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Mannose receptor dependent uptake of ricin A_1 and A_2 chains by macrophages

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Abstract

The ricin A chain, the toxic subunit of ricin, consists of two forms which differ in sugar content. The major component A_1 contains one high mannose chain while the minor component A_2 contains an additional high mannose chain. Endocytosis of this toxin occurs in macrophages via the mannose receptor. To study the role of the sugar residues in ricin A chain cytotoxicity, we have purified the two forms by ion-exchange chromatography. The uptake of A_1 and A_2 by a macrophage cell line was concentration and time dependent. The total amount of A_2 internalized was approximately twice the amount of A_1, indicating a higher affinity of A_2 for the mannose receptor. Ricin A_2 was four times more toxic to macrophages than A_1, in agreement with the higher affinity of A_2 compared to the A_1. These experiments suggest that the high mannose chains on the A chain promote mannose-receptor-mediated endocytosis by providing the initial binding to the cell surface. Once the toxin is accumulated inside the cell however, the carbohydrates do not seem to influence intracellular transport and/or translocation of the ricin A chain into the cytoplasm.

Keywords: Plant lectins; Toxins; Macrophages; High mannose chains

1. Introduction

The ricin A chain, the toxic subunit of the plant toxin ricin, is a glycoprotein which exists in two forms which differ in sugar content. The major component A_1 (M_r 30,000) comprises 64% of the total and contains a single high mannose oligosaccharide linked to Asn-10. The minor component A_2 (M_r 32,000) contains an additional high mannose chain linked to Asn-236 [1,2]. Highly purified ricin A chain intoxicates macrophages

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both in vitro [3] and in vivo [4,5], following binding and endocytosis via the macrophage specific mannose receptor. Once internalized, the toxin is first rapidly cleaved by endosomal proteases [6] and then transported to the intracellular compartment from which translocation occurs [7–10]. In the cytoplasm, the ricin A chain or a peptide thereof [11] enzymatically inactivates protein synthesis by removing a single adenosine residue from 28S rRNA [12,13]. The translocation mechanism, as well as the intracellular compartment from which this toxin translocates into the cytoplasm, is still unclear.

Previous reports have shown that periodate treatment of ricin, which leads to selective oxidation of the terminal sugar moieties of its oligosaccharide chains, results in a loss of cellular toxicity but has no effect on cell binding affinity or A chain activity in vitro [14,15]. These authors concluded that the carbohydrate in ricin may facilitate intoxication.

In this paper we directly examine the role of high mannose oligosaccharides on the ricin A chain in intracellular transport and translocation. We have purified the components A_1 and A_2 by ion-exchange chromatography and characterized their uptake and cytotoxicity in macrophages. The experiments presented indicate that the mannose-receptor-mediated uptake of the ricin A chain is dependent on the number of carbohydrate chains present on the toxin molecule. The ability of the ricin A chain to intoxicate macrophages correlates with the total amount of toxin accumulated inside the cell. These results suggest that the high mannose chains promote receptor-mediated endocytosis by providing the initial binding to the cell surface. Once the toxin is accumulated inside the cell the carbohydrates do not seem to facilitate intracellular transport and/or translocation of the ricin A chain into the cytoplasm.

2. Experimental

Materials.—Castor beans (*R. communis*) were obtained from A.H. Hummert's Seed Company, St. Louis, MO. Carrier-free sodium ^{125}I- and ^{3}H-leucine were obtained from Amersham. Both ricin A_1 and ricin A_2 chains were radioiodinated using the chloramine T method [16]. Rabbit reticulocyte lysate was purchased from Promega. All other chemicals were standard reagent grade and were obtained from Sigma, St. Louis, MO.

Cells.—J774-E clone, a murine macrophage cell line (mannose-receptor positive) [17], was maintained in alpha modification of Eagle's Medium (αMEM) [1], 10% fetal calf serum, 60 μM thioguanine, penicillin, and streptomycin.

