Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 1082-1088

RESISTANCE OF TETRAHYMENA TO RICIN, A TOXIC ENZYME FROM RICINUS communis

CRAIG G. WILDE, SOPHIE BOGUSLAWSKI AND L. L. HOUSTON

Department of Biochemistry
University of Kansas
Lawrence, Kansas 66045

Received November 5, 1979

The protozoan Tetrahymena pyriformis was found to be resistant to the toxic action of ricin in vivo. Isolated Tetrahymena ribosomes were strongly resistant to the A subunit of ricin when tested in a cell free protein synthesis system under different conditions and also lacked the ability to bind A chain stoichometrically. This suggests that Tetrahymena is resistant in vivo because it contains a ribosome which is not susceptible to the toxic action of ricin.

Recent work has established the fact that ricin inhibits translational activity by modifying the 60S ribosomal subunit (see reference 1). The molecular lesion introduced by ricin is not known and is probably similar to that introduced by abrin, a toxic protein from the seeds of Abrus precatorius, the rosary pea (1). Ricin contains two disulfide linked polypeptide chains; one (A chain) efficiently inhibits mammalian ribosomes and the other (B chain) binds to galactosyl residues on cell surface receptors. The A chain alone (M_r = 30,000) is able to inhibit cell free protein synthesis, but the B chain (M_r = 34,000) is required to deliver the A chain through the plasma membrane in order to inhibit intracellular translation (1).

Although ricin is an enzyme which requires no cofactors, ricin and the A chain bind stoichiometrically to rat liver ribosomes (2). The site of A chain binding is on the 60S subunit, consistent with the site of the enzymatic target. The binary EF-2 • GDP(NH)P complex and tRNA block both the enzymatic action of A chain and prevent its binding to 60S rat liver subunits (3). These facts suggest that the enzymatic target and the site of A chain binding are the same. It has been difficult to identify any covalent lesion introduced into the ribosome, such as by proteolytic (4), ribonuclease (5) or phosphatase (6) activity, which could account for the enzymatic action of ricin.

E. coli ribosomes are resistant to ricin and they do not bind A chain (2). Efforts to produce mutants of mammalian cells with ribosomes resistant to ricin have not yet been

0006-291X/79/231082-07$01.00/0

successful. Because comparative studies can often be productive, we set out to find a eucaryotic ribosome which was completely insensitive to ricin.

METHODS

Ricin$_1$ and ricin$_1$ A chain were isolated as previously described (7). The A chain was labeled with 1 mole of [^{3}H] N-ethylmaleimide (NEM) per mole of protein (8). Tetrahymena pyriformis was grown in 1% proteose peptone-0.1% yeast extract at room temperature with gentle shaking and harvested by centrifugation at 500 x g. Cells were suspended in 20 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.6, 100 mM KCl, 3.5 mM magnesium acetate, 6 mM mercaptoethanol, 0.25 M sucrose and 1 mg/ml heparin and lysed by sonication. The crude sonicate was centrifuged 10 minutes at 9000 x g at 4°C and the supernatent centrifuged at 20,000 x g for 20 minutes. The supernatant was passed through a 1.7 x 17 cm column of Sepharose CL6B equilibrated with 20 mM HEPES, pH 7.6, 100 mM KCl, 4 mM magnesium acetate and 6 mM mercaptoethanol. The excluded volume was used as a source of ribosomes in subsequent experiments.

Growth curves were obtained using the chemically defined media of Kidder and Dewey (9). Cells were fixed in 2% formalin and counted in a hemacytometer or their turbidity measured at 540 nm.

Cell free protein synthesis was carried out in 50 µl volumes as described (10). Soluble factors were from rat liver S-100. Binding of [^{3}H] NEM-labeled A chain was measured as before (8).

RESULTS AND DISCUSSION

Ricin does not exert its toxic effect on Tetrahymena raised in defined medium (Figure 1). The doubling time of 11.5 hours in the absence of ricin was actually reduced

