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Effect of Trinitrophenylation of Amino Groups on Biological Activities of Ricin D

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Modification of amino groups with 2, 4, ii-trinitrohenzenesulfonic acid (TNBS) remarkably reduced the toxicity of ricin D to mice, whereas cytoagglutinating and carbohydrate-binding activities were fully retained until the modification of 8 moles (73 %) of the free amino groups, indicating that the free amino groups exposed on the surface of ricin D molecule contribute to the toxic action but are not essential for the binding to oligosaccharides on cell surface.

Three trinitrophenyl (TNP) amino groups out of eight in ricin D were found to be distributed to the A chain and five TNP-amino groups were deduced to be distributed to the B chain.

The inhibitory activity of the TNP-ricin D to cell-free protein synthesis was more than 50 % of that of native ricin D, indicating that amino groups in the A chain do not contribute much to the inhibition of protein synthesis. Digestibility of the TNP-ricin D by protease and its incorporation into cells also were the same as the native ricin D.

These results demonstrate that the trinitrophenylation is one of the useful means of reducing the toxicity of ricin D without impairing the B chain's ability to bind to the cell surface and the A chain's ability to inhibit protein synthesis. A possible contribution of amino groups to the transport mechanism of ricin D to the cytoplasm are discussed in the results.

INTRODUCTION

Ricin D, one of the highly purified toxic proteins present in castor beans (***Ricinus*** *communis*), has been found to be composed of two types of polypeptide chains differing in their biological functions : the A chain inhibits protein synthesis and the B chain agglutinates animal cells by binding to galactose containing oligosaccharide on the cell surface (Olsnes and Pihl, 1973). Two polypeptide chains are held together by a single disulfide bridge easily cleaved by reduction, and high toxicity of ricin D is elicited by cooperative action of the two chains (Funatsu and Funatsu, 1977).

Chemical modification studies on ricin D are directed toward elucidating the role of individual amino acids residues involved in toxic action as well as cytoagglutination. Maleylation of amino groups of ricin D resulted in an inactivation of both toxic action and cytoagglutination (Funatsu et al., 1977), whereas trinitrophenylation led to a decrease of its toxicity to mice without affecting the cytoagglutinating activity (Taira et al., 1978), suggesting that free

amino groups exposed on the surface of ricin D molecule are not essential for the cytoagglutinating action but contribute to the toxic action.

The present paper describes details of modifications of free amino groups of ricin D with 2, 4, 6-trinitrobenzenesulfonic acid and properties of the modified ricin D.

MATERIALS AND METHODS

Materials

Ricin D was prepared as described previously (Hara et al., 1974). 2, 4, 6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Nakarai Chemicals Co. All reagents used were of analytical grade unless otherwise specified.

Trinitrophenylation

Trinitrophenylation of the ricin D was carried out according to the method of Okuyama and Satake (1960) as previously described (Taira et al., 1978). TNBS solution (3.1 mg/0. 5 ml) was added at a concentration of 3-fold moles per free amino groups to 8 ml of 0.2 % ricin D solution in 0. 1 M sodium borate buffer, pH 8.5, or 0. 1 M sodium phosphate buffer, pH 7.4. After incubation at 30°C or 0°C for various times under stirring. 1 ml of the reaction mixture was poured into a Visking dialysis tube and 1 ml of ice-cold 5 mM phosphate buffer, pH 7.2, containing 0.9 % NaCl (PBS solution) was added. The solution was dialyzed against PBS solution in a cold room overnight. The dialysate was filled up to 4ml with PBS solution and the produced precipitate was removed by centrifugation. The number of TNP-amino groups in the TNP-ricin D was calculated by the difference in optical density at 345 nm between TNP- and native ricin D with a molar extinction coefficient of 1.1×10^4.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (1969). Proteins were dissolved in 20 μl of 10 mM phosphate buffer, pH 7.2, containing 25 % glycerol and 1 % SDS. After incubation at room temperature overnight, the protein solution was subjected to electrophoresis.