Toxin purification.—Ricin toxin was prepared as described [18], and the A chain was isolated following disulfide reduction [3]. The A_1 and A_2 chains were separated as previously described [19]. Briefly, 30 mg of highly purified ricin A chain was extensively dialyzed against the CM-Sephadex starting buffer (5 mM Na-phosphate buffer pH 6.5 containing 0.1% 2-mercaptoethanol) and then applied to a CM-Sephadex column

[1] Abbreviations used: αMEM, minimal essential medium with Earle's salts; BSA, bovine serum albumin; HBSA, Hank's balanced salt solution with 1% BSA; PBS, Dulbecco's phosphate buffered saline, TCA, trichloroacetic acid.

equilibrated with the starting buffer. Bound toxin was eluted with a linear gradient of NaCl from 0 to 0.2 M in the same buffer. Fractions containing A_2 (first peak) and A_1 (second peak) were pooled and concentrated to approximately 0.5 mg/mL by ultrafiltration in a Sartorius apparatus. The toxins were dialyzed against phosphate-buffered saline prior to experiments.

Assay of enzymatic activity.—The biological activity of the purified toxins was tested on rabbit reticulocyte lysate (Promega) according to the manufacturer's procedure.

Uptake and degradation of toxins.—Ligand uptake studies were performed as described previously [11]. Cells were incubated in Hank's balanced salt solution containing 1% bovine serum albumin (HBSA) and varying amounts of either ^{125}I-ricin A_1 chain or ^{125}I-ricin A_2 chain (specific activity 1×10^6 cpm/μg) for 10 min at 37 °C. The cells were then washed once with ice cold Dulbecco's phosphate-buffered saline (PBS), twice with 0.5 M NaCl, 0.2 M acetic acid, pH 2.5, twice with HBSA and lysed with 0.1 M NaOH. Cell lysates were assayed with a gamma counter to determine the cell-associated ligand. Non-specific uptake was determined in all uptake experiments by including 2 mg/mL yeast mannan in control wells [20]. To assess the rates of toxin internalization and degradation, aliquots of the uptake medium were removed after different times of uptake, and proteins were precipitated with trichloroacetic acid (TCA). Both soluble and precipitable radioactivity were then quantitated in a gamma counter. Duplicate values were determined for all uptake experiments.

Cytotoxicity studies.—Cells were incubated with various dilutions of toxins for 18 h at 37 °C. The media was then removed and the cells were further incubated for 1 h in a leucine-free medium containing 1 μCi/mL of ^{3}H-leucine. The cells were washed with ice cold HBSA, with 10% (w/v) TCA and then solubilized in 0.1 M NaOH. Aliquots of the cell lysates were assayed in a liquid scintillation counter. The results are expressed as percentage of the radioactivity incorporated into control cells not treated with toxins.

3. Results and discussion

Macrophages actively internalize glycoproteins with terminal D-mannosyl and L-fucosyl groups. The ricin A is a mixture of two differently glycosylated forms, the A_1 component (M_r 30,000) of sugar composition $(GlcNAc)_2(Man)_{3-4}(Xyl)(Fuc)$ and the A_2 component (M_r 32,000) which contains an additional sugar of composition $(GlcNAc)_2(Man)_{4-6}$ [1,2]. The high mannose oligosaccharides on the protein molecule allow the ricin A chain to bind to and kill macrophages following receptor-mediated endocytosis via the mannose receptor [3–5,21]. In order to characterize the uptake and cytotoxicity of the two forms of the toxin in macrophages we separated the two components by ion-exchange chromatography. The highly purified ricin A chain was loaded on a CM-Sephadex column and the bound toxin eluted with a linear gradient of NaCl. Fractions corresponding to two different peaks were pooled and analysed by SDS-PAGE. As shown in Fig. 1, the first peak contained almost exclusively A_2 (lane 3) and the second peak contained A_1 (lane 2). To examine if the additional high mannose oligosaccharide present on the A_2 chain was affecting the activity of the toxin by altering its conformation, we tested the purified components in a cell-free protein

