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Purification of the *Clostridium spiroforme* Binary Toxin and Activity of the Toxin on HEp-2 Cells

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The two components Sa (M_r, 44,000) and Sb (M_r, 92,000) of *Clostridium spiroforme* toxin were identified and characterized. Serological data permitted the identification of two groups of actin ADP-ribosylating clostridial toxins. The first consists of only *C. botulinum* C2. The second group includes spiroforme toxin, iota toxin of *C. perfringens* E, and an enzyme called CDT found in one strain of *C. difficile*, antibodies against which cross-react with all of the members of both groups. *C. spiroforme* toxin acted on cells by disrupting microfilaments by ADP-ribosylation of G actin. Toxicity was not blocked by 10 or 20 mM ammonium chloride and was only moderately inhibited by 30 mM NH_4Cl. Inhibition of coated-pit formation in HEp-2 cells by potassium depletion strongly protected against the effect of *C. spiroforme* toxin. Toxicity was not blocked by incubation of HEp-2 cells and spiroforme toxin at 15°C. These results suggest that this new binary toxin enters cells via the coated-pit–coated-vesicle pathway and might reach the cytoplasm at the same time as or before transfer to early endosomes.

Several *Clostridium* species produce binary toxins (composed of two independent proteins) which ultimately disrupt the microfilaments of animal cells. The molecular basis of this effect is ADP-ribosylation of G actin which impedes assembly of this molecule into F actin (1, 20, 25). These binary toxins are divided into two groups based on their immunological reactivities (23). The first group contains the C2 toxin of *C. botulinum* types C and D, while the second consists of *C. perfringens* type E iota toxin (33) and *C. spiroforme* toxin (23, 32, 35). In addition, we have recently found an actin ADP-ribosylating protein in a strain of *C. difficile* (24). This last molecule immunologically cross-reacted with the ADP-ribosylating moieties of the iota-spiroforme group of binary toxins (24).

C. spiroforme binary toxin is composed of two independent polypeptide chains called Sa (light chain) and Sb (heavy chain) (23, 34); these are not associated by either covalent or noncovalent bonds. In this they resemble *C. botulinum* C2 toxin (19). The light chain of *C. spiroforme* toxin has been shown to be an ADP-ribosyltransferase which covalently modifies G actin (23, 32). The heavy chain, on the other hand, is required for penetration of Sa into the cytosol (23, 32, 35). Like C2 toxin component II, Sb must undergo limited proteolysis to be functionally active (27, 35).

In this paper, we describe the purification of the Sa (ADP-ribosylating) and Sb (binding) components of *C. spiroforme* binary toxin. We also studied the interaction of this toxin with cultured HEp-2 cells and investigated its uptake by an endocytotic process.

MATERIALS AND METHODS

Bacterial strains and toxin production. *C. spiroforme* NCTC 11493 (Rhône-Mérieux collection no. 15991) was grown in a 10-liter fermentor under anaerobic conditions (80% nitrogen–20% hydrogen in the gas phase). The fermentor was inoculated with 1 liter of an overnight bacterial culture. The medium used was the clostridial toxin production medium described by Sterne and Batty (34). The pH was automatically maintained at 7.4. After 48 h of growth at 37°C, the mouse lethality of the supernatant fluid was measured; it was usually 30 to 40 minimal lethal doses/ml. The bacterial supernatant fluid was concentrated by ultrafiltration through an S1Y10 membrane (Amicon Corp., Danvers, Mass.) to a volume of 600 ml. Ricin and diphtheria toxins were purified in our laboratory as previously described (17).

Preparation of antisera. New Zealand White rabbits (2 to 3 kg) were injected subcutaneously with 50 to 70 μg of pure component Sa or Sb of *C. spiroforme* contained in 2 ml of phosphate-buffered saline. An equal volume of complete Freund adjuvant was added for the primary injection. A booster injection with the same volume and protein concentration but with incomplete adjuvant was given 30 days later. The rabbits were bled 10 days after the booster injection. Antisera were kept at 4°C with 0.2% sodium azide.

Horse anti-diphtheria toxin serum (3,900 flocculation U/ml) was obtained from Institut Pasteur Production (Marnes, France). Rabbit serum against ricin toxin was prepared in our laboratory as described previously (17).

ADP-ribosyltransferase assays. In vitro ADP-ribosylation assays were performed with G actin isolated from *Xenopus laevis* oocytes as described previously (24, 26). The mixture for polyacrylamide gel electrophoresis (PAGE) assay (total volume, 20 μl) contained 7 μg of oocyte actin, 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 (specific activity, 30 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.). Between 1 and 10 μl of a fraction to be tested for enzymatic activity was added. After incubation for 1 h at 37°C, sample buffer was added and the preparation was fractionated by SDS-PAGE and processed by autoradiography.

Immunoblotting procedure. The immunoblotting method of Burnette et al. (4) was used. Proteins separated by sodium dodecyl sulfate (SDS)-PAGE were transferred electrophoretically to nitrocellulose (BA85; Schleicher & Schuell, Cera Labo, Paris, France). The nitrocellulose was incubated for 1 h in phosphate-buffered saline containing 5% milk and

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then incubated overnight at room temperature with specific antibodies diluted in the same solution. Bound antibodies were detected with [^{125}I]-labeled protein A, followed by autoradiography.