SDS-urea-polyacrylamide gel electrophoresis

Gel eletrophoresis in dissociating media was carried out essentially by the procedure of Swank and Munkres (1971). Proteins were dissolved in 20 μl of 1 % SDS, 8 M urea and 0.01 M H_3PO_4 adjusted to pH 6.8 with tris, incubated 10 min at 60°C, and stored overnight at room temperature prior to electrophoresis. Electrophoresis was performed at pH 6.8 using 12.5 % polyacrylamide gel (1 : 10 crosslinkage). Proteins were detected by staining with Coomassie Brilliant Blue.

Amino acid analysis

The protein was hydrolyzed with 5.7 N HCl containing 0.05 % 2-mer-

captoethanol in an evacuated sealed tube at 108°C for 24 hr. Amino acid composition was analyzed with a JLC-6AH amino acid analyzer.

Affinity chromatography on Sepharose 4B

Affinity chromatography of TNP-ricin D was performed as previously described (Funatsu et al., 1977). After dialysis against 0.2 M NaCl-5 mM phosphate buffer, pH 7.1, TNP-ricin D was applied to a column of Sepharose 4B equilibrated previously with the same buffer and developed with the same buffer at 7°C. The adsorbed protein was eluted with 0.1 M lactose in the same buffer.

DEAE-cellulose column chromatography

The protein was dialyzed against 5 mM Tris-HCl buffer, pH 7.6, and applied to a DEAE-cellulose column previously equilibrated with the same buffer. After washing the column with the same buffer, the adsorbed protein was eluted with a linear gradient of NaCl from 0 to 0.4 M in the same buffer.

Reduction and carboxymethylation

Reduction and carboxymethylation of TNP-ricin D was carried out in 0.35 M Tris-HCl buffer, pH 8.6, principally by the method of Crestfield et *al.* (1963). To 50mg of TNP-ricin D in 12 ml of Tris-HCl buffer, pH 8.6, 0.3 ml of 5 % EDTA and 0.1 ml of 2-mercaptoethanol were added, and the mixture was allowed to stand for 2 hr at room temperature. After reduction, 0.268 g of monoiodoacetic acid dissolved in 1 ml of 1 M NaOH was added, and the mixture was maintained at room temperature for 15 min in the dark. The reduced-carboxymethylated TNP-ricin D was separated from excess reagents by filtration through a column of Sephadex G-25 in deionized water.

Digestion with protease

Digestion of the protein was performed with trypsin, chymotrypsin or nagarse. Ten microliters of the protease solution (0.2 % in 50mM Tris-HCl buffer, pH 8.0) was added to 1 ml of TNP-ricin D solution (0.1% in 50 mM Tris-HCl buffer, pH 8.0) and the reaction mixture was incubated at room temperature for 1 hr.

Cytoagglutinating activity

Cytoagglutinating activity was measured using sarcoma 180 ascites (SA) tumor cells as previously described (Funatsu ***et al.,*** 1976). An amount of 0.2 ml of TNP-ricin D solution with various concentrations, which had been previously dialyzed against PBS solution, was added to 0.2 ml of SA cell suspension (1×10^7 cells/ml). After incubation at room temperature, the agglutination potency was measured under a microscope and expressed as the minimum amount necessary to agglutinate a mojor part of the cells in small aggregates.

Toxicity

Toxicity of TNP-ricin D was determined using male pure-bred mice (ddN)

weighing 20 to 25 g as previously described (Ishiguro *et al.*, 1964). After intraperitoneal injection into mice, the results were observed at intervals of 24 hr. The half lethal dose at 48 hr (MLD_{48}) was adopted as a measure of toxicity.

Inhibitory activity to cell-free protein synthesis

The activity of modified ricin D to inhibit protein synthesis in a cell-free system was determined using crude rabbit reticulocyte lysate principally according to the method of Woodward *et al. (1974).* Twenty microliters of TNP-ricin D solution (in 20 mM Tris-HCl buffer, pH 7. 8) and 20 μl of ^{3}H-L-leucine solution (10 &i/ml) were added to 150 μl of the synthesizing system consisting of the unfractionated lysate, amino acid mixture, creatine phosphate, creatine kinase, ATP, GTP and hemin. After incubation at 30°C for 60 min, 50 μl of the reaction mixture was plated on a glass fibre disc (Whatman GF/C, 25 mm). The disc was dipped in 10 % (W/V) trichloroacetic acid (TCA), heated at 90°C to 100°C for 15 min and then washed with 5 % TCA solution. Hemin in the reaction mixture was decolorized by treating with bleaching solution for 2 hr. The disc was dried by washing first with ethanol-ether (1 : 1) and then with ether. The radioactivity was measured in a liquid scintillation spectrometer Aloka LSC-602.