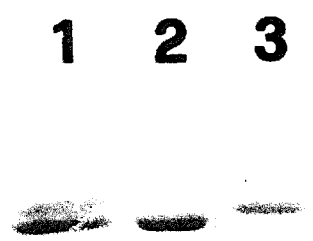

Fig. 1. SDS-PAGE of ricin A, A_1, and A_2 chains on 12.5% polyacrylamide gel. Lane 1, ricin A chain; lane 2, ricin A_1 chain (CM-Sephadex peak 2); lane 3, ricin A_2 chain (CM-Sephadex peak 1).

translation assay. As shown in Fig. 2, ricin A_1 and ricin A_2 showed identical enzymatic activity indicating that the different glycosylation pattern has no influence on in vitro toxicity. These results are in agreement with earlier experiments where it was shown that chemical destruction of the oligosaccharide on the ricin A chain did not alter its enzymatic properties [14,15].

The mannose receptor contains multiple ligand binding sites, and its binding affinity depends on the number of exposed D-mannose units present on a ligand [22,23]. It seemed likely then, that A_1 would bind less avidly to cells than A_2. To test this hypothesis we characterized the uptake of both ligands by the macrophage cell line J774-E clone, which expresses high levels of mannose-receptor activity [17]. Fig. 3 shows the concentration dependence of the ^{125}I-ricin A_1 and A_2 chain uptake. The

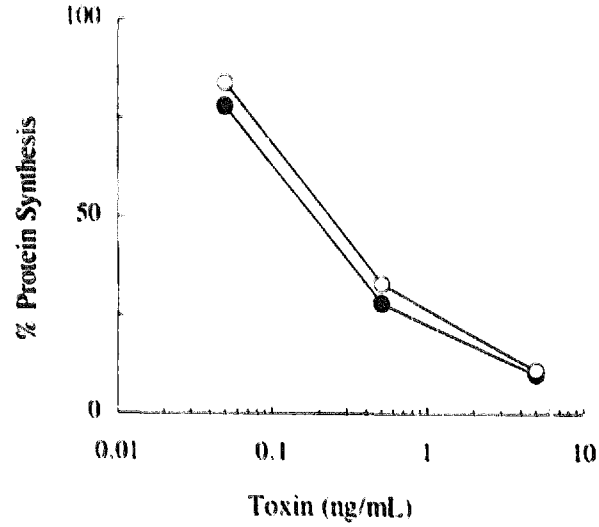

Fig. 2. Activity of ricin A_1 and A_2 in a cell-free protein translation assay. Different concentrations of ricin A_1 (●) and A_2 (○) chains or H_2O as control were incubated for 1 h at 30 °C with rabbit reticulocyte lysate, supplemented with an aminoacid mixture minus leucine, RNasin, Brome Mosaic Virus mRNA and ^{3}H-leucine. TCA precipitable counts were measured with a liquid scintillation counter.

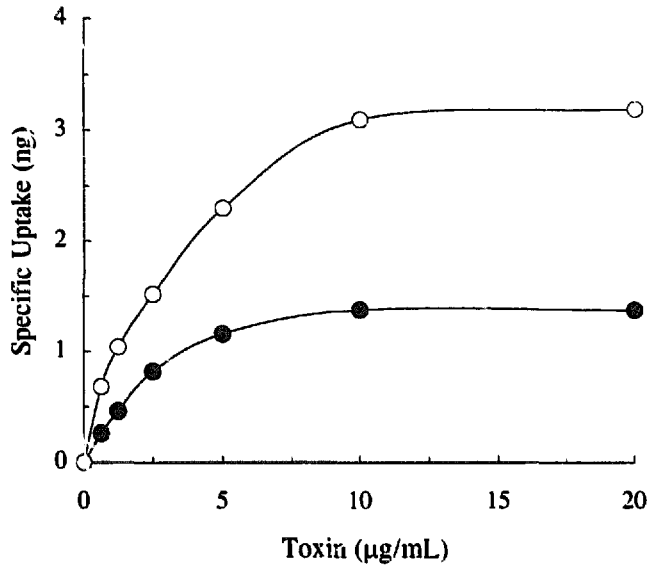

Fig. 3. Concentration dependence of ricin A_1 and A_2 uptake. J774-E clone cells were incubated in a medium containing varying amounts of ^{125}I-A_1 (●) and A_2 (○) at 37 °C for 10 min to permit toxin internalization. Cells were then acid washed to remove non-internalized toxin from the plasma membrane, lysed with 0.1 M NaOH and counted to measure the amount of ligand still cell-associated. Non-specific uptake, estimated by adding yeast mannan to the assay, was less than 20% of total uptake.