Cell culture assays. Cells were cultivated in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum. Vero (African green monkey kidney) cells were used for cytotoxic assays. Vero cells were plated into a 96-well Falcon tissue culture plate (Becton Dickinson Labware, Oxnard, Calif.) and grown for 24 h to form monolayers. Serial twofold dilutions of samples containing the toxin, activated by trypsin (0.02% chymotrypsin-free trypsin [Calbiochem-Behring, La Jolla, Calif.] at pH 7.5 for 30 min at 37°C, followed by addition of 0.04% [final concentration] soybean trypsin inhibitor [Sigma Chimie, L'Isle d'Abeau, France]), were prepared in DMEM and added to the monolayers. The cells were observed at 24, 48, and 72 h after inoculation for morphological alteration. One cytotoxic unit was defined as the reciprocal of the highest dilution which produced a cytopathic effect on 100% of the cells. HEp-2 (human carcinoma) cells were used for cell biology studies. They were cultivated either in 24-well tissue culture plates (Falcon) or on cover slips in the same medium used for Vero cells. HEp-2 cell cultures were routinely checked and determined to be free of mycoplasma organisms by the Laboratory of Mycoplasma (Institut Pasteur, Paris, France). ADP-ribosylation in vitro of actin was used to quantify the effects of *C. spiroforme* toxin on cells. Briefly, the cell monolayers from 24-well tissue culture plates were first washed with cold DMEM, followed by incubation with various dilutions of *C. spiroforme* toxin. For studies on the effects of temperature and NH_4Cl or potassium depletion, the protocol was the same. After incubation at 37°C (the time of incubation described in each figure legend), the cells were washed three times with ice-cold DMEM and detached with a rubber policeman in 0.5 ml of 100 mM HEPES buffer (pH 7.4). The cells were then spun down in a microcentrifuge, and the pellets were suspended in 50 μl of 100 mM HEPES buffer (pH 7.4). The cells were broken by three cycles of freezing and thawing. In vitro ADP-ribosylation by the *C. spiroforme* Sa chain was then performed on cell extracts (the mixture contained 20 μl of cell extracts, 2.5×10^5 cpm of [^{32}P]NAD and 0.5 μg of Sa). After 1 h at 37°C, the samples were analyzed by SDS-PAGE and autoradiography. The actin bands were excised from the dry gel and the radioactivity was counted. The results are expressed as percentages of the control (not treated with toxin) values. Toxicity on HEp-2 cells, induced by ricin and diphtheria toxins, was studied as previously described (17).

Protocol for hypotonic shock of cells. The method described by Moya et al. (17) was used to deplete HEp-2 cells of K^+ ions. Cells were grown for 48 h on 24-well Falcon tissue culture plates. The medium was discarded, and each monolayer was washed twice with 50 mM sodium HEPES buffer (pH 7.4) with 100 mM NaCl. The cells were hypotonically shocked for 5 min by incubation in DMEM-water (1:1), followed by incubation for the indicated time in isotonic K^+-free medium (50 mM sodium HEPES [pH 7.4], 100 mM NaCl, 1 mM $CaCl_2$).

Immunofluorescence studies. After 48 h of growth on cover slips, HEp-2 cells were treated as described in the figure legends and then fixed for 20 min at room temperature in 10 mM sodium phosphate buffer (pH 7.4) containing 3% (vol/vol) paraformaldehyde, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$. Fixed cells were washed twice with 10 mM sodium phosphate buffer (pH 7.4). Each monolayer was then permeabilized with 2 ml of 0.1% (vol/vol) Triton X-100 in 10 mM sodium phosphate buffer (pH 7.4). For experiments with F actin, each cover slip was placed on 30 μl of 10-fold-diluted *N*-(7-nitrobenz-2-oxa-1,3-diazo-4-yl) phallacidin (NBD-phallacidin; Molecular Probes, Junction City, Oreg.). For microtubule staining, a monoclonal anti-α-tubulin antibody (Amersham, Buckinghamshire, England) was used, followed by incubation with rhodamine-labeled rabbit anti-mouse antibodies (Sigma). The cover slips were washed and mounted on glass slides, examined, and photographed under a fluorescence microscope.

RESULTS

Purification of *C. spiroforme* binary toxin components Sa and Sb. Purification of the two components of *C. spiroforme* binary toxin, Sa and Sb, was achieved as follows. Culture supernatant fluid (total volume, 10 liters) was concentrated by filtration on a membrane (Amicon S1Y10; cutoff, 10,000 daltons) to a volume of 600 ml, after which 20 ml of the concentrate (0.45 mg of protein per ml) was dialyzed against 50 mM Tris hydrochloride (pH 7.5; buffer A) and loaded on an Ultrogel AcA 34 (IBF, Paris, France) column (100 by 2.6 cm) equilibrated with buffer A. ADP-ribosyltransferase activity was monitored in each fraction to detect Sa activity. Sb activity was determined by its ability (after trypsinization) to induce rounding up of Vero cells when associated with Sa. Active samples were pooled and applied to a DEAE-Sepharose CL6B column (15 by 1 cm; Pharmacia, Paris, France) equilibrated with buffer A.