Labelling with ^{125}I

Labelling of the protein with ^{125}I was carried out according to Hunter and Greenwood (1962). One mg of protein in 100 μl of 10 mM phosphate buffer, pH 7.0 was added to 60 μl of 0.5 M phoshate buffer, pH 7.2 containing 0.2 mCi of carrier-free Na^{125}I (NEN) and then 100 μl of chloramine-T solution (100 μg) was added. After 1 min at room temperature with stirring, 100 μl (500 μg) of sodium metabisulfite was added to stop the reaction. After addition of 100 μl (1 mg) of KI, ^{125}I-labeled protein was purified by gel filtration on a Sephadex G-75 column (1.0 x30 cm) equilibrated with PBS solution.

Irreversible binding of ^{125}I-labeled protein to cells

From HeLa cells growing as a monolayer in glass dishes (5.3 x 10^5 cells/dish), the culture medium was removed and replaced by 1 ml of serum-free minimum essential medium containing 5 μg of ^{125}I-labeled native ricin D (specific activity : 111600 cpm/μg) or TNP-ricin D (specific activity : 54100 cpm/μg). In the control, the same medium containing 0. 1 M lactose was used. After incubation at 37°C for various times, the medium was replaced by 2 ml of PBS solution containing 0. 1 M lactose and the cells were incubated for 15 min at 37°C. After washing with 2 ml of fresh PBS solution containing 0.1 M lactose, the cells were dissolved in 2 ml of 0. 1 M KOH solution. The radioactivity of the solution was counted in a well type scintillation counter Aloka JDC-5.

RESULTS AND DISCUSSION

Effect of trinitrophenylation on toxicity and cytoagglutinating activity of

ricin D

The modification of free amino groups in ricin D by 3 fold molar excess of TNBS at pH 7.4 or 8.5 and at 0°C or 30°C is shown in Fig. 1. The degree of trinitrophenylation increased progressively with increasing pH and temperature.

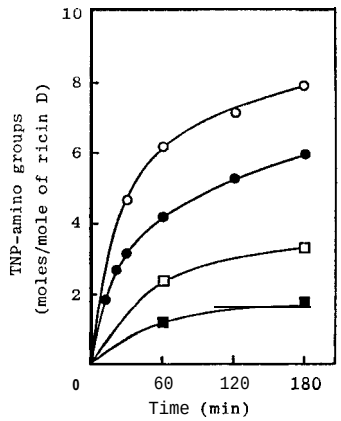

Fig. 1. Time course of trinitrophenylation of ricin D. Ricin D was trinitrophenylated with 3 fold moles of TNBS per free amino groups. ○—○, at pH 8.5 and 30°C; ●—●, at pH 7.4 and 30°C; □—□, at pH 8.5 and 0°C; ■—■, at pH 7.4 and O'C.

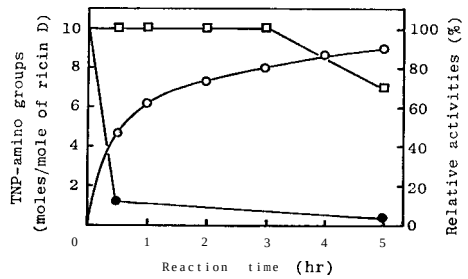

Fig. 2. Effect of trinitrophenylation on toxicity and cytoagglutinating activity of ricin D. Trinitrophenylation of ricin D was carried out with 3 fold moles of TNBS at pH 8.5 and 30°C. Toxicity to mice and cytoagglutinating activity to SA cells of modified ricin D were examined as described in MATERIALS AND METHODS. ○—○, TNP-amino groups; ●—●, toxicity ; ❑ —□, cytoagglutinating activity.

