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Lectin-deficient ricin toxin intoxicates cells bearing the D-mannose receptor

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Abstract

Ricin toxin with genetic or chemical modification of lectin sites has been previously reported to show markedly reduced cytotoxicity to cells following uptake by several receptors including the mannose receptor. Investigators have hypothesized that an intracellular galactoside-binding function was required for optimal intracellular targeting of ricin for these receptors. We have prepared insect-derived mutant ricin toxin B chain (RTB) with modifications of three lectin site domains (1α, 1β, and 2γ) yielding a 1000-fold reduced galactoside avidity. After reassociation with plant RTA, the recombinant heterodimer and plant ricin were tested for cytotoxicity on mammalian cells expressing (mouse peritoneal macrophages, J774E cells, and MMR61 cells) or not expressing (KB cells) the D-mannose receptor. Receptor expression was confirmed by immunofluorescence microscopy. Lactose was included in the media to block cell-surface galactoside binding, and mannan was added as a control in each experiment to confirm mannose receptor-specific targeting. Plant ricin A chain (RTA) and *E. coli*-derived RTA were also tested for cytotoxicity on J774E and KB cells. Both wild-type and lectin-deficient ricin displayed mannose-receptor mediated cell cytotoxicity. This is the first report of a genetically modified ricin showing that RTB intracellular galactose binding activity is not required for ricin cytotoxicity. Sensitivity of mannose-receptor bearing cells, but not control cells, to mannosylated RTA, but not unglycosylated RTA, confirmed these observations. These results imply fusion toxins employing ricin can be prepared with maximal reductions in normal tissue binding. © 1997 Elsevier Science Ltd.

Keywords: Ricin; Mannose receptor

Abbreviations: RTA, ricin A chain; RTB, ricin B chain; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; K_a, association constant; BSA, bovine serum albumin

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1. Introduction

Ricin, the heterodimer 64 kDa glycoprotein synthesized in castor bean seeds, is one of the most potent toxins known to man. The toxin consists of a 33 kDa galactoside-specific lectin B chain (RTB) disulfide linked to a 31 kDa rRNA *N*-glycosidase A chain (RTA) [1]. RTB initiates mammalian cell binding to complex cell-surface glycoproteins and triggers receptor-mediated endocytosis [2]. After entering endosomes, ricin traffics to the Golgi [3]. Subsequently, the protein is routed to a translocation competent compartment where reduction and RTA translocation to the cytosol occurs [4]. Cytosolic RTA catalytically inactivates protein synthesis by depurinating a critical adenine base from the elongation factor binding site on an rRNA stem-loop structure [5]. A single molecule of RTA in the cytosol can lead to cell apoptosis [6].

To take advantage of the extreme potency and unique mechanism of action, ricin moieties have been covalently coupled to tumor-selective monoclonal antibodies and administered systemically to patients with refractory malignancies [7]. These immunoconjugates have been prepared with either RTA alone or affinity cross-linked ricin. Monoclonal antibody-RTA conjugates reactive with a number of cell surface antigens have been less potent than plant ricin, conjugates prepared with intact toxin, or RTA conjugates mixed with purified RTB or RTB immunoconjugates [8–10]. This discrepancy has been ascribed to an intoxication enhancement function of RTB.

The intoxication enhancement function of RTB has been suggested to be an intracellular galactose-binding activity that facilitates ricin transfer to a translocation-competent compartment [11]. Four studies support this hypothesis. In one study, ricin was linked to monophosphopentamannose and *O*-acetylated with *N*-acetylimidazole [12]. The modified ricin conjugate showed