The column flowthrough was shown to contain all of the ADP-ribosyltransferase Sa activity, whereas elution of the DEAE-Sepharose CL6B column with buffer A containing 0.1 M NaCl yielded the Sb component of *C. spiroforme* toxin. The Sa and Sb components were then purified as follows. Pooled fractions containing Sa were dialyzed against 50 mM Tris hydrochloride buffer (pH 8.5; buffer T) and applied to a DEAE-Sepharose column (15 by 1 cm) equilibrated with buffer T. The column was washed with buffer T until no protein could be eluted, and then buffer T containing 100 mM NaCl was applied and Sa was recovered in the column fractions. Pooled fractions from the DEAE-Sepharose CL6B column containing Sb were dialyzed against distilled water and purified by preparative flat-bed isoelectric focusing with 5% LKB Ampholine (pH 4 to 6) as previously described (24). Yields of 200 μg of purified Sa and Sb were obtained from 4.5-mg samples of the bacterial supernatant proteins. Figure 1 shows a silver-stained SDS-PAGE gel of Sa purified by this protocol. The molecular weight of Sa was found to be 44,000. The Sb component was found to have a molecular weight of 92,000 and, upon treatment with trypsin, to run on PAGE at a molecular ratio of 76,000 (Fig. 1). It is worthy of note that even in the absence of trypsin treatment, a small amount of Sb in its activated form (M_r, 76,000) was already present (Fig. 1). The isoelectric points of Sa and Sb were determined by analytical isoelectric focusing in a thin-layer polyacrylamide gel (pH 3.5 to 9.5) (LKB) performed as recommended by the manufacturer (data not shown). The pI of Sb was 4.7 (before or after trypsin treatment), whereas that of Sa was 6.2.

To confirm that purified Sa was an ADP-ribosyltransferase, the following experiment was done. An SDS-PAGE gel was loaded with purified Sa, and after electrophoresis, the gel was sliced into 20 portions and the protein contained in each slice was eluted with a buffer containing an excess of bovine serum albumin. Each fraction was then tested for

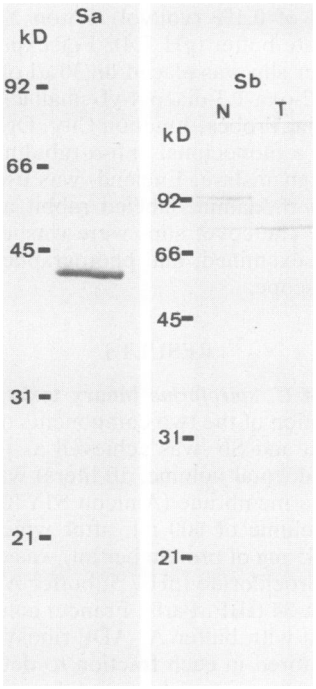


FIG. 1. SDS-PAGE of purified *C. spiroforme* Sa and Sb chains. About 1 μg of each toxin was layered on an SDS–12% PAGE gel. After electrophoresis, the gel was stained by the silver stain technique. Panels: Sa, toxin component Sa; Sb, toxin component Sb; N, Not trypsinized; T, after trypsinization; kD, kilodaltons.

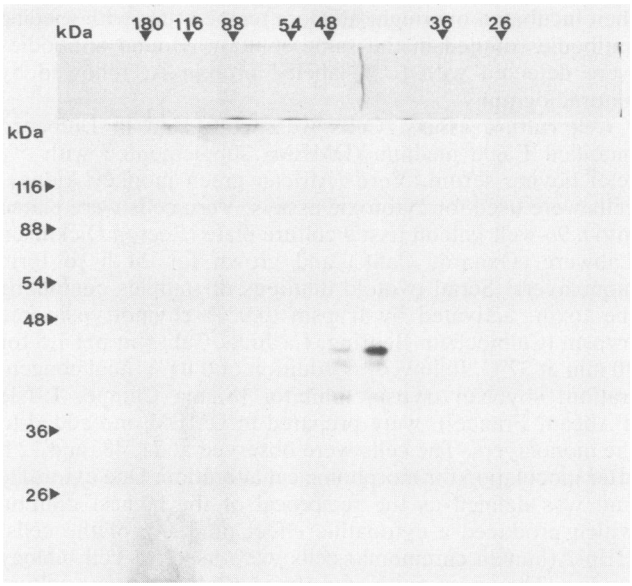


FIG. 2. Evidence that 44,000-M_r protein Sa is enzymically active. A 2-μg sample of purified Sa was subjected to SDS-7.5 to 15% PAGE (the sample was not boiled with application buffer before electrophoresis). After completion of the run, the gel was sliced into 20 portions. The proteins in each slice were eluted into 0.5 ml of 5 mM HEPES buffer (pH 7.2) containing 10 mg of bovine serum albumin per ml. A 15-μl sample of each eluate was tested for ADP-ribosyltransferase by SDS-PAGE (see Materials and Methods), followed by autoradiography. The panel at the top represents a stained (silver method) duplicate gel. kDa, Kilodaltons.