At pH 8.5 and 30℃, the degree of trinitrophenylation increased steeply for about one hour and then gradually. At this condition the effect of trinitrophenylation on the toxicity to mice and the cytoagglutinating activity to SA cells of ricin D were examined (Fig. 2). A molecule of ricin D contains eleven amino groups : nine lysyl residues plus two N-terminal amino groups (Funatsu

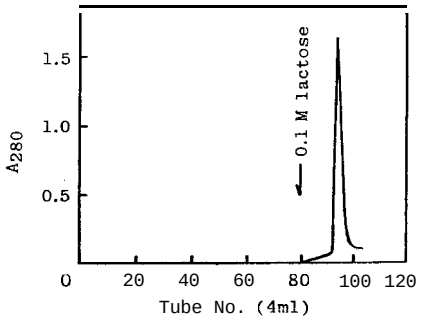

Fig. 3. Affinity chromatography of TNP-ricin D on a Sepharosc 4B column. TNP(8)-ricin D was applied to a column (2× 19 cm) of Sepharose 4B equilibrated with 0.2 M NaCl-5mM phosphate buffer (pH 7. 1) and washed with the same buffer. The adsorbed protein was eluted with 0.1 M lactose in the same buffer. Temperature was 7°C.

et *al.*, 1978, 1979). The toxicity was decreased to 12.5 % of that of ricin D by the modification of 4. 5 moles (41 %) of the free amino groups, and to 4. 6 % by the modification of 9 moles (82 %) of the free amino groups. This result suggests that free amino groups exposed on the surface of ricin D molecule contribute to the toxic action. However, the cytoagglutinating activity was fully retained until about 8 moles (73 %) of the free amino groups were modified and decreased slowly by the further modification. The TNP-ricin D containing 8 TNP-amino groups (TNP(8)-ricin D) bound to the Sepharose 4B matrix as native ricin D and was eluted with 0. 1 M lactose solution (Fig. 3), indicating that the free amino groups apparently are not involved in the carbohydrate-binding.

Distribution of TNP-amino groups amongst the peptide chains of ricin D

In order to know the number of TNP-amino groups on two constituent polypetide chains of TNP-ricin D, the A chain was isolated from TNP-ricin

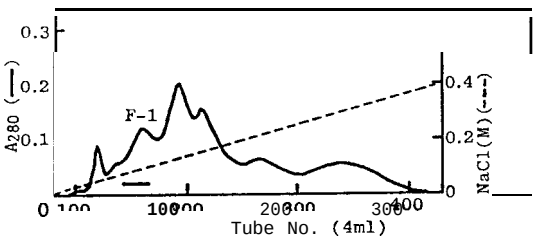

Fig. 4. DEAE-cellulose column chromatography of the reduced-carboxymethylated TNP-ricin D. Reduced-carboxymethylated TNP(8)-ricin D was applied to the column (1. 2x30 cm) of DEAE-cellulose equilibrated with 5 mM Tris-HCl buffer (pH 7.6) and eluted with a linear gradient of NaCl from 0 to 0.4 M in the same buffer. The fraction F-l indicated by horizontal bar, in which A chain was contained, was pooled.

D obtained by trinitrophenylation at pH 8.0 and 30°C for 3 hr and the number of TNP-amino groups in the A chain was determined. After reduction and carboxymethylation of TNP(8)-ricin D, the product was applied to DEAE-cellulose column. The fraction F-l indicated by horizontal bar in Fig. 4, in which the A chain was contained (Fig. 6), was pooled and the contaminated TNP-ricin D was removed by affinity chromatography on Sepharose 4B. TNP-A chain was eluted without adsorption on Sepharose 4B as shown in Fig. 5 and 6. TNP-amino groups in the isolated A chain was estimated to be 3.37 moles per mole of A chain on the basis of the optical density of 0.305 at 345

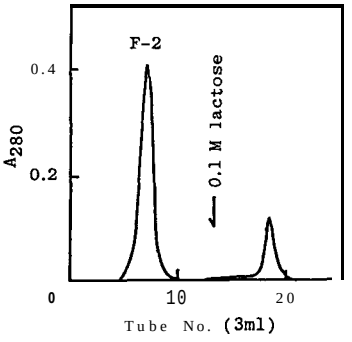

Fig. 5. Affinity chromatography of fraction F-l obtained in Fig. 4 on a Sepharose 4B column. Chromatographic conditions were the same as in Fig. 3, except the column size was 1.2 ×21 cm.

