Differential Effects of Nitrated Ricin and Nitrated and Dithionite-Reduced Ricin on Protein-Synthesis Inhibition and Transmembrane Transport in Eukaryotic Cells

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Ricin₁ was nitrated with tetranitromethane and reduced with sodium dithionite. Of the 8.0 nitro groups incorporated, 3.2 were on the A chain and 5.1 were on the B chain. Nitrated ricin₁ was somewhat less active than nitrated and reduced ricin₁ in inhibiting protein synthesis *in vitro*, but both were highly inhibitory. However, the modified toxins were less than 1% as active as ricin in inhibiting protein synthesis in cultured cells. Indirect immunofluorescence assays demonstrated that both modified toxins were specifically bound to the cell surface and could be displaced by galactose.

Ricin is an extremely potent enzyme capable of killing cultured eukaryotic cells at concentrations as low as 1–0.1 μ M. The toxin has two dissimilar, specialized polypeptides held together by a single disulphide bond and is apparently the product of activation of a larger less-toxic protein (*Ricinus communis* agglutinin) (Cawley *et al.*, 1978). Cell death caused by ricin is the direct result of the ricin A-chain inflicting some type of damage on the 60S ribosomal subunit, which results in blockage of translation (Olsnes & Pihl, 1976). Although *Ricinus communis* agglutinin is not very toxic to cells or inhibitory to translation *in vitro*, the isolated A-chain of this agglutinin is nearly as inhibitory as ricin A-chain to rat liver ribosomes (Cawley *et al.*, 1978). The inactivation of ribosomes by ricin A-chain is rapid and results in decreased activity of functions associated with elongation factor-2 (Olsnes & Pihl, 1976). The nature of the damage to the ribosome is unknown. Even though ricin is an enzyme, stoichiometric binding of ricin and ricin A-chain to the ribosome has been observed (Hedblom *et al.*, 1976, 1979).

In order for the A-chain to gain access to the cytoplasm, the B-chain, a polypeptide which binds to *N*-acetylgalactosamine- or galactose-containing receptors, must be present. Whether both chains enter the cytoplasm or the B-chain remains at the cell surface bound to its receptor(s) is a matter for speculation. Studies directed towards understanding the events leading to the transport of the A chain across the plasma membrane have suffered from the use of much larger concentrations of toxin than are needed for efficient killing. The high concentration of toxin required for detection may mask specific

transport systems which are present in small quantities and lead directly to ribosome inhibition.

One method for studying such transport systems is the development of mutant strains of cells that are resistant to the effects of ricin (Gottlieb & Kornfeld, 1976; Meager *et al.*, 1976; Sandvig *et al.*, 1978). Another approach is to investigate the effects of chemical modification of ricin in the hope of being able to separate transport from ribosome inactivation.

Materials and Methods

Ricin₁, one of the three different ricin species in castor beans (*Ricinus communis*), ricin A-chain and ricin B-chain were purified as previously described (Cawley *et al.*, 1978).

Nitration of ricin was accomplished by dissolving 2.4 mg of ricin₁/ml in 0.1 M-Tris/HCl, pH 9.0, at 22°C and adding various amounts of 0.85 M-tetranitromethane (Aldrich Chemical Co.) in 95% (v/v) ethanol. The reaction was carried out for 20 min and the excess reagent was separated from the modified protein on a Sephadex G-25 column equilibrated with 0.1 M-Tris/HCl, pH 9.0 at 4°C.

Reduction of the nitrotyrosyl derivatives to aminotyrosyl derivatives was accomplished by the addition of 40-fold molar excess (over nitrotyrosine) of sodium dithionite. The proteins were freed of excess reagents on a Sephadex G-25 column. Nitrotyrosyl-ricin₁ refers to nitrated ricin₁, and aminotyrosyl-ricin₁ refers to nitrated and dithionite-reduced ricin₁.

The extent of nitration was measured spectrophotometrically by using the absorption coefficient

$2200\text{ M}^{-1}\cdot\text{cm}^{-1}$ at the 381 nm isosbestic point (Riordan *et al.*, 1967). Dithionite reduction was followed by the loss of the nitrotyrosine spectrum and was assumed to be complete when the 381 nm absorbance returned to the baseline value established with an appropriate control protein. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Poly(U) translation was measured as described by Cawley *et al.* (1977). Protein synthesis in mouse L cells was measured as follows. Mouse L cells (N.C.T.C. clone 929) were grown to near-confluence in a T-25 Falcon flask in 20 ml of chemically defined serum-free medium (Kitos *et al.*, 1962). The toxins were diluted in the medium and 4 ml samples were added to cells in triplicate and the cells incubated for 3 h. Then $1\mu\text{Ci}$ of [^3H]leucine was added to 1 ml of leucine-free medium and this mixture was incubated with the toxin-treated cells for 1 h. The flasks were then chilled on ice, drained, rinsed with 0.9% NaCl, and the cells removed with trypsin. An equal volume of 10% (v/v) trichloroacetic acid was used to precipitate the labelled proteins and the precipitate collected by centrifugation (45000g, 10 min). The precipitate was washed once with 5% trichloroacetic acid, mixed with 0.4 ml of Protosol and counted for radioactivity in a toluene-based scintillant (10 ml; 5 mg of 2,5-diphenyloxazole/ml of toluene).