ADP-ribosyltransferase activity by using *X. laevis* actin as a substrate (24). Actin was labeled only in slices containing Sa protein (Fig. 2).

Immunoreactivity of Sa and Sb. Rabbit antibodies raised against the purified Sa component cross-reacted by immunoblot with the ADP-ribosyltransferase moieties of *C. perfringens* iota toxin (ia) and CDT (the transferase isolated from *C. difficile* 196 [24]; Fig. 3A). However, no cross-reactivity with component I of *C. botulinum* C2 toxin was found (Fig. 3A).

Correspondingly, rabbit antibodies raised against purified Sb (trypsin treated) chain reacted by immunoblot with the *C. perfringens* iota toxin-binding component (ib) but not with C2 toxin component II (Fig. 3B).

Toxicity of *C. spiroforme* binary toxin for mice and Vero cells. The toxicity of *C. spiroforme* binary toxin for mice was tested with Sa in combination with trypsin-activated component Sb. Component Sa or Sb alone was devoid of toxicity. Sa and Sb together were highly toxic for mice (10 minimal lethal doses/μg of protein, tested intraperitoneally). The same result was observed when *C. spiroforme* toxin toxicity for Vero cells was tested. Cells rounded up only when they were exposed to the combination of Sa and Sb (32 cytotoxic U/μg of Sa plus Sb in equimolar amounts).

Effect of *C. spiroforme* binary toxin on HEp-2 cells. HEp-2 cells were used in this study because their receptor-mediated endocytosis by coated pits can be efficiently blocked by potassium depletion (10, 17). Therefore, it was of interest to test *C. spiroforme* toxin on these cells to study its mechanism of entry into the cytoplasm. We first determined whether *C. spiroforme* toxin was able to specifically disrupt cytoskeletal filaments, such as F actin or microtubules, in HEp-2 cells. HEp-2 cells were treated with 10^{-7} M *C.*

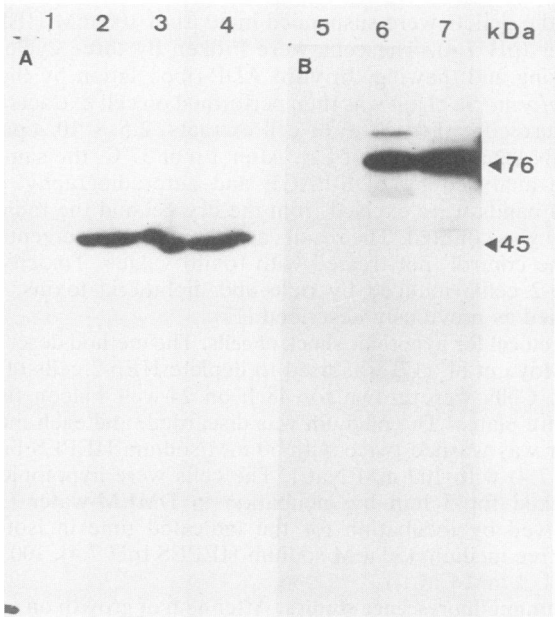


FIG. 3. Western blot (immunoblot) analysis of clostridial binary toxins using rabbit sera raised against purified *C. spiroforme* Sa and Sb components (trypsin activated). (A) Blot with anti-Sa diluted 4,000-fold. Lanes: 1, C2 component I; 2, iota ia; 3, CDT; 4, Sa. (B) Blot with anti-Sb diluted 4,000-fold. Lanes: 5, C2 component II; 6, iota ib; 7, Sb. kDa, Kilodaltons.

spiroforme toxin (to monitor the cytopathic effects easily by microscope; Sa plus Sb in equimolar amounts) for 4 h and then stained by an immunofluorescence technique for tubulin and with *N*-(7-nitrobenz-2-oxa-1,3-diazo-4-yl) phallacidin (a fluorescent molecule derived from phalloidin) for F actin. When HEp-2 cells were intoxicated, F actin (seen as stress fibers) disappeared totally (Fig. 4C and D). Microtubules, on the other hand, although very concentrated in the cytoplasm compared with control cells, were still present (Fig. 4A and B).

We then examined the effect of ammonium chloride, a drug well known to raise endosomal, trans-Golgi, and lysosomal pHs (14), on toxicity. HEp-2 cells were treated for 30 min with medium containing 10, 20, or 30 mM NH_4Cl. *C. spiroforme* toxin (Sa plus Sb in equimolar amounts) was then added to the cell culture at various concentrations in the presence of NH_4Cl (10, 20, or 30 mM) and incubated at 37°C. After 6 h, the cells were harvested and tested for in vitro actin ADP-ribosylation by the *C. spiroforme* toxin Sa component as described in Materials and Methods. Ammonium chloride afforded no protection against *C. spiroforme* toxin at 10 or 20 mM (Fig. 5). However, at 30 mM, slight protection (especially at a 10^{-8} M toxin concentration) was observed (Fig. 5). We must stress that at 30 mM NH_4Cl a 70% reduction of actin ADP-ribosylation was observed in control preparations (not treated with toxin). We do not know the mechanism of action of NH_4Cl on the size of the cellular G actin pool. However, this large reduction of actin ADP-ribosylation in vitro by 30 mM NH_4Cl makes interpretation of protection against *C. spiroforme* toxin difficult.

