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FORMATION OF A HYBRID TOXIN FROM RICIN AGGLUTININ AND A NON-TOXIC MUTANT PROTEIN OF DIPHTHERIA TOXIN

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SUMMARY

CRM45, a non-toxic mutant protein of diphtheria toxin, is treated with glutaraldehyde and conjugated to ricin agglutinin. The hybrid protein thus obtained is purified by gel filtration and affinity chromatography. The toxicity of the purified hybrid toxin is about 8-10 times grater than that of ricin agglutinin when tested in mice and cultured L cells.

INTRODUCTION

Diphtheria toxin, with a molecular weight of 62,000 daltons, is a single polypeptide chain containing two disulfide bridges. Following mild treatment with trypsin in the presence of certain thiols (1,2), it splits into two fragments, A and B, of about 22,000 and 40,000 daltons respectively. Fragment B binds to susceptible mammalian cell surfaces and introduces fragment A into the cell cytoplasm (3). Fragment A is a potent NAD:elongation factor 2-ADP ribosyl(NAD:EF2-ADPR) transferase (4,5). Mutant protein of diphtheria toxin, CRM30 or CRM45 has the same enzymatic activity per molecule as fragment A or diphtheria toxin (8,9). Thus, it stops protein synthesis in eukaryotic cell extracts. However, it is not toxic to susceptible cultured cells and animals because it cannot bind to the cell surface. When CRM45 proteins are enclosed with HVJ(Sendai virus) envelopes, the CRM45 can be introduced into mammalian cell cytoplasm and then kill the cell (10).

It has been reported that a kind of lectin is incorporated into mammalian cells (11). At the cell surface, ricin agglutinins bind to

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saccharides which contain terminal non-reducing galactose (12). They are then incorporated into cells presumably by endocytotic activity induced by the lectin. The toxicity of the agglutinin when tested in mice is found to be about 1/2000 of that of ricin toxin (13).

In order to study the entry mechanism into the cell of diphtheria toxin, these enzymically active, but non-toxic, CRM45 molecules are treated with glutaraldehyde and are then conjugated to ricin lectin. This hybrid toxin (CRM45 plus ricin lectin) is incorporated into the cell by the action of the cell in response to the lectin moiety. The hybrid is more toxic to L cells, which are resistant to diphtheria toxin, than ricin agglutinin or diphtheria toxin.

MATERIALS AND METHODS

Purification of ricin agglutinin and CRM45: From decorticated ricinus seeds ricin toxin and agglutinin were extracted and fractionated with ammonium sulfate. The crude fraction thus obtained was applied to a Sepharose 4B column, to which the ricin toxin and and agglutinin bound. They were then eluted using galactose. The ricin toxin and agglutinin, thus isolated(12), were separated by gel filtration using Bio-Gel P200. The agglutinin was again purified by the Bio-Gel.

CRM45 proteins were produced by C7(β45) and subsequently purified by DE52 (7).

The purity of the ricin agglutinin (120,000 daltons) and the CRM45 (45,000 daltons) was demonstrated by electrophoresis. They were run on SDS polyacrylamide gels where a single sharp band was observed for each.

Conjugation procedures: Two mls of CRM45 (1.8 mg/ml) were treated with glutaraldehyde at a concentration of 0.08% for 5 hours at room temperature. The treated CRM45 was dialysed overnight against 2 liters of 0.15 M NaCl to remove the glutaraldehyde. Three tenths of an ml (0.3 ml) of ricin agglutinin (3.2 mg/ml) was added to the dialysed CRM45 solution. The mixture was then dialysed against 0.1M carbonate-bicarbonate buffer (pH 9.4) at 4 C overnight. Lysine was added to the dialysed mixture to a final concentration of 0.02M. The mixture was agitated for about 2 hours and then dialysed against two changes of 2 liters phosphate buffered saline (PBS) at 4 C overnight.

Assay for NAD:ADPR transferase activity: The assay was carried out on 5, 10 and 20 µl samples using a slightly modified version of the method described by Gill and Pappenheimer(4).

Haemagglutinating activity: The activity was determined using rabbit erythrocytes and expressed as HA /ml.