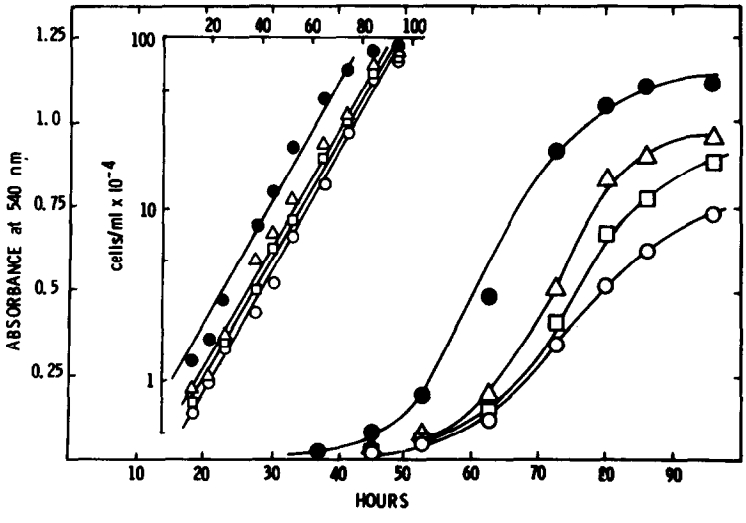
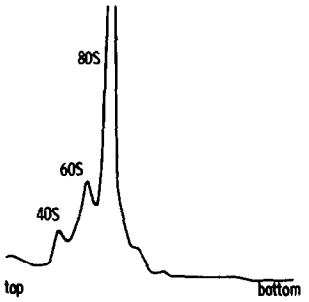
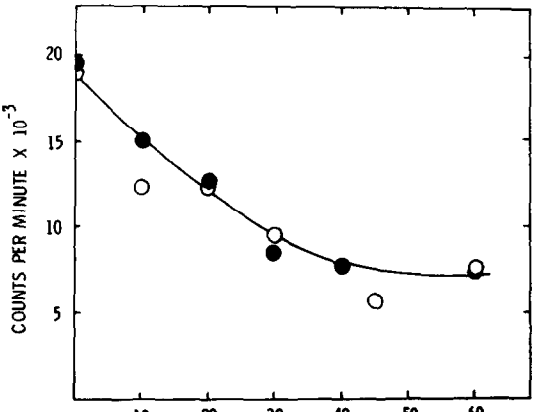


Fig. 1: Effect of ricin concentration on the growth of Tetrahymena. Tetrahymena were grown in a shallow layer (10% of flask volume) of chemically defined medium. Samples were withdrawn at indicated times, cells were fixed in 2% formalin and counted in a hemacytometer. Absorbance at 540 nm was read in a Spectronic 20 at indicated times. (○) control; (□) 1 µg/ml ricin; (△) 10 µg/ml ricin; (●) 100 µg/ml ricin.



(2)



(3)

Fig. 2: Sucrose density gradient profile of Tetrahymena ribosomes. Ribosomal material (2.5 A_{260} units) was applied to 10-45% sucrose gradients and centrifuged for 50 minutes at 50,000 rpm in a Beckman SW 50.1 rotor. Absorbance was monitored at 260 nm.

Fig. 3: Effect of ricin A chain on cell free protein synthesis with Tetrahymena ribosomes. Ribosomes were preincubated for increasing lengths of time in buffer containing 31.6 µg/ml ricin A chain (●). A separate control was determined for each time (○). After preincubation, factors required for protein synthesis were added and tubes were incubated 50 minutes at 37°C. Protein synthesis was assayed as cpm [^{3}H] phenylalanine incorporated into hot trichloroacetic acid precipitable material. All points are the average of duplicates.

nearly 20% to 9 hours in the presence of 100 µg/ml of ricin, a concentration which would completely inhibit the growth of cultured HeLa cells (11). The more rapid growth in the presence of high ricin concentrations suggests that Tetrahymena is able to utilize ricin as a carbon and nitrogen source. These data imply that ricin is taken up by the protozoa; however, the resistance may lie in either degradation of ricin before it reaches the ribosome, or the target ribosomes may not be susceptible to the toxin, or both.

In order to investigate the sensitivity of Tetrahymena ribosomes to ricin, a cell free protein synthesis system was used to provide free access of ricin A chain to the ribosomes. Sucrose gradient profiles showed that the preparations contained mainly 80S monosomes and free ribosomal subunits (Figure 2). A small shoulder of dimers was observed, but no larger forms. We found that ribosomes isolated without quickly separating low molecular weight components by gel filtration, were always inactive. This may be due to the high activity of proteases and nucleases in Tetrahymena preparations (12), which might degrade polysomes. The presence of these degradative enzymes may also explain why soluble factors from rat liver were found to be much more effective in stimulating cell free protein synthesis than factors from Tetrahymena.

