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ADP-RIBOSYLATION IN CULTURED CELLS TREATED WITH CLOSTRIDIUM DIFFICILE TOXIN B

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In cultured fibroblasts intoxicated with Clostridium difficile toxin B, a radioactive moiety was transferred from [^{14}C-adenosine] NAD, but not from [^{14}C-nicotinamide] NAD, into a cellular protein (MW 90.000). No labeling was detected in toxin-treated cultures not yet showing any toxin-induced cytopathogenic effect, whereas maximal labeling was obtained in cultures with about half of the cells showing a cytopathogenic effect. The radioactivity was removed from the substrate by treatment with snake venom phosphodiesterase. The results suggest that ADP-ribosylation of a cellular protein occurs in toxin B-treated cells and that this reaction may be responsible for development of the cytopathogenic effect.

An increasing number of bacterial toxins are found to possess ADP-ribosyltransferase activity: diphtheria toxin, Pseudomonas exotoxin A and exoenzyme S, cholera toxin, E.coli LT, Salmonella enterotoxin, pertussis toxin and botulinum toxin type C_2 (1,2,3). These toxins catalyze a transfer of ADP-ribose from NAD to a cellular substrate (1), a reaction which constitutes the molecular basis for their different cytotoxic effects. The substrates for ADP-ribosylation are different cellular proteins, such as elongation factor 2, or regulatory subunits of adenylate cyclase (1). These observations prompted us to find out whether toxin B from Clostridium difficile has a similar activity.

C.difficile toxin B is involved in the etiology of antibiotic-associated colitis (4). In toxin-treated cultured cells, a characteristic cytopatho-

ABBREVIATIONS

LF=flocculating unit, CPE=cytopathogenic effect, TCD_{50}=50% tissue culture dose, TCA=trichloroacetic acid.

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genic effect (CPE) appears after a latency period, during which the toxin is internalized via endocytosis (5) and processed in the lysosomes (6). The intracellular molecular target for toxin B has not yet been identified, although some part of the microfilament system has been suggested as a likely target (7).

MATERIALS AND METHODS

Chemicals. Eagle minimal essential medium, calf serum and trypsin were obtained from Flow Laboratories, Irvine, Scotland. [U-^{14}C] adenosine (specific activity 527 mCi/mmole), [carbonyl-^{14}C] nicotinamide (spec. act. 55 mCi/mmole), and nicotinamide [U-^{14}C] adenine dinucleotide (NAD) (spec. act. 260 mCi/mmole) were purchased from Amersham International plc, Buckinghamshire, England, and [adenylate-^{32}P] NAD (spec. act. 33.2 Ci/mmole) from NEN Chemicals GmbH, Dreieich, Federal Republic of Germany. Snake venom phosphodiesterase I was obtained from Worthington Biochem. Corp., Freehold, New Jersey 07728. Acrylamide (>99.9% purity) was from BioRad, Richmond, California 94804.

Toxins. Toxin B was purified from dialysis cultures of a clinical isolate of C.difficile as previously described (5). Diphtheria toxin (300 LF/mg) was kindly provided by P. Askelöf and P. Gillenius, National Bacteriological Laboratory, Stockholm, Sweden. The toxins were reduced by incubation with 1 mM dithiotreitol for 15 min at room temperature.

Cultivation of cells. Human diploid embryonic lung fibroblasts (line MRC-5) were cultivated as previously described (8) in Eagle minimal essential medium supplemented with 10% newborn calf serum, 5 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml), hereafter referred to as growth medium. The cells were free of mycoplasma infection as determined by Hoechst staining (9) and cultivation.

The unit of toxin activity was the 50% tissue culture dose (TCD_{50}), i.e., the toxin dilution inducing within 20 h a characteristic actinomorphic cytopathogenic effect in 50% of cells exposed under standardized conditions (8). The amount of protein corresponding to 1 TCD_{50} varied from 0.1 to 1 ng in different preparations. Exposure of cells to a dose of 1000 TCD_{50} for 2 h resulted in a CPE in all cells.

Assay of ADP-ribosylation in intact cells. Confluent monolayers of cells in six-well plates were incubated at 37°C with 1 µCi [^{14}C] adenosine, [^{14}C] nicotinamide or [^{14}C-adenine] NAD in 1 ml growth medium per well. After 1 h, toxin B (1000 TCD_{50}) or diphtheria toxin (30 LF) was added and the incubation continued for 2 h. Extracellular radioactivity was carefully removed by rinsing twice with Hank's balanced salt solution. The cells were solubilized in 0.1% SDS, whereupon the cells from two wells (corresponding to 210-250 µg protein) were pooled and protein precipitated with trichlo-

roacetic acid (TCA) as previously described (8). The amount of protein was determined by the method of Lowry et al. (10).