Determination of toxicity: Two hundred L cells were suspended in 2 ml of MEM medium containing 10% calf serum. These suspensions were added to 30 mm petri dishes and incubated at 37 C. After 6 hours, various concentrations of sample (toxin, agglutinin or hybrid toxin) were added to the petri dishes containing the L cells. They were then incubated an additional 9 hours. At the end of this time the medium was exchanged for medium containing 0.01M lactose. The next day the medium was exchanged again for fresh medium without lactose. After 7 days the cells were fixed with methanol and stained with Giemsa. The number of colonies

on each petri dish were counted and the results obtained are the average of duplicate dishes for each sample and for the control dishes. The control dishes contained 139 and 142 colonies each an average of 141 colonies.

To prepare samples for the incubation and testing above, each sample was adjusted to a final concentration of 20 μg/ml. This concentration was used for the dilutions.

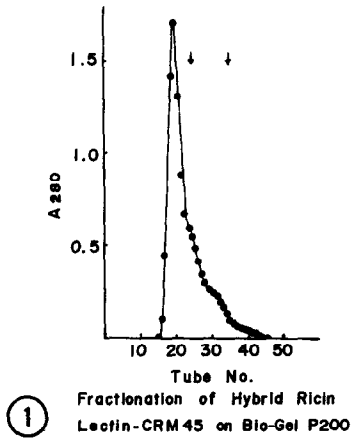
The difinition used in this paper for the minimum lethal dose is: That amount of toxin required to kill a 26-30 g mouse on the fourth day after intraperitoneal injection.

RESULTS AND DISCUSSION

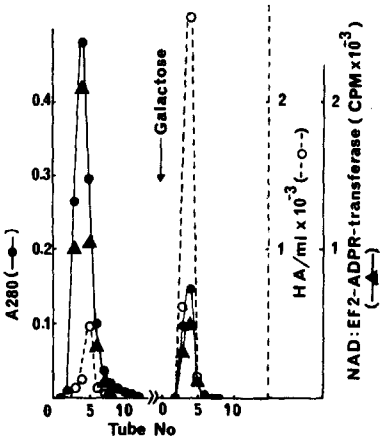
1. Purification of hybrid toxin composed of CRM45 and ricin agglutinin.

Purified CRM45 proteins are treated with glutaraldehyde and then conjugated to purified ricin agglutinin at a molar ratio of about 10: 1, as described in Materials and Methods. These are then applied to a Bio-Gel P200 column to separate free ricin agglutinin and free CRM45 from the newly formed hybrid toxin. In Fig 1, at an absorbancy of 280 nm, a single prominent peak with two shoulders is seen. The peak is found in tubes 17-21, which correspond to the void volume. The two shoulders are found in tubes 23-24 which corresponds to CRM45. NAD:EF2-ADPR transferase activity, haemagglutinatinating activity, and toxicity in mice are associated with the prominent peak.

As has been previously reported, a Sepharose 4B column contains β-galactosyl residues. These can bind the ricin agglutinin moiety of the hybrid toxin. Once bound, the hybrid toxin(tubes 17-20) can be further purified using a Sepharose 4B column. As seen in Fig 2, the first peak, which passed through the column, with an absorbancy at 280 nm has a high enzymic activity of NAD:EF2-ADPR transferase but low haemagglutinating activity. This fraction is composed mainly of aggregate CRM45 which is unconjugated with ricin agglutinin. The toxicity of this fraction, as tested in mice, is much lower than that of the peak fraction from the gel filtration of Fig 1. The next peak is eluted with 0.1M galactose. Both haemagglutinating activity and the enzymic activity are found in this eluted fraction. When this fraction is run on a 7.5% SDS polyacryl-



① Fractionation of Hybrid Ricin Lectin-CRM45 on Bio-Gel P200



② Fractionation of Hybrid Ricin Lectin-CRM45 on Sepharose 4B

Fig 1. Fractionation of Hybrid Toxin of CRM45 and Ricin Agglutinin by Bio-Gel P200.