AKR SL3 cells, a mouse leukaemia cell line grown in suspension, were prepared for assay by washing the cells in RPMI 1640 medium containing no methionine (RPMI-Met⁻), incubating the cells in RPMI-Met⁻ medium for 45 min and adding measured volumes of the cells to small glass test tubes. The number of cells in each test tube was usually about 0.5×10^6 in 0.3 ml. The test toxins were diluted in RPMI-Met⁻ medium and added to the cells. After 85 min of incubation, $10\mu\text{l}$ of RPMI-Met⁻ medium containing 6×10^6 c.p.m. of [^{35}S]methionine (914 Ci/mmol) was added and incubation continued for an additional 30 min. The cells were collected by washing on to Gelman glass-fibre filters with 0.9% NaCl, protein was precipitated on the filter with 5% trichloroacetic acid and the filters were washed with 5% trichloroacetic

acid, then with 95% ethanol, and counted for radioactivity. Controls incorporated 70000 c.p.m. in this assay. Because ricin can probably bind to serum glycoproteins, serum was not added to the cells in either assay.

Results

Nitration of ricin₁ was carried out with 4-, 8-, 16- and 32-fold molar excess (over tyrosine) of tetranitromethane for 20 min. Under these conditions, a maximum of 8.0 nitrotyrosyl groups were formed. Ricin contains 27 tyrosine residues, of which 15 are in the A-chain and 12 in the B-chain (Olsnes & Pihl, 1976). It appears that approximately two-thirds of the tyrosine residues were not exposed to the solvent and could not be nitrated. To investigate the distribution of these eight nitrotyrosine residues in ricin, the A- and B-chains were separated after mercaptoethanol reduction (Cawley *et al.*, 1978) and the nitrotyrosine content of each was determined. The sample used for this analysis was modified by using a 16-fold molar excess of tetranitromethane. Of the 8.0 nitro groups introduced into this derivative, 3.2 were found in the A chain and 5.1 in the B chain. Since the sum of the two chains was very close to the value obtained for the intact nitrated protein and all values were near integers, it is likely that all of the exposed tyrosine residues in both chains were fully modified and that the preparation did not contain a large population of positional isomers with varying degrees of nitration.

The effect of nitration and reduction had little effect on the ability of ricin to inhibit polyphenylalanine synthesis *in vitro* by rat liver ribosomes (Table 1). However, nitrotyrosyl-ricin₁ was not as effective as ricin₁ or aminotyrosyl-ricin₁ in inhibiting poly(U) translation.

To determine whether these derivatives were active *in vivo*, small quantities were injected intraperitoneally into mice. The LD₅₀ for ricin in mice has been estimated to be about $0.2\mu\text{g}$ (Olsnes & Pihl, 1973), but inoculation of $0.1\mu\text{g}$ of our preparation results in death of all the mice within 2 days. Mice were injected with $10\mu\text{g}$ of nitrotyrosyl-ricin₁ or aminotyrosyl-ricin (100 times the 100%-lethal dose of ricin) lived for 1 month without showing any ill

Table 1. *Inhibition of rat liver ribosome activity by modified ricin derivatives*
Protein synthesis *in vitro* using rat liver ribosomes and poly(U) translation was measured.

Derivative	Concn. of protein ($\mu\text{g}/\text{ml}$)	Synthesis (% of control)		
		10	20	50
Ricin ₁		33	10	10
Nitrotyrosyl-ricin ₁		100	75	62
Aminotyrosyl-ricin ₁		51	10	14

effects. Mice inoculated with 10 μ g of ricin₁ died within 24 h.

The fact that the modified toxins, although active in cell-free preparations, were not able to exert their lethal potential if membrane transport was required was confirmed by using cultured cells to assay protein synthesis. Fig. 1 clearly indicates that

Fig. 1. Effect of modified derivatives on protein synthesis in cultured cells

Ricin₁ (○), nitrotyrosyl-ricin₁ (◐) or aminotyrosyl-ricin₁ (●) was incubated at various concentrations with AKR SL3 cells (a) or mouse L cells (b) and the trichloroacetic acid-precipitable radioactivity measured as described in the Materials and Methods section.

nitrotyrosyl- and aminotyrosyl-ricin₁ were not efficient inhibitors of protein synthesis if transmembrane transport was required to gain access to the ribosome. Both mouse L and AKR SL3 leukaemia cells were inhibited by ricin₁ at nearly the same concentration (1–3 ng/ml). No inhibition of protein synthesis by either modified derivative was apparent at concentrations 100 times that effective with ricin₁. Much larger concentration could, however, achieve some protein-synthesis inhibition. When 30 μ g of either nitrotyrosyl- or aminotyrosyl-ricin/ml was used, protein synthesis was inhibited by about 50%.

