RICIN DOES NOT ACT AS AN ENDONUCLEASE ON L CELL POLYSOMAL RNA

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Received November 3,1975

SUMMARY: The ability of ricin to act as an endonuclease was examined using labelled polysomes isolated from mouse L cells grown in [^{32}P] orthophosphate. No change was detected in the electrophoretic mobility of any ribosomal RNA species whether L cells were first treated with ricin or the isolated polysomes were treated with ricin. No trichloroacetic acid soluble counts over control levels were released by ricin treatment of isolated polysomes and no changes were observed in sucrose density gradient polysome profile with ricin treatment. The action of ricin on polysomes which results in inhibition of translation does not appear to involve endonuclease activity.

Ricin, isolated from Ricinus communis, is a highly toxic glycoprotein of approximately 60,000 molecular weight which inhibits protein synthesis of eucaryotic, but not procaryotic, cells (1). Ricin and another plant lectin, abrin, are composed of two polypeptide chains held together by a disulfide bond. The B chain shows specificity for carbohydrate moieties and is responsible for the reaction of the lectin with cell surface receptors in the membrane. The A chain is capable of inhibiting protein synthesis in vitro, but requires the B chain before it can express its toxic effect in vivo (2). It is not known if homology exists at the amino acid sequence level between abrin and ricin; however, hybrids will form (3). In spite of the apparent similarity of diptheria toxin and ricin in structure, no functional homology exists in the mechanism of protein synthesis inhibition and hybrids will not form (3). Ricin (4) and diptheria toxin (5) have been used to prevent the proliferation of tumor cells when injected into test animals, although a recent report disputes this claim for diptheria toxin (6).

Evidence has been presented showing that the site of action of ricin may be on the 60S ribosomal subunit and on elongation factor 2 (7). Other evidence suggests an additional effect on elongation factor 1 dependent binding of amino acyl tRNA to ribosomes (8). Because of the small quantity of ricin required for the observation of toxic effects, an enzymatic activity has generally been attributed to ricin. It has also been suggested that ricin may be a nuclease which can cleave 28s ribosomal RNA in a manner similar to colicin E3 (9). We have investigated this latter possibility using polysomes from L cells which were grown in [^{32}P] orthophosphate.

METHODS

Cell Culture System: NCTC clone 929 mouse fibroblast cells (L-cells) were grown as previously described in 250 ml milk dilution bottles in a modified Waymouth's MD705/1 media (10). These served as inoculum for "roller bottles"; a 1.9 liter bottle (11 cm diameter at its base and 19 cm tall) with a surface area of approximately 1800 cm^2. Each milk dilution bottle was used to inoculate 3 roller bottles with 13 mls of inoculum. Fresh media (100 ml) was then added and the bottles placed on a roller apparatus at 1/3 RPM. After 3 to 4 days, the cells were drained and refed with 150 ml of fresh media. After an additional 3 days the cells were exposed to [^{32}P] phosphate in order to label RNA in the polysomes.

Preparation of ribosomal RNA containing ^{32}P: L-cells were grown as described and transferred to roller bottles for 6 days. The media was drained and replaced with 200 ml of fresh media which contained no phosphate. After 2 hours of phosphate starvation, this media was replaced with 50 ml of media to which had been added 1.4 ml of 2.05 mM [^{32}P] orthophosphate containing approximately 10 mCi ^{32}P. After 5 hours, the cells were harvested and the polysomes were isolated.

Assay for release of soluble counts from labelled ribosomes: Seven A_{260} units of labelled polysomes were added at 37°C either to ricin A chain at a final concentration up to 34 µg/ml or to an equal volume of PBS which served as a control. Fifty microliter samples were removed at various times up to 120 minutes and placed in 50 µl of 10% TCA. An additional 1.5 ml of 5% TCA was immediately added and the samples were filtered on a 25 mm Millipore filter. The filters were then washed twice with 5 mls of 5% TCA. The initial filtrate and the washes were counted separately.

Examination of total RNA on polyacrylamide-SDS gels: Three to four A_{260} units of polysomes were incubated at 37°C for 15 minutes, in the presence and absence of ricin (final concentration of 34 µg/ml) in a total volume of 0.8 ml. Total RNA was then isolated from the polysomes by cold phenol extraction. A solution containing 0.1 M NaCl, 0.5% SDS and 50 mM Tris chloride, pH 7.6, was added to bring the polysome sample up to 10 ml. The aqueous phase was extracted three times with an equal volume of fresh 88% (v/v) phenol. The RNA was precipitated from the aqueous layer with 2.5 volumes of cold absolute ethanol. After allowing 4 or more hours for precipitation, the precipitate was collected by centrifugation and resuspended in buffer containing 0.01 M

