDT (track 1), purified C2 toxin component I (track 2), C2 toxin component II (track 3), a preparation containing C. difficile toxin B (track 4), a preparation containing C. difficile toxin A (track 5). Panel a shows the gel stained by the silver strain technique (16). (C) Western blot analysis of rabbit serum directed against CDT on various purified proteins or Clostridium culture supernatants. Panel a shows the gel stained by the silver stain technique. Panel b shows the blot with anti-CDT (diluted 1,000-fold); tracks 1 through 3 represent purified CDT, C2 component I, C2 component II, respectively; tracks 4 to 13 represent Clostridium culture supernatants of C. difficile CD196, C. botulinum D1873, C. botulinum C468, C. perfringens E (NCIB 10748), C. difficile VPI 10453, C. difficile CD660, C. difficile CD1063, C. difficile CD201, and C. difficile CD126, respectively.

TABLE 1. ADP-ribosylation of actin by C2 component I or CDT of Vero cells treated in culture with *C. difficile* A, B, or C2 toxins

ADP-ribosyltransferase	ADP-ribose incorporated (cpm) in vitro by cells treated with:			
	Toxin A	Toxin B	C2 toxin	None
None	138	140	135	138
CDT	3,243 (6)[a]	1,704 (50)	292 (91)	3,452
C2 component I	3,096 (0)	1,612 (44)	165 (94)	2,891
Diphtheria toxin fragment A	692	492	520	547

[a] Values in parentheses represent percentages of reduction with respect to untreated control cells.

extracts were ADP-ribosylated by C2 toxin light chain or CDT, fractionated by SDS-PAGE, and processed for autoradiography. The radioactive spots corresponding to actin were cut out from the gel and counted for radioactivity. In Table 1, we report the results of such an experiment. Treatment of cells with toxin A did not significantly change the amount of actin available for ADP-ribosylation by C2 or CDT. On the other hand, Vero cells cultivated with C2 toxin had virtually no actin which could be ADP-ribosylated in vitro either by C2 toxin or CDT (Table 1). There was a clear-cut decrease (ca. 50%) in the amount of available actin when the Vero cells had been treated with *C. difficile* toxin B.

DISCUSSION

We have found that 1 *C. difficile* strain (CD196) out of 15 strains tested thus far produces a potent ADP-ribosyltransferase which covalently modifies cell actin in a similar way to *C. botulinum* C2 toxin light chain. CDT was purified to homogeneity by an immunoaffinity procedure. It has been reported recently that another toxin (iota) secreted by *C. perfringens* type E strains has a molecular architecture and an enzymatic activity similar to C2 toxin, although it ADP-ribosylates alpha actin, in addition to beta and gamma actin (37). The enzyme that we have isolated from CD196 has a molecular weight of 43,000 and thus is smaller than the light chain of C2 toxin (45 or 55 kDa [results of this study and reference 22]) or iota toxin (48 kDa [33]). The isoelectric point of CDT was found to be 7.8, which is very different from that of C2 toxin component I (pI 4.3) (28) or iota toxin light chain (pI 5.2) (32, 33). Antibodies raised against CDT did not react with C2 toxin component I, and antibodies directed against C2 toxin light chain did not recognize CDT. However, anti-CDT recognized a 43-kDa protein in the culture supernatant of *C. perfringens* type E, indicating that CDT could have some antigenic epitopes in common with iota toxin light chain. It has already been shown that *Clostridium spiroforme* also produces an iota-like toxin (2). Therefore, CDT could belong to the family of iota or *C. spiroforme* binary toxins but is very different from the *C. botulinum* C2 molecule. However, thus far, we have not been able to ADP-ribosylate muscle alpha actin by CDT (data not shown), as was reported for iota toxin i_a chain (37). Simpson et al. (31) have shown that iota toxin light chain and C2 toxin component II do not complement. We have obtained the same result with CDT and C2 component II. This result indicates that the two light chains, C2 component I and CDT, are different. Also different are the conditions of production of CDT. C2 toxin is produced only during sporulation of *C. botulinum* type C or D strains (17), but the production of iota toxin does not seem to depend on sporu-

lation (2). In the case of CDT, no sporulation was required to obtain the enzyme in the culture medium, which again suggests that CDT is more like iota toxin than like C2.

Several hypotheses could explain the presence of an ADP-ribosyltransferase in *C. difficile* that acts in a similar way to C2 toxin light chain. The first hypothesis would be that this strain elaborates a binary toxin, such as iota or C2. However, no toxic activity (either on cultured cells or upon injection in mice) could be found when crude culture supernatant from CD196 was trypsinized. This result indicates that CD196 does not secrete the equivalent of C2 toxin component II, which is activated by trypsin. The second possibility is that CDT might represent the active enzymatic component of *C. difficile* A or B toxin, which in most strains was latent but in CD196 had an enzymically active conformation for some reason. Indeed, ADP-ribosylation of G-actin induces rounding up of cells, and the cytopathic effects of C2 toxin are related to those observed with either *C. difficile* A or B toxin. Furthermore, C2 toxin has an enterotoxic activity (19). It is tempting to think that one or both of the *C. difficile* A and B toxins could exhibit an ADP-ribosyltransferase activity. Florin and Thelestam (6) reported that *C. difficile* B toxin caused the ADP-ribosylation of a cellular protein of about 90 kDa. However, ADP-ribosylation of this protein was also evident in untreated control cells (6). Using *X. laevis* oocytes loaded with [^{32}P]NAD, we have tested if microinjected *C. difficile* A or B toxins could label any oocyte protein. We never found a specific effect of the toxins, but we sometimes found a 80-kDa band which was labeled even in mock-injected oocytes. Such endogenous labeling of this protein occurred with [^{32}P]ADP-ribose, as well as with [^{32}P]NAD. We are inclined to think that the labeled protein observed by Florin and Thelestam is the same 80-kDa molecule that we found in oocytes and further, that it could correspond to the Sp83 stress-inducible molecule which Carlsson and Lazarides (4) have shown to be ADP-ribosylated by free ADP-ribose.

Direct experiments show that CDT is not a portion of either *C. difficile* A or B toxin. Strains of *C. difficile* producing the A and B toxins (such as the VPI 10463) displayed no ADP-ribosylating activity and had nothing in their culture supernatants that was recognizable by anti-CDT. In addition, antibodies raised against CDT did not neutralize the toxicity of *C. difficile* A or B toxins toward cultured cells.

We suppose, therefore, that CDT belongs to another binary toxin (maybe of the *C. perfringens* iota toxin family) which is not known so far and whose light chain gene has been introduced into CD196. This would imply that the genes for the light chain and the heavy chains of this binary toxin are the products of separate noncontiguous DNA fragments, allowing individual transfer into recipient cells. In relationship with this hypothesis, it is known that the genes coding for the three proteins which composed the toxins of *Bacillus anthracis* (protective antigen PA, lethal factor LF, and edema factor EF) are separate and noncontiguous (27).

A final interesting observation is that Vero cells treated in culture with cytotoxin B had not, as expected from the results of Pothoulakis et al. (24) and Mitchell et al. (15), an increase in G-actin which could be ADP-ribosylated by CDT or C2 toxin light chain. Instead, we found a decrease in the content of G-actin which could be ADP-ribosylated in vitro. We do not know, at the present time, if this smaller amount of G-actin is the result of previous ADP-ribosylation or is due to another covalent modification or change in the

conformation of the G-actin pool caused either directly or indirectly by the action of *C. difficile* toxin B.

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