Because the lack of inhibition of protein synthesis *in vivo* or in cultured cells could possibly be due to modification of the B-chain-dependent cell-surface binding, an indirect immunofluorescence assay was used to detect binding to AKR SL3 cells. The cells were treated with (1) ricin₁ or modified ricin₁, (2) rabbit antiserum to ricin, and (3) antiserum to rabbit immunoglobulin G raised in goats and conjugated to fluorescein (Hyland Laboratories). Fig. 2 shows that both nitrotyrosyl-ricin₁ and aminotyrosyl-ricin₁ bind to AKR SL3 cells in this assay. Therefore the lack of toxicity *in vivo* or in cultured cells cannot be attributed to grossly impaired binding of the derivatives or to loss of the inherent enzymic activity of ricin A-chain.

In order to examine the specificity of binding 20 mM-galactose was included in the incubation of ricin or modified ricin₁ with cells. Galactose completely prevented binding of the toxins as measured by the immunofluorescence assay, but 20 mM-glucose had no effect.

Because immunofluorescence is not quantitative, subtle differences in binding cannot be ruled out. However, there appeared to be no large differences in the fluorescence intensity between any of the

Fig. 2. Binding of modified derivatives to AKR SL3 cells

Indirect immunofluorescence assays were performed on cells to which had been added ricin₁ (a), nitrotyrosyl-ricin₁ (b) and aminotyrosyl-ricin₁ (c). Cells were incubated with 15 μ g of protein in 0.3 ml of RPMI 1640 medium for 20 min at 0°C, washed once with RPMI 1640 medium and incubated for 20 min with antibody to ricin raised in rabbits and washed once. Fluorescein-conjugated antibody to rabbit immunoglobulin was added for 20 min and the cells were centrifuged and washed. Most cells were found in small clumps of a few cells.

derivatives when the same quantities of protein were applied to the cells. The modified derivatives did appear to be somewhat less stable than ricin₁ to freezing and thawing (storage at -80°C) when the indirect immunofluorescence assay was repeated at bimonthly intervals for 3 months. These data also indicate that the antigenicity of the ricin derivatives was not significantly changed by nitration and reduction.

Discussion

From the results of Nicolson *et al.* (1975) and others (Olsnes & Pihl, 1976), the events that lead to inhibition of protein synthesis have been postulated to include (1) binding of ricin to randomly distributed receptors on the cell surfaces, (2) ricin-induced receptor aggregation to form clusters and caps, (3) endocytosis of ricin-receptor complexes and (4) fusion of the vesicles with lysosomes and/or release of ricin from the endocytotic vesicles into the cytoplasm. Although there is no doubt that these events occur, the fact that some mutant cell lines are capable of internalizing as much ricin as do normal cells (Sandvig *et al.*, 1978) suggests that endocytosis may not be the only path of entry into the cell, nor the most important way; furthermore, ricin internalized by endocytosis is still separated from the cytoplasm by the vesicle membrane. Another more specific mechanism may be operating, which may be the more potent delivery system. Because of the extremely small quantities of toxin needed to exert a biological effect, it is difficult to study this question.

If transport were not severely curtailed, entry of only a few molecules of the extremely potent modified ricin₁ derivatives would in time completely terminate protein synthesis. We assume that neither derivative is released into the cytoplasm to any significant extent during the time of treatment. The data presented here indicate that nitration of ricin leads to separate effects on transport and protein-synthesis inhibition.

The fact that nitrotyrosyl-ricin₁ was somewhat less active than aminotyrosyl-ricin₁ might be due to the larger bulk of the nitro group, which is decreased by conversion into the amino group. Concomitant with reduction is a change in the pK of the tyrosine hydroxy group, which may also affect the biological activity.

Because galactose inhibits binding of the modified toxins and glucose does not, it appears that the modified toxins are specifically bound to their normal receptors. Since binding of the modified derivatives occurs, but does not result in the death of the cell, it is suggestive that some subsequent step in

transport, separate from simple binding, is inhibited. It is possible that these derivatives are still internalized by endocytosis and the apparent resistance of the cells could also be due to the rate at which transport occurs. Inhibition of protein synthesis in cultured cells observed at very high concentrations could have been due to non-specific pinocytosis. Although it is possible that the resistance to inhibition can be explained by increased degradation by cellular proteinases, it is not likely because of the short incubation time used and the extreme resistance of ricin to proteolytic degradation (Olsnes & Pihl, 1976). Modification could conceivably affect the transport mechanism by preventing either required conformational changes that involve tyrosine residues or perhaps the hypothesized release of the A-chain from the B-chain at the cell surface.

These derivatives should prove useful in investigating the transport mechanism of ricin and may contribute to understanding some of the molecular details of the process.

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