sodium acetate, 0.1 M NaCl and 1 mM EDTA adjusted to pH 5.1 with acetic acid. For gel electrophoresis, a 25 µg sample was made 20% in glycerol and layered on a cylindrical gel 10 cm long made with 2.6% acrylamide and 0.13% bisacrylamide. The gel had been pre-soaked in the reservoir buffer and pre-electrophoresed for 1 hour. The reservoir buffer, which was also used to make the gel, consisted of 0.04 M Tris acetate, pH 7.8, 0.02 M sodium acetate, 0.002 M sodium EDTA and 0.1% SDS (11). The gels were polymerized with 0.086% ammonium persulfate and 0.086% N,N,N',N',-tetramethylenediamine. The gels were run at room temperature for 3 to 4 hours at 30 V until the tracking dye was about 2 cm from the bottom. The gels were immediately scanned at 260 nm, then stored at -20°C overnight, cut into 2 mm slices and counted in Brays scintillation solution. Other samples were analyzed using slab gel electrophoresis and the radioactivity detected by autoradiography. The buffers and gel concentration used were those employed by Meyhack et al (12) to observe the cleavage of 16s RNA by E. Coli colicin E3.

Isolation of polysomes from rat liver and L cells: Isolation of rat liver polysomes was done according to the magnesium precipitation method of Palmiter (13) except that 4 mls of Buffer A per gram of liver was used instead of 9 mls and the final pellet was suspended in a buffer consisting of 20 mM Tris chloride (pH 7.5), 0.1 M NH_4Cl, 5 mM magnesium acetate and 1 mM dithiothreitol according to Staehelin and Falvey (14) at a concentration of 130-170 A_{260}/ml. Isolation of polysomes from L cells followed Palmiter.

Assay for in vitro toxicity: Preparation of high speed supernatant (S-100) of rat liver was done according to Staehalin and Falvey (14). The in vitro polypeptide synthesis reaction mixture contained the following in a total volume of 0.4 ml; 7.5 A_{260} units of rat liver polysomes, 30 µl S-100, 60 µg yeast tRNA, 1 mM ATP, 0.2 mM GTP, 10 mM PEP, 80 units pyruvate kinase, 200 µg polyuridylic acid, 50 mM potassium acetate, 1 mM $CaCl_2$, 15 mM magnesium acetate, 5 mM B-mercaptoethanol, and 25 mM Tris acetate adjusted to pH 7.5 with acetic acid. Ricin was added in 5 mM sodium phosphate containing 0.2 M NaCl, pH 7.2 (PBS); equal amounts of PBS were added to control and experimental tubes. At zero time, 7.5 µCi [^{3}H] phenylalanine were added. At different times 50 µl samples were quenched with 50 µl of 10% TCA followed by additional 1.5 ml of 5% TCA and incubated for 10 minutes in a water bath at 100°C. The hot-acid-insoluble material was collected on a Whatman Type A glass fiber filter, dried and counted by liquid scintillation. Ricin was isolated using affinity chromatography employing lactose attached to Sepharose 4B.

RESULTS AND DISCUSSION

The density gradient centrifugation patterns of polysomes isolated from ricin treated L cells in the presence and absence of lactose, polysomes treated directly with ricin, and control polysomes were nearly identical and no significant differences could be demonstrated (Fig. 1).

Cells grown for 5 hours in [^{32}P] orthophosphate served as a source of labelled polysomes. The polysomes isolated from these cells contained between 4.5 and 7.9 x 10^4 counts per minute per A_{260} unit for four different preparations. Incubation of the [^{32}P] labelled polysomes with large concen-

trations of purified A chain which result in the almost immediate termination of protein synthesis by the *in vitro* assay, failed to release TCA-soluble counts from the labelled RNA. Time points up to 120 minutes showed no difference within experimental error. Only about 420 cpm were found in the TCA-supernatant after 60 minutes of A chain treatment when [^{32}P] polysomes containing 1.54 x 10^5 cpm were incubated with 34 µg/ml of ricin chain. Controls without A chain contained 500 cpm in the TCA supernatant. The release of nucleotides by exonucleolytic action would amount to only about 50 cpm/nucleotide and would be difficult to detect.