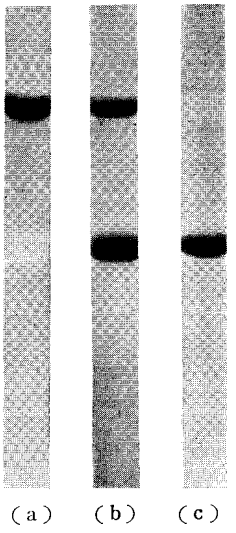

Fig. 6. SDS-polyacrylamide gel electrophoresis of the fractions obtained Fig. 4 and 5. The fractions obtained in Fig. 4 and 5 were subjected to SDS-polyacrylamide gel electrophresis. Gel concentration was 10 %. (a) ricin D, (b) fraction F-l, and (c) fraction F-2.

nm for 8.21 mM of TNP-A chain obtained by amino acid analysis. These results indicate that all three amino groups in the A chain of ricin D were trinitrophenylated and it was deduced that five free amino groups out of eight amino groups in the B chain would be trinitrophenylated.

Digestibility of TNP-ricin D by protease

In order to determine if the loss of toxicity for mice is not due to a conformational change of the molecule resulting in high digestibility by protease, TNP(8)-ricin D was submitted to proteolytic digestion. After incubation with trypsin, chymotrypsin or nagarse at room temperature for 1 hr, 50 μl of the digest was added to 50 μl of 20 mM Tris-H_3PO_4 buffer, pH 6.8, containing 2 % SDS and 10 M urea, heated at 60°C for 10 min and left overnight at room temperature. Forty μl of the digest was subjected to gel-electrophoresis. As shown in Fig. 7, no significant differences were observed between native and TNP-ricin D.

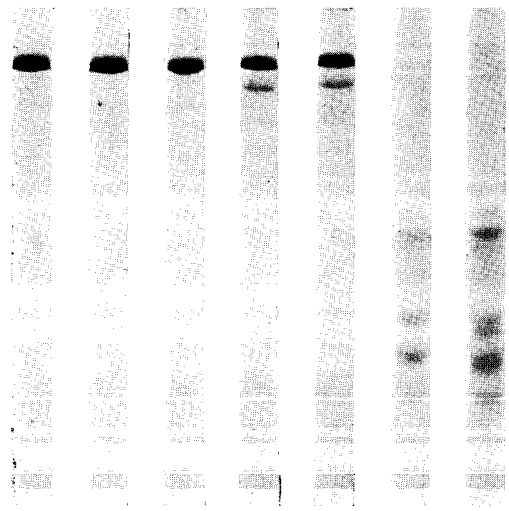

Fig. 7. SDS-urea-polyacrylamide gel electrophoresis of the proteolytic digests of native ricin D and TNP-ricin D. Native ricin D and TNP(8)-ricin D were digested with proteases for 1 hr at pH 8.0 at room temperature. Digests were subjected to SDS-urea-polyacrylamide gel electrophoresis. Gel concentration was 12.5 %. (a) native ricin D, (b) tryptic digest of native ricin D, (c) tryptic digest of TNP-ricin D, (d) chymotryptic digest of native ricin D, (e) chymotryptic digest of TNP-ricin D, (f) nagarse-digest of native ricin D, and (g) nagarse-digest of TNP-ricin D.

Inhibitory activity of TNP-ricin D to cell-free protein synthesis

If ricin intoxication in *vivo* is due to the inactivation of ribosome 60S subunit by its A chain, toxicity of TNP-ricin must decrease concomitantly