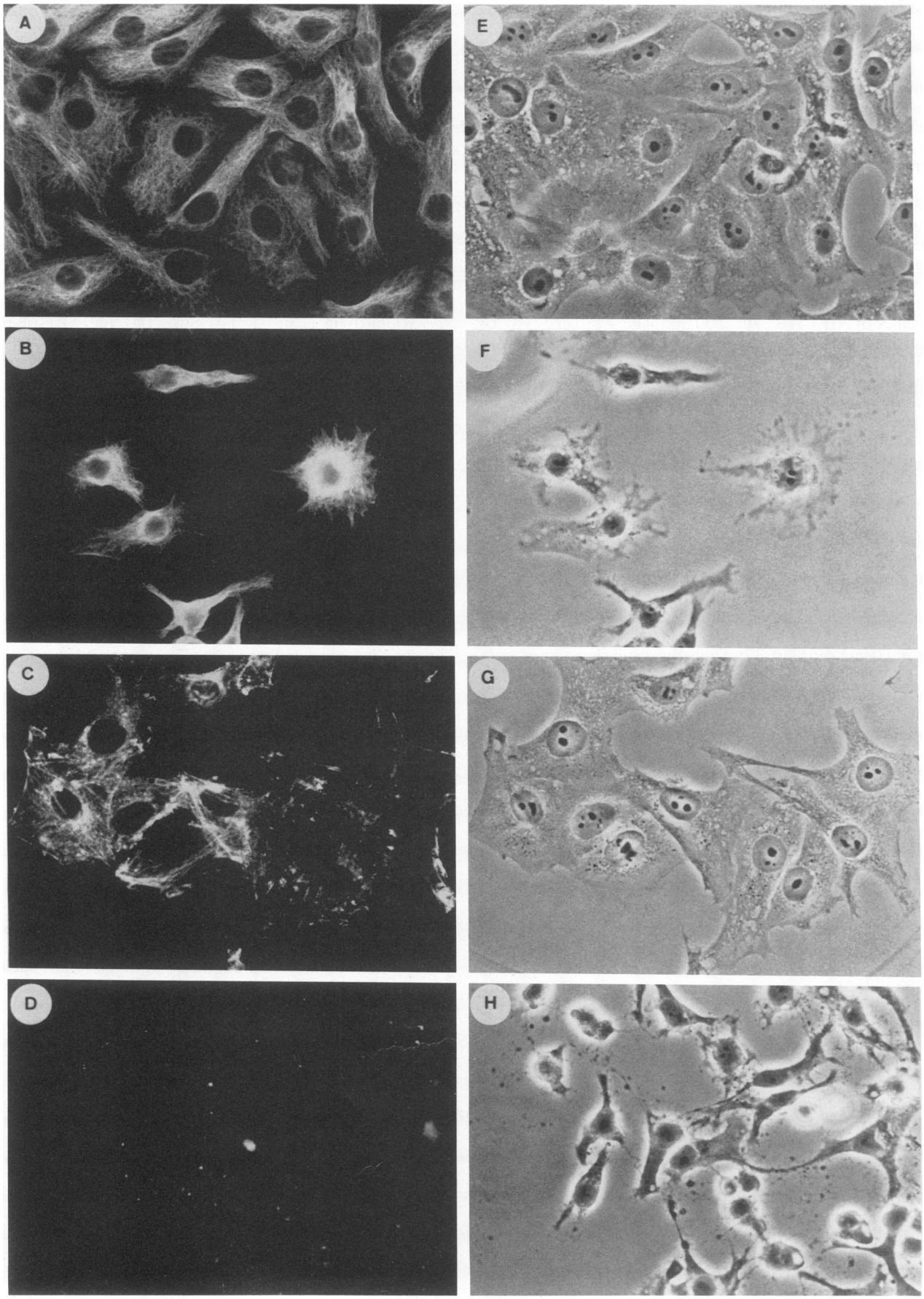
The role of coated pits in the entry of *C. spiroforme* toxin into HEp-2 cells was examined by using the fact that depletion of potassium totally blocks the formation of coated pits in HEp-2 cells (10, 17). Diphtheria and ricin toxins were used to monitor the effects of potassium depletion (17). HEp-2 cells were depleted of potassium and incubated for 30 min (to minimize the effects of lack of potassium on cell protein synthesis [17]) with various doses of *C. spiroforme*, diphtheria or ricin toxin. After this step, the cells were carefully washed with medium without potassium and incubated with DMEM containing antibodies against the *C. spiroforme* Sb component, diphtheria toxin, or ricin toxin to block further entry (control experiments confirmed that the antibodies were effective). In this way, only molecules taken up during the potassium depletion step (in the absence of coated pits) could intoxicate the cells. The effect of *C. spiroforme* toxin on actin ADP-ribosylation was blocked (as was the cytotoxicity of diphtheria toxin [Fig. 6B]) when coated-pit formation was inhibited (Fig. 6A). As we have already reported (17), ricin toxin toxicity was not affected by inhibition of receptor-mediated endocytosis involving clathrin (Fig. 6B).