Assay of ADP-ribosylation in cell lysates. Pelleted cells (3 x 10^5 cells per sample, corresponding to 210-250 µg protein) were lysed by addition of 40 µl assay buffer, as defined by Pappenheimer et al. (11), but containing 0.25 µCi [^{14}C-adenine]NAD or [^{32}P]NAD per sample. Toxins were added (see above), and after incubation for 15 min at 37°C the protein was precipitated.

SDS-polyacrylamide gel electrophoresis. TCA-precipitated protein pellets were boiled in preparation buffer and analyzed by 7-20% gradient gel electrophoresis according to Laemmli (12). After staining with Coomassie brilliant blue R-250, the gels were prepared for fluorography according to Chamberlain (13) and dried. Fuji X-ray RX films were exposed to the gels for 1-3 weeks at -70°C before development. All experiments were performed at least twice. The experiment shown in Fig. 1, lanes 1-3, was performed 15 times.

Treatment with snake venom phosphodiesterase. TCA-precipitated protein pellets from toxin-treated cells were dissolved in 0.11 M Tris-HCl, pH 8.9, containing 0.11 M NaCl and 15 mM $MgCl_2$, and phosphodiesterase (25 U) was added. After incubation for 2 h at 37°C protein was again precipitated.

RESULTS AND DISCUSSION

When cell lysates are incubated with NAD, labeled anywhere in the ADP-ribose part, and a toxin possessing ADP-ribosylating activity, e.g. diphtheria toxin, a label is transferred from NAD to a cellular substrate (11) (Fig. 1, lane 4). Since C.difficile toxin B is assumed to need proteolytic activation in the lysosomes in order to intoxicate cells (6) and the exact type of activation is not yet unravelled, we considered intact cells more suitable for detecting a potential ADP-ribosylating activity of this toxin. NAD is taken up very slowly by intact cells (14), but precursors such as adenosine and nicotinamide are easily taken up and incorporated into NAD intracellularly (15,16). Since diphtheria toxin only accepts intact NAD as a donor of ADP-ribose (17), it was used as a positive control to ensure that the precursors were in fact incorporated into intracellular NAD (as shown for [^{14}C]adenosine in Fig. 1, lane 3).

When intact cells, preincubated with [^{14}C]adenosine (Fig. 1, lane 1) to label intracellular NAD, were exposed to toxin B until a CPE was developed

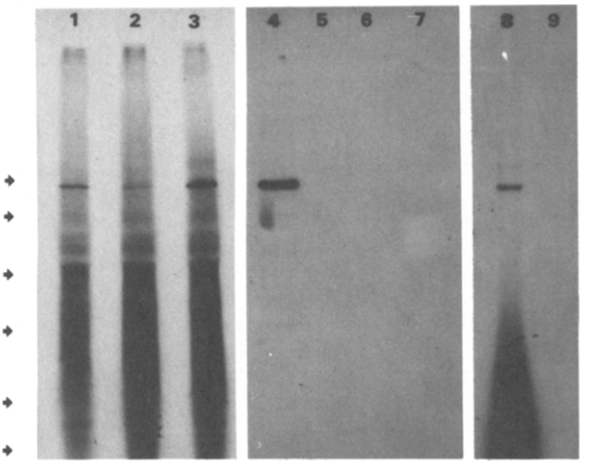

Fig. 1.

Autoradiograms of TCA-precipitated protein from cells preincubated with [^{14}C] adenosine and treated with toxin for 120 min (lanes 1,2,3,8,9) or cell lysates incubated with [^{14}C-adenine] NAD and toxin for 15 min (lanes 4,5,6,7). The pellet in lane 9 was treated with phosphodiesterase. Toxins: unreduced toxin B (lanes 1,6,8,9), reduced toxin B (lane 7), unreduced diphtheria toxin (lane 3), reduced diphtheria toxin (lane 4), no toxin (lanes 2,5). A direct comparison of the labeling intensity induced by the two toxins is not relevant, since large amounts of diphtheria toxin were used in order to get a satisfactory positive control. Molecular weight markers (from top to bottom): phosphorylase b (94.000), albumin (67.000), ovalbumin (43.000), carbonic anhydrase (30.000), trypsin inhibitor (20.100) and α-lactalbumin (14.400).

in all cells, a radioactive label was transferred to a protein (MW approx. 90.000). A labeled protein was also observed after toxin-treatment of cells preincubated with [^{14}C-adenine]-labeled NAD (data not shown), probably due to extracellular hydrolysis of NAD to adenosine (14). Preincubation of cells with [^{14}C] nicotinamide before toxin-treatment did not result in any labeled protein, although [^{14}C] nicotinamide was taken up into the cytosol (data not shown).