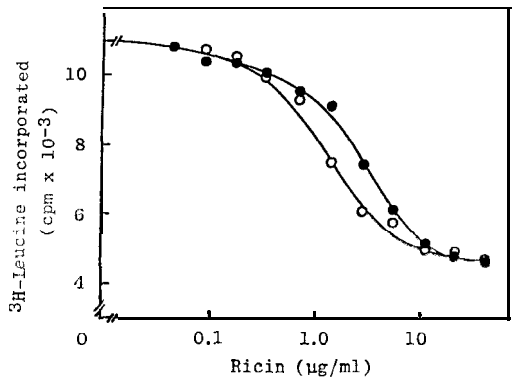

Fig. 8. Inhibition of protein synthesis in a cell-free system by native ricin D and TNP-ricin D. Twenty microliters of 20 mM Tris-HCl buffer (pH 7.8) containing increasing amounts of ricin D and 20 μl of ^{3}H-L-leucine solution (10 μCi/ml) were added to 160 μl of the cell-free system of protein synthesis using crude rabbit reticulocyte lysnte. The radioactivity of ^{3}H-L-leucine incorporated into protein after incubation at 30°C for 60 min was measured as described in MATERIALS AND METHODS. ○—○, native ricin D; ●--●, TNP-ricin D.

with inhibitory activity of protein synthesis. Inhibitory activity of TNP(8)-ricin D in cell-free system using crude reticulocyte lysate was examined and the result is shown in Fig. 8. Inhibitory activity of TNP-ricin D as judged by the amount necessary to inhibit 50% of leucine incorporation was more than 50% of that of native ricin D. This data is not in agreement with the decrease of toxicity for mice and indicates that amino groups in the A chain do not contribute much to the inhibition of protein synthesis in cell-free system. Since the fact that the cytoagglutinating activity of TNP-ricin D is as high as that of native ricin D indicates that TNP-ricin D retains binding ability to the cells, the discrepancy between toxicity for mice and inhibitory activity of protein synthesis in a cell-free system may be due to the difference in events that occur in the cell membrane after the binding of TNP-ricin D to the cell membrane.

Incorporation of TNP-ricin D into cells

In order to compare the incorporation kinetics of native and TNP(8)-ricin D into the cells, irreversible binding of the two ricins to the cells was examined using ^{125}I-labeled ricin D and HeLa cells. As shown in Fig. 9, ricin D bound to the cells at 0°C was almost completely removed by washing with lactose, whereas ricin D incubated with HeLa cells at 37°C was irreversibly bound to the cells. The amounts of native and TNP-ricin D bound to the cells after 1 hr incubation were calculated to be 93. 5 ng and 117. 8 ng, respectively, indicating that the TNP-ricin D is incorporated into the cells in the same manner as native ricin D.

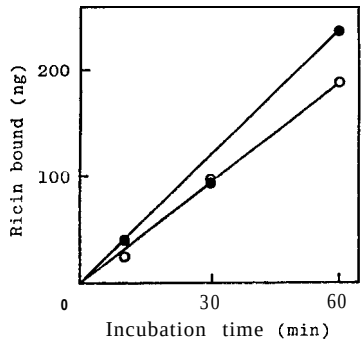

Fig. 9. Irreversible binding of ^{125}I-labeled native ricin D and TNP-ricin D to HeLa cells. After incubation of ^{125}I-labeled native ricin D or TNP(8)-ricin D (5 μg) with HeLa cells (5.3×10^5 cells/dish) at 37°C, the radioactivity of ^{125}I-labeled ricin D irreversibly bound to the cells was counted as described in MATERIALS AND METHODS. ○—○, native ricin D; ●—●, TNP-ricin D.

The steps in the intoxication of cells by ricin D are thought to include (1) binding of ricin D to receptors on the cell surfaces, (2) internalization of ricin D into cell, (3) splitting into ricin's constituent polypeptide chains by reduction of inter-chain disulfide bridge and (4) inactivation of 60S ribosomal subunit by A chain (Nicolson *et al.*, *1975:* Oda and Funatsu, 1979; Sandvig and Olsnes, 1982). Although there is no doubt that these events occur, the data of irreversible binding to the cells, as well as those of the inhibitory activity to cell-free protein synthesis, were not in agreement with the low toxicity of TNP-ricin D for mice, and suggested that the toxicity of ricin D was not necessarily proportional to the amount of the incorporated ricin D. Since ricin D internalized by endocytosis is still separated from the cytoplasm by the vesicle membrane, it is possible that there is an unknown mechanism in transport of the internalized ricin D to the cytoplasm. The contribution of amino groups in ricin D to this mechanism seems to be one possibility.

This trinitrophenylation is one of the useful means of reducing the toxicity of ricin D without impairing the B chain's ability to bind to the cell surface and without impairing the A chain's ability to inhibit protein synthesis.

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