Finally, we examined the possibility that, once taken up into vesicles, *C. spiroforme* binary toxin could travel to the trans-Golgi network (8), the site at which ricin toxin is thought to cross the membrane to the cytosol (38, 39). A temperature below 20°C (38, 39) has been shown to block the traffic from endosomes to the trans-Golgi network, probably by disrupting the microtubule meshwork which tracks vesicles between endosomes to the trans-Golgi network area. A temperature of 15°C does not block receptor-mediated endocytosis (i.e., the vesicular traffic between plasma membrane to endosomes [12]). HEp-2 cells were incubated for 10 h at 15°C with *C. spiroforme*, diphtheria, or ricin toxin. The same experiments were done in parallel with cells incubated at 37°C. After incubation (at 15 or 37°C), the in vitro ADP-ribosylation of actin was tested and protein synthesis (for diphtheria and ricin toxins) was estimated. Even when incubated with cells at 15°C *C. spiroforme* binary toxin was able to reduce the G actin available for ADP-ribosylation in vitro, although less efficiently (Fig. 7A). The same results were obtained with diphtheria toxin (Fig. 7B). At 15°C, ricin toxin was without effect on HEp-2 cell protein synthesis (Fig. 7B), as previously shown by van Deurs et al. (38).

DISCUSSION

In the present work, we describe a method to purify the two components, Sa and Sb, of the *C. spiroforme* binary toxin. Component Sa has an M_r of 44,000 and a pI of 6.2, it is an ADP-ribosyltransferase which modifies G actin (23, 32). Antibodies raised against Sa cross-reacted with *C. perfringens* iota toxin chain ia and with CDT, the ADP-ribosyltransferase isolated from *C. difficile* 196 (24). No cross-reactivity of Sa was observed with C2 toxin component I. The Sb component of *C. spiroforme* toxin has an M_r of 92,000, and when treated with trypsin, it shifted to a molecule with an M_r of 76,000. It was reported by Ohishi (18) that activation of *C. botulinum* component II was always accompanied by a change in its molecular weight from 101,000 to 88,000. Therefore, a polypeptide of comparable size (M_rs, 13,000 for C2 component II and 16,000 for Sb) is lost upon activation in both actin ADP-ribosylating clostridial toxin-binding components. We did not determine whether Sb could be oligomerized upon trypsinization, as was reported for C2-II (18). The isoelectric point of Sb, either at its native molecular weight or after trypsin treatment, was found to be 4.7, indicating that the lost polypeptide did not bear a net electric charge. In agreement with our findings on Sa, no immunological relatedness with C2 component II was found, but antibodies raised against Sb cross-reacted with the *C. perfringens* E toxin ib chain. In this study, we found that a combination of Sa and Sb at a molar ratio of 1:1 was toxic for mice (intraperitoneal minimal lethal dose, 100 ng, a dose very close to that of C2 toxin [19]).

C. spiroforme binary toxin was active on cultured cells (Vero or HEp-2) and induced disappearance of F actin without modifying microtubules. More interesting was the finding that ammonium chloride, a chemical known to increase the pHs of endosomes and lysosomes (12, 13), did not block the cytotoxic effect of *C. spiroforme* toxin (except for moderate inhibition at 30 mM). Ammonium chloride (a lysomotropic amine) is well known to block the toxic effects of several bacterial and plant toxins (diphtheria toxin, modeccin, and *Pseudomonas aeruginosa* exotoxin A). It is thought that toxins that are blocked by weak bases, such as ammonium chloride, must reach an acidic cell compartment to be translocated (or to translocate a fragment) into the cytosol (21). These acidic compartments are endosomes, trans-Golgi, or (of course) lysosomes (2, 15, 37). A new acidic cell compartment required for sorting lysosomal proteins by using mannose-6-phosphate receptors has been previously described (7), but no toxin has been shown to be routed to this organelle. Ricin is known not to be affected by lysomotropic amines; instead, these drugs increase its toxicity for cells (14), and recently this toxin has been shown to reach the trans-Golgi compartment (38, 39). The results we obtained with ammonium chloride suggest that for *C. spiroforme* toxin, an acidic compartment is not required for translocation of the molecule through the membrane. A comparable nonacidic mechanism for the entry of *Bordetella pertussis* adenylate cyclase has been reported recently by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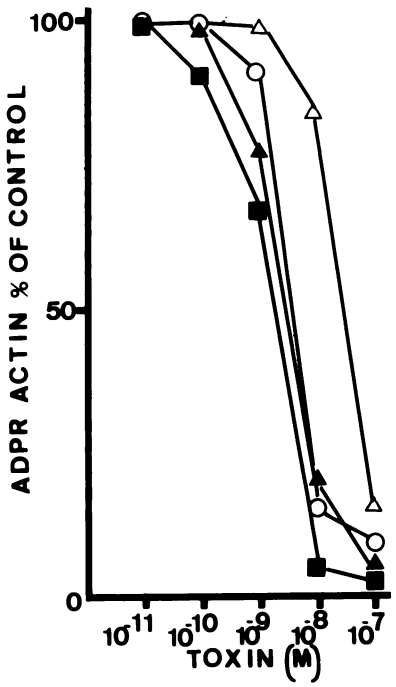