Exposure of precipitated protein from toxin B-treated cells to snake venom phosphodiesterase (18) resulted in loss of the label (Fig. 1, lane 9). Since this enzyme splits the pyrophosphate-bond in covalently ADP-ribosylated proteins (19), this finding implies that at least adenosine and the two phosphate groups were transferred from NAD to a cellular substrate. In analogy with other bacterial toxins (1), we propose that the moiety transferred is ADP-ribose. A determination of whether the ribose

residue connected to nicotinamide was transferred would require a precursor specifically labeling NAD in this part, or cellular uptake of intact NAD prelabeled in this part.

The protein labeled in toxin B-treated cells (Fig. 1, lane 1) comigrated with the protein labeled in cells treated with diphtheria toxin (Fig. 1, lane 3). Also in control cells not treated with toxin, a weak label was observed in a comigrating protein (Fig. 1, lane 2). This label invariably was significantly less intensive, and probably represented some endogenous ADP-ribosylation reaction (1,20,21).

No label was detectable upon incubation of cell lysates with either unreduced or reduced toxin B and [^{14}C-adenine] NAD (Fig. 1, lanes 6 and 7) or [^{32}P] NAD (data not shown). This result was as expected, since toxin B needs to be activated by lysosomal proteases (6).

In toxin B-treated cells not yet showing any CPE, the label was of the same intensity (Fig. 2, lane 2) as in cells not treated with toxin (Fig. 2, lane

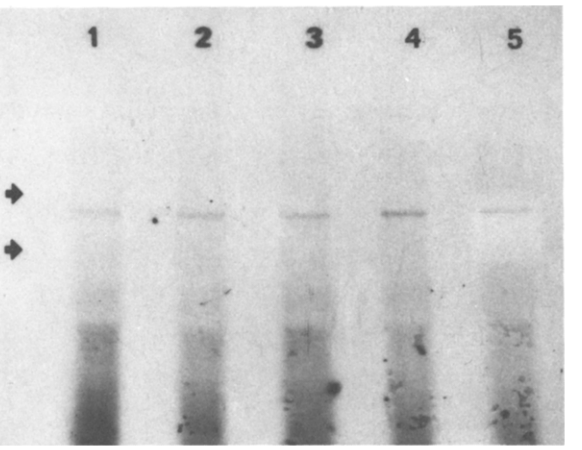

Fig. 2.

Autoradiograms of TCA-precipitated protein from cells preincubated with [^{14}C] adenosine and treated with toxin B for different time periods: 30 min; the cells did not show any CPE (lane 2), 60 min; the cells were on the verge to develop a CPE (i.e. at the end of the latency period) (lane 3), 90 min; 50% of the cells showed a CPE (lane 4), 120 min; 100% of the cells showed a CPE (lane 5). Cells not treated with toxin in lane 1. Molecular weight markers: phosphorylase b (94.000), and albumin (67.000).

1). An increase in the labeling intensity occurred in cells on the verge to develop a CPE, i.e. at the very end of the latency period (Fig. 2, lane 3). The labeling was maximal in cultures showing a CPE in 50% of the cells (Fig. 2, lane 4). These results show a correlation in time between ADP-ribosylation and appearance of the CPE and it is reasonable to assume that these two events are related. The ADP-ribosylation reaction might constitute the molecular basis for the CPE.

However, there is no evidence that toxin B is in itself an enzyme. It might stimulate an endogenous ADP-ribosyltransferase. Moreover, since it has not yet been possible to purify toxin B to homogeneity, it cannot be excluded that the ADP-ribosylation was caused by another factor from C.difficile, contaminating our preparations of toxin B. These points will be clarified when highly purified and activated toxin B can be used in an in vitro assay for ADP-ribosylating activity. In further studies we will focus on identifying the mechanism of proteolytic activation of toxin B as well as the cellular substrate for ADP-ribosylation in toxin B-treated cells.

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