non-specific uptake was measured in the presence of excess yeast mannan, a competitive ligand for the mannose receptor. For both the A_1 and A_2 chains the amount of ligand internalized reaches apparent saturation with increasing ligand concentration. The total amount of A_2 internalized over a period of 10 min at 37 °C was, depending on the experiment, approximately two to three times the amount of A_1, confirming a higher affinity of A_2 for the mannose receptor. A double reciprocal plot of the data yielded a K_{uptake} of 1×10^{-7} M for A_1 and 8.6×10^{-8} M for A_2. The K_{uptake} is defined as the concentration of ligand required to produce a half maximal uptake.

We have previously shown that endosomal proteolysis, which occurs very rapidly after endocytosis, modulates ricin A chain cytotoxicity [11]. We suggested that selective cleavage of the toxin may be required to remove high mannose oligosaccharides from the protein molecule, thus facilitating translocation across membranes. In this view the additional oligosaccharide present on A_2 could represent a steric hindrance to toxin translocation if not removed by proteases, resulting in a decreased cytotoxicity of A_2 compared to A_1. To estimate if the carbohydrate chains had an effect on the rate of internalization and degradation of the toxins, the cells were incubated with ^{125}I-A_1 or A_2 and at various times both the cell-associated ligand and the trichloroacetic acid soluble ligand in the media were measured. For both A_1 and A_2 chains the amount of cell-associated ligand shows apparent saturation at 10 min (Fig. 4A) as proteolytic fragments are generated and released into the medium as TCA soluble products (Fig. 4B). The total uptake, determined by combining the cell-associated ligand with the digested ligand, was linear for up to 30 min. The degraded ligand for both toxins was calculated to be 70% of the total toxin taken up by the cells. These experiments show

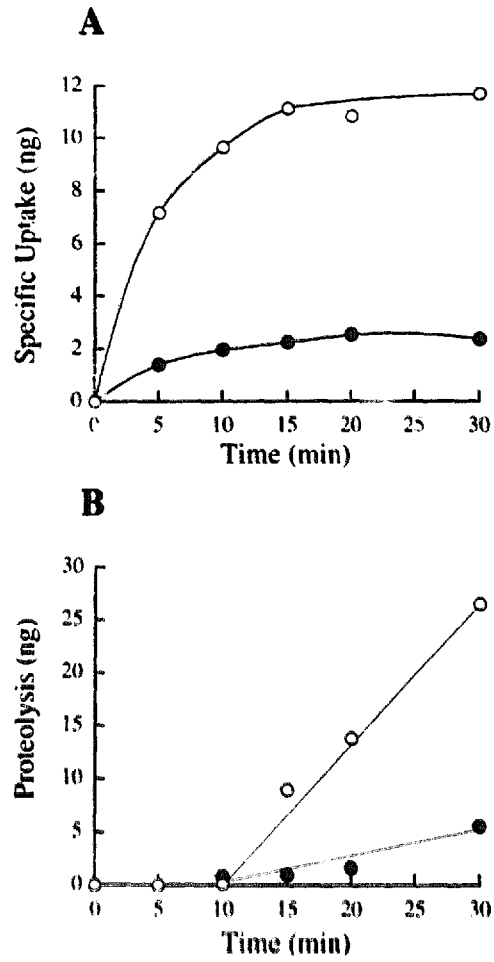

Fig. 4. Time dependence of ricin A_1 and A_2 uptake. J774-E clone cells were incubated for various times in a medium containing 5 μg/ml radiolabeled A_1 (●) or A_2 (○). A, cells were acid washed to remove residual toxin from the plasma membrane, lysed and counted to measure cell-associated radioactivity. B, to determine the amount of ligand taken up and degraded, the incubation media were collected at various times and TCA precipitated. Proteolytic fragments of the ligands are detected as TCA soluble radiolabel. Non-specific uptake and degradation were determined by adding yeast mannan to the assay.

that endosomal processing occurs at the same rate regardless of the presence of one or two sugar chains.