FIG. 5. Effect of NH_4Cl on intoxication of HEp-2 cells by *C. spiroforme* toxin. HEp-2 cells grown in 24-well microplates were incubated with 10, 20, or 30 mM NH_4Cl for 30 min, and then *C. spiroforme* toxin dilutions were applied to the monolayer. After 6 h at 37°C, the cells were processed for in vitro ADP-ribosylation (ADPR) of actin by the Sa chain of *C. spiroforme* toxin as described in Materials and Methods. Controls without toxin but with ammonium chloride at different concentrations were run at the same time. HEp-2 cells were used with *C. spiroforme* toxin without NH_4Cl (■), with 10 mM NH_4Cl (▲), with 20 mM NH_4Cl (○), and with *C. spiroforme* toxin with 30 mM NH_4Cl (△).

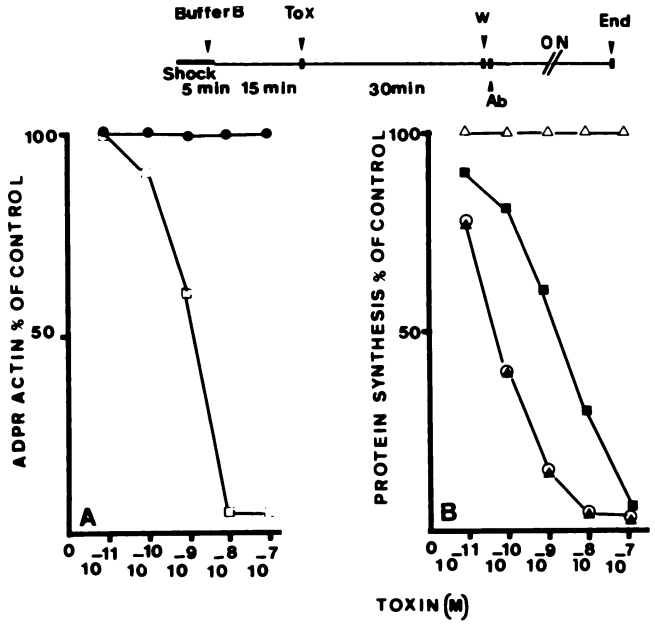

FIG. 6. Effects of *C. spiroforme* toxin on HEp-2 cell actin ADP-ribosylation (ADPR) after intracellular K^+ depletion; comparison with diphtheria and ricin toxin effects on protein synthesis. Experiments were conducted as represented in the schematic drawing at the top. After isotonic K^+-free medium (buffer B), various concentrations of *C. spiroforme* toxin (A) were added to the cells. After 30 min at 37°C, cells were carefully washed three times with buffer B (W) and then incubated overnight in DMEM containing 50 μg of rabbit anti-*C. spiroforme* toxin component Sb serum per ml (A) with 20 flocculation U of horse anti-diphtheria toxin serum per ml or 10 μl of rabbit anti-ricin serum per ml (B) (Ab). ADP-ribosylation of actin in vitro (A) was performed as described in Materials and Methods. Protein synthesis (B) was assayed by incorporation of [^{14}C]leucine as previously described (17). Panel A symbols: ●, cells without K^+; □, cells with K^+. Panel B symbols: △, diphtheria toxin on cells without K^+; ▲, ricin toxin on cells without K^+; ■, diphtheria toxin on cells with K^+; ○, ricin toxin on cells with K^+.

Gordon et al. (6). However, *Bacillus anthracis* adenylate cyclase cell intoxication was found to be acid dependent (6).

At 15°C, HEp-2 cells had their G actin ADP-ribosylated by *C. spiroforme* binary toxin. This result suggests that the toxin may not have to move from the cell periphery to the organelles close to the nucleus (lysosomes, mannose-6-phosphate receptor uncoupling compartment, or trans-Golgi), since a temperature below 20°C is supposed to affect the traffic of vesicles between late endosomes and the trans-Golgi network and has been shown to block ricin toxin effects (38, 39).