When Tetrahymena ribosomes were assayed in the presence of up to 200 µg/ml of ricin A chain, no decrease in the translation of polyuridylic acid was observed. Concentrations of 0.1 ng/ml of A chain are effective against rat liver ribosomes (7). Since it is known that ricin inhibition is time dependent, Tetrahymena ribosomes were incubated with A chain at 37°C and assayed for their protein synthesis ability. Unfortunately, Tetrahymena ribosomes were not stable at 37°C and controls gradually lost their ability to polymerize polyphenylalanine. However, the inclusion of A chain in the incubation produced no further decline in protein synthesis ability (Figure 3). Inclusion of a wide range of ricin concentrations with Tetrahymena ribosomes in a 10 minute preincubation did not inhibit protein synthesis (Table 1). The highest concentration used is more than 20,000 times that needed to inhibit 50% of rat liver ribosomal protein synthesis. In view of these data and the extreme resistance of ricin to proteases (1), it is unlikely that the lack of sensitivity of ribosomes is due to rapid proteolysis of ricin.

High concentrations of Mg^{2+} have been shown to have significant protective and restorative effects on rat liver and wheat germ ribosomes which have been treated with A chain (8). In this work Tetrahymena ribosomes showed a high Mg^{2+} optimum (18 mM) for polyuridylic acid translation. This raises the possibility that the resistance of Tetrahymena ribosomes may be due, at least partially, to high Mg^{2+} required for protein synthesis. To examine this possibility, ribosomes were incubated with ricin A chain and assayed for protein synthetic activity in the presence of 5-25 mM Mg^{2+} (Figure 4). No

Table 1. Effect of Increasing Concentrations of Ricin A Chain on Protein Synthesis. Tetrahymena ribosomes were preincubated at 37°C with ricin A chain. After 10 minutes, factors required for protein synthesis were added and incubated for 50 minutes at 37°C. Values are the average of triplicate assays. The Mg^{2+} was 18 mM in the assay.

concentration of ricin A chain µg/ml	cpm incorporated into (^{3}H) polyphenylalanine
0.0	30,049
0.0002	29,046
0.002	31,082
0.02	30,749
0.2	28,763
2.0	33,813
20.0000	31,539
200.	30,950
2000.	32,296

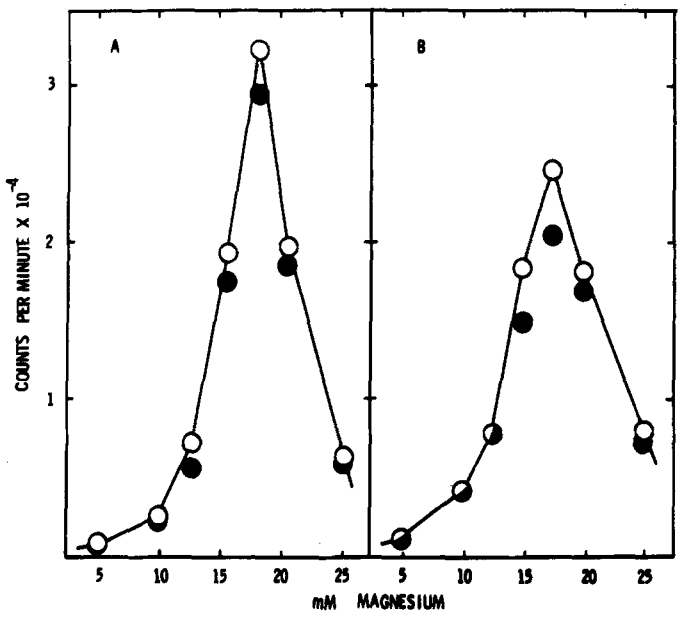


Fig. 4: Effect of Mg^{2+} on ricin ricin chain inhibition. Tetrahymena ribosomes were preincubated 10 minutes at 37°C in the presence of 20 µg/ml (A) or 400 µg/ml (B) ricin A chain. Both preincubation and the subsequent polyphenylalanine synthesis assay were carried out at the indicated Mg^{2+} concentrations with (●) and without (○) A chain.

effect of Mg^{2+} on inhibition of polyphenylalanine synthesis was seen in the presence of either 20 µg/ml or 400 µg/ml A chain. The slightly lower level of synthesis seen at all Mg^{2+} concentrations using 400 µg/ml A chain was not considered significant in light of the massive dose of A chain used. In other experiments, the difference was even less than shown in Fig. 4B. The low protein synthesis activity at Mg^{2+} concentrations below about 10 mM makes it difficult to draw any conclusions, but wheat germ ribosomes which are totally resistant to 2 µg/ml A chain at 17 mM Mg^{2+} are 70% inhibited at 10 mM Mg^{2+} (8). It is possible to examine the protective effects of Mg^{2+} below 10 mM by preincubating ribosomes in low Mg^{2+} buffer and assaying incorporation into polyphenylalanine at optimal Mg^{2+}. Experiments of this kind also failed to show any effect of 6-18 mM Mg^{2+} on A chain inactivation of Tetrahymena ribosomes. Therefore it is unlikely that Mg^{2+} plays a significant role in the resistance of Tetrahymena ribosomes to ricin. In view of these data we conclude that Tetrahymena are resistant to the effects of ricin because their ribosomes are resistant.