It is possible, on the other hand, that the high mannose chains are necessary for targeting the toxin to the intracellular compartment from which translocation into the cytoplasm may occur. Our uptake studies have shown that macrophages take up more A_2 than A_1 (Figs. 3 and 4). The cellular toxicity of the ricin A chain would then be expected to be mainly due to the more glycosylated form of the toxin. Studies performed in mice with immunotoxins prepared with A_1 and A_2 and monoclonal antibodies have shown that immunotoxins derived from A_1 accumulated less in the liver than those derived from A_2 and were cleared more slowly from the blood [24,25] confirming the role of the sugars in endocytosis. To see if the high mannose chains influenced the

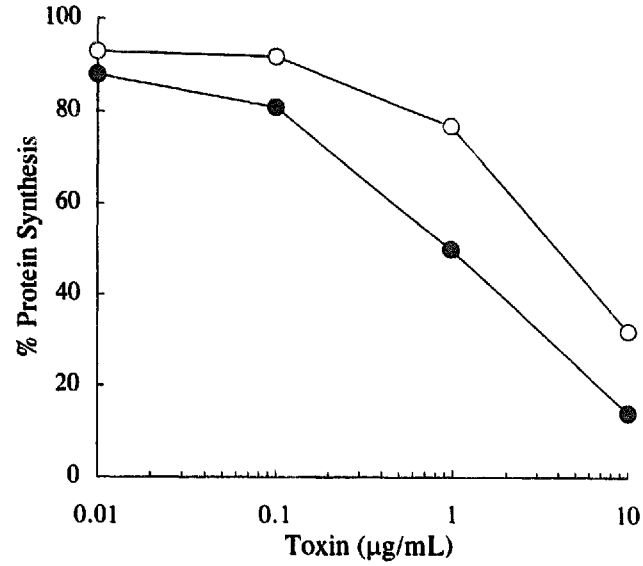

Fig. 5. Cytotoxicity of A_1 and A_2 on mouse macrophages. J774-E clone cells (mannose-receptor positive) were incubated with varying concentrations of A_1 (●) or A_2 (○) for 18 h at 37 °C. The ability of cells to synthesize proteins was then measured by adding a medium containing ^{3}H-leucine for 1 h. Cells were then washed and the TCA precipitable proteins collected and assayed for radioactivity. The results are expressed as percentage of the radiolabelled proteins synthesized in control cells in absence of toxin.

intracellular targeting and/or translocation of the toxin subsequent to initial binding we measured the cytotoxic activity of the two forms of the ricin A chain. As shown in Fig. 5, each toxin caused a dose dependent inhibition of ^{3}H-leucine incorporation into proteins upon continuous incubation with the cells for 18 h. Yeast mannan almost completely inhibited the toxic effect of both toxins (data not shown) confirming the role of mannose-receptor-mediated endocytosis in ricin A chains toxicity. Cytotoxicity of A_2 is four times higher than that of the A_1 chain. The concentration at which protein synthesis was inhibited by 50% (IC_{50}) is 4 μg/mL for A_1 and 1 μg/mL for A_2. This result is in agreement with the two to three times higher uptake of A_2 compared to A_1 and suggests that the difference in amount taken up is responsible for the higher toxicity of A_2 relative to A_1. The data presented here show that both the ricin A_1 and A_2 chains bind to the mannose receptor on the cell surface of macrophages. The binding and uptake of both ligands is dependent on the number of high mannose oligosaccharides present on the protein molecule. Once accumulated inside the cell, endosomal proteolysis occurs for both the A_1 and A_2 chains at the same rate. The carbohydrates on the protein molecules do not seem to further facilitate intracellular transport and/or translocation of the ricin A chain into the cytoplasm.

Acknowledgements

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