Inhibition of coated-pit formation by potassium depletion seemed to block the cytotoxicity induced by *C. spiroforme* toxin, as it does for diphtheria toxin (17). Such treatment has no effect on ricin toxin (10, 17). It has been inferred from previously reported data that diphtheria toxin must use coated pits (16) for productive entry into cells (17), whereas ricin toxin was supposed to use an alternative pathway of endocytosis (smooth pits and smooth vesicles; 10, 17, 30). On this basis, *C. spiroforme* toxin could behave like diphtheria toxin (i.e., entry via coated pits and coated vesicles). However, we are not entirely certain that potassium depletion is without effect on toxin binding to the cell membrane. Indeed, incubation of HEp-2 cells at 4°C with *C. spiroforme* toxin in medium with or without potassium, followed by repeated washing and incubation at 37°C in the presence of antibodies against the *C. spiroforme* Sb toxin chain, did not induce a profound cytopathic effect (data not shown). Therefore, it is difficult to determine the influence of potassium depletion on *C. spiroforme* toxin binding.

On the basis of the data presented in this paper, we can tentatively suggest the location for cell entry of *C. spiroforme* binary toxin. Since the toxin requires neither an acidic pH nor vesicle traffic from the periphery to the cell center and yet does seem to require the coated-pit–coated-vesicle pathway of endocytosis, it might well enter the cytosol from uncoated vesicles (coated vesicles which have lost their clathrin coat [22]) or during fusion of uncoated vesicles with

FIG. 4. Immunofluorescence studies of the effects of *C. spiroforme* toxin on microtubules and F actin organization. HEp-2 cells were treated with 10^{-7} M *C. spiroforme* binary toxin (Sa/Sb molar ratio, 1:1) for 4 h at 37°C. The cells were then fixed, stained, and photographed as described in Materials and Methods. Panels: A, control cells stained for tubulin; E, phase contrast of panel A; B, cells treated with *C. spiroforme* toxin and stained for tubulin; F, phase contrast of panel B; C, control cells stained for F actin; G, phase contrast of panel C; D, cells treated with *C. spiroforme* toxin and stained for F actin; H, phase contrast of panel D.

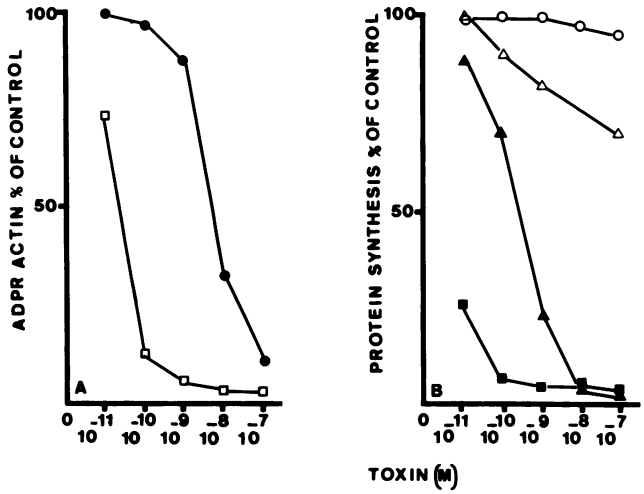

FIG. 7. Effects of low temperature (15°C) on actin ADP-ribosylation (ADPR) of HEp-2 cells by *C. spiroforme* toxin; comparison with diphtheria and ricin toxin effects on protein synthesis. HEp-2 cell monolayers after 48 h of growth were washed three times with ice-cold DMEM (to block endocytosis). The toxin dilutions were first incubated with cells at 4°C in DMEM adjusted to pH 7.4 with 10 mM HEPES buffer. The microplates were then wrapped in sealed plastic bags and incubated for 10 h in a water bath (at either 37 or 15°C). After incubation, cell monolayers were washed three times with ice-cold DMEM and incubated at 4°C for 20 min in DMEM containing antibodies specific to each toxin (as described in the diagram at the top of Fig. 6). For ADP-ribosylation in vitro of actin (A), the cells were processed as described in Materials and Methods. For estimation of protein synthesis (B), cell monolayers were incubated further for 10 min at 37°C with 0.5 μCi of [^{14}C]leucine per ml and processed as previously described (17). Panel A symbols: □, *C. spiroforme* toxin on cells at 37°C; ●, *C. spiroforme* toxin on cells at 15°C. Panel B symbols: ○, ricin toxin on cells at 15°C; △, diphtheria toxin on cells at 15°C; ▲, ricin toxin on cells at 37°C; ■, diphtheria toxin on cells at 37°C.

early endosomes (31). This location of toxin entry would differ from that of diphtheria toxin in an acidic environment (5, 9, 11, 28) (early endosome [21]) or that of ricin toxin (trans-Golgi [38, 39]) and could therefore be comparable to that of shigella toxin, which is not inhibited by lysosomotropic amines (27) and requires coated pits (29). This site of cell penetration would be quite useful for a toxin that ultimately acts on G actin, since it is known that formation of F actin from G actin is achieved mainly in the periphery of the cell (subcortical actin web) by gelsolin or villin (36) and in the vicinity of the cytoplasmic domain of integrins, the specialized transmembrane proteins involved in binding of the extracellular matrix (3). Therefore, in this cell domain, there is probably a higher concentration of G actin molecules. In that way, the ADP-ribosylation of G actin by Sa could be quite efficient.

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