CRM45 proteins were treated with 0.08% glutaraldehyde and then dialyzed against 0.15M NaCl. The treated CRM45 and ricin agglutinin were mixed and conjugated as described in Materials and Methods. The mixture was apllied to a Bio-Gel P200 equilibrated with PBS. Ten drop fractions were collected and the absorbance of each fraction was measured at 280 nm. Arrows denote the positions corresponding to ricin agglutinin and CRM45, respectively.

Fig 2. Purification of Hybrid toxin of CRM45 and Ricin Agglutinin by Affinity Chromatography of Sepharose 4B.

Fractions 17-20 of Fig 1 were pooled and applied to a Sepharose column equilibrated with PBS. The column was washed with 20 ml of PBS and the hybrid toxin was eluted with 0.1M galactose in PBS. Twenth drop fractions were collected. Aliquots of each fraction were used to determine haemagglutinating activity, NAD:EF2-ADPR transferase activity, and absorbance at 280 nm.

amide gel for 5 hours, a protein band is observed only at the top of the gel. This eluted eluted fraction is mainly hybrid toxin. The enzymatic activity of CRM45 is decreased to about 0.6% of the original activity following glutaraldehyde treatment. Based upon the enzymatic activity, the data from both haemagglutinating and enzymatic activities indicates that the hybrid toxin consists of about 65% CRM45 and 35% agglutinin. Therefore, one ricin agglutinin molecule binds, on an average, five CRM45 molecules which seem to be in an aggregate form, and one mg of the hybrid toxin is equivalent to about 4 μg of diphtheria toxin as the enzymatic activity.

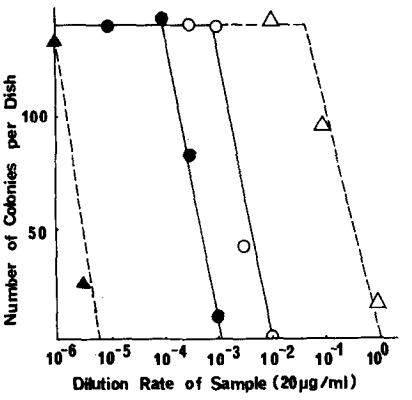


Fig 3. Toxicity of Hybrid Toxin, Ricin Agglutinin, Ricin Toxin and Diphtheria Toxin for L cells.

Effects of the hybrid toxin, ricin agglutinin, ricin toxin and diphtheria toxin on colony formation of L cells were determined as described in Materials and Methods. Each sample was adjusted to a final concentration of 20 μg/ml. This concentration was used for the dilutions. Hybrid Toxin (—●—), Ricin Agglutinin (—○—), Ricin Toxin (--▲--). and Diphtheria Toxin (--△--).

2. Toxicity of the hybrid toxin to L cells.

The toxicity of the hybrid toxin is tested using mouse L cells known to be at least 10,000 times as resistant to diphtheria toxin as HeLa cells. Fig 3 shows that the toxicity of the hybrid toxin is about 8-10 times greater than that of ricin agglutinin. Also, it shows that the toxicity of the hybrid toxin is about 1,000 times greater than that of diphtheria toxin. Ricin agglutinin toxicity, alone, is about 2,000 times less than the ricin toxin.

The toxicity of the hybrid toxin is also tested in mice. About 10 -15 μg of the hybrid toxin is found to kill mice. This is also about 7-10 times more effective than ricin agglutinin. The toxicity of ricin agglutinin is about 1/2,000 of that of ricin toxin in mice. It has been reported that the toxicity of ricin agglutinin, when tested on protein synthesis of cells in culture, is found to be about 1/30 that of ricin toxin(13). Under our experimental conditions, however, the toxicity in mice agreed well with the toxicity in colony formation of

cultured mouse L cells. Since 1/3 of the hybrid molecule is composed of agglutinin protein, the CRM45 moiety of the hybrid increases the toxicity of the agglutinin alone by about 30 times. Although the CRM45 of the hybrid has lower enzymatic activity than the original CRM45, The CRM45 may have become stable to proteolytic enzymes in cells as a result of the glutaraldehyde treatment. In fact, the enzymatic activity of the CRM45 treated with glutaraldehyde is more stable than the original.

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