Previous studies have supported the proposal that the ability of a ribosome to bind A chain correlates with its susceptibility to enzymatic inactivation. The data in Table II

Table 2. Binding of [^{3}H] NEM Labeled Ricin A Chain to Ribosomes. Ribosomes were incubated 10 minutes at 37°C with [^{3}H] NEM labeled A chain, collected by centrifugation and the sedimented radioactivity counted following a previously described procedure (6). The extent of ribosome recovery was determined from the A_{260} remaining in the supernatant.

ribosomes (pmol)	Mg^{2+} (mM)	%ribosomes pelleted	cpm
0	7.5	-	738
18	5.5	64	1424
18	7.5	63	1177
18	10.5	66	1569
18	15.5	62	1077
18	18.5	68	1039

show that Tetrahymena ribosomes fail to bind labelled A chain at all Mg^{2+} concentrations used. Using this particular labelled A chain preparation, 15 pmoles of Tetrahymena ribosomes bound only 300-700 cpm over background compared to 10,000 cpm over background for an equivalent amount of rat liver 60S ribosomal subunits.

Data are now available to show that three classes of eucaryotic ribosomes exist which can be differentiated by their susceptibility to ricin A chain. Some ribosomes, such as rat liver ribosomes, are extremely sensitive to inhibition while other eucaryotic ribosomes, such as wheat germ ribosomes, are relatively resistant — they can be inhibited with sufficiently high A chain concentrations. A third class, represented by Tetrahymena ribosomes, appears to be completely resistant as judged by both in vitro protein synthesis and by A chain binding studies. Procaryotic ribosomes also belong in the third class. It is clear that ricin resistance does not partition along strictly eucaryotic/procaryotic lines. If the reason for the resistance of Tetrahymena ribosomes to ricin were known, we might gain more insight into the mechanism by which ricin inactivates translation.

ACKNOWLEDGEMENT

This investigation was supported by Grant Number CA 16206 awarded by the National Cancer Institute, DHEW. LLH is a recipient of a Research Career Development Award from the National Cancer Institute.

REFERENCES

1. Olsnes, S. and Pihl, A. (1976) Receptors and Recognition Series B: The Specificity and Action of Animal, Bacterial and Plant Toxins (Cuatrecasas, P., Ed.) pp. 131-173, Chapman and Hall, London.

2. Hedblom, M.L., Cawley, D.B., and Houston, L.L. (1976) Arch. Biochem. Biophys. 177, 46-55.
3. Cawley, D.B., Hedblom, M.L., and Houston, L.L. (1979) Biochemistry, 12, 2648-2654.
4. Lugnier, A.A.J., Dirheimer, G., Madjar, J.J., Reboud, J.P., Howard, G.A. (1976) FEBS Lett. 6, 343-347.
5. Mitchell, S.J., Hedblom, M., Cawley, D., and Houston, L.L. (1976) Biochem. Biophys., Res. Commun. 68, 763-769.
6. Houston, L.L. (1978) Biochem. Biophys. Res. Commun. 85, 131-139.
7. Cawley, D.B., Hedblom, M.A., and Houston, L.L. (1978) Arch. Biochem. Biophys. 190, 744-755.
8. Hedblom, M.L., Cawley, D.B., Boguslawski, S. and Houston, L.L. (1979) J. Supramol. Struct. 9, 253-68.
9. Kidder, G.W., and Dewey, V.C. (1957) Arch. Biochem. Biophys. 66, 486-492.
10. Cawley, D.B., Hedblom, M.L., Hoffman, E.J., and Houston, L.L. (1977) Arch Biochem. Biophys. 182, 690-695.
11. Olsnes, S., Sandvig, K., Refnes, K., and Pihl, A. (1976) J. Biol. Chem. 257, 3985-3992.
12. Muller, M., Baudhuin, P., and DeDuve, C. (1966) J. Cell Physiol. 68, 165-176.