The possibility that ribosomal RNA species were cleaved into larger TCA-insoluble fragments was investigated using gel electrophoresis in sodium dodecyl sulfate. The profiles of scanning cylindrical gels at 260 nm (Fig. 2) showed no differences in the position of 28s and 18s RNA from poly-

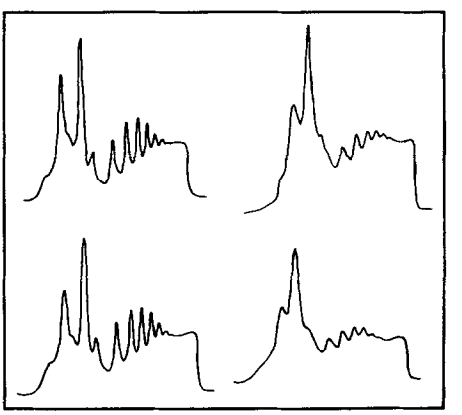

Fig. 1. Profiles of ricin treated polysomes. Ricin was added with or without 0.5 M lactose three hours prior to [^{32}P] orthophosphate labelling. Polysomes were then purified according to Palmiter (13). Alternatively, purified polysomes were inoculated in the presence or absence of ricin for 30 minutes at 37°C. Three A_{260} units of polysomes in a volume of 0.2 ml were layered on a 4.8 ml 10-45% sucrose gradient containing 50 mM Tris chloride, 50 mM KCl and 5 mM $MgCl_2$ and centrifuged at 4°C for 30 minutes at 50,000 RPM in a Beckman SW 50.1 rotor. Top left: control polysomes incubated at 37°C for 30 minutes. Bottom left: polysomes treated with 34 µg/ml ricin. Top right: polysomes from ricin (10 µg/ml) treated L cells. Bottom right: polysomes from ricin treated L cells protected with lactose.

somes treated with ricin, from L cells treated with ricin and from isolated RNA treated with ricin (not shown). Only single peaks were observed for 28s and 18s RNA when co-electrophoresis was carried out on RNA isolated from [^{32}P] polysomes from ricin treated and untreated [^{32}P] labelled L cells. The system used here has been shown to be sensitive to the change brought about by the E. coli colicin E3 catalyzed cleavage of E. coli ribosomal RNA in which approximately 50 nucleotides are removed from the 5' end of 16s RNA (15).

A second gel system has been used which employs a higher acrylamide concentration in an effort to observe any small fragment released from [^{32}P] phosphate labelled RNA. Ricin was incubated at concentrations from 6.8 to 272 µg/ml with [^{32}P] labelled polysomes at 37°C for as long as 90 minutes after which the samples were denatured in sodium dodecyl sulfate and 2 mM EDTA and run on slab gel electrophoresis. Ricin at 10 µg/ml almost immediately inhibits polyuridylic acid directed translation. The position of the RNA species within the gel was determined by autoradiography. Short exposures of the X-ray film were used to examine the 28s and 18s RNA since most of the radioactivity was found there. These species were well resolved. Longer exposures were used, which completely obliterated any detail around the 28s

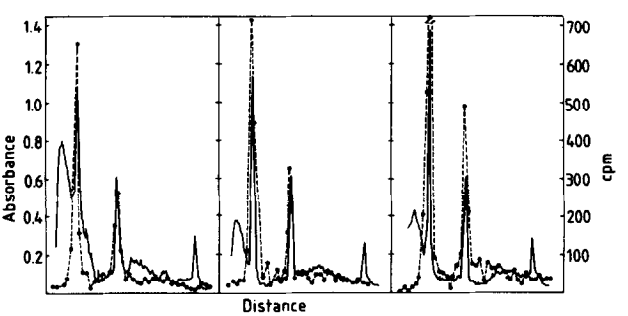

Fig. 2. Profiles of ribosomal RNA on sodium dodecyl sulfate gel electrophoresis. Total RNA was extracted from ricin-treated polysomes, 25 µg samples were placed on each gel, and scanned at 260 nm (solid line) immediately after electrophoresis. Gels were then frozen, cut into 2 mm slices and counted (dotted line). Left: RNA from untreated polysomes. Middle: RNA from ricin-treated polysomes. Right: Co-electrophoresis of RNA (15 µg) from untreated polysomes and RNA (10 µg) from ricin-treated polysomes.

and 18s region, in an effort to find a small RNA species migrating near the 5s RNA species. In four separate experiments, no RNA species was detected other than the 28s, 18s and 4-5s RNA species that were present in the controls. No small piece of labelled RNA was found moving in front of the 5s region.

The toxicity of ricin does not depend on the addition of cofactors to cell free protein synthesis systems. This has led to suggestions of protease or nuclease functions for ricin as well as other activities affecting ribosomal activity (7,8,9). Whatever the nature of ricin action is on eucaryotic polysomes, it does not involve the cleavage of substantial parts of 28s or 18s ribosomal RNA, nor does it cleave mRNA so that polysomal structure is destroyed. We have not observed any change in the electrophoretic mobility of 5s RNA species. Endonucleolytic cleavage within the interior parts of 28s and 18s would have been easily observed; however, it still remains a possibility that a small number of nucleotides could be removed from the 3' or 5' end of ribosomal RNA exonuclease action. Any exonucleolytic activity would probably be of a very selective and limited nature.

ACKNOWLEDGEMENT

The help of Dr. Paul A. Kitos in providing tissue culture facilities is appreciated. This work was supported by grants from the National Cancer Institute CA00114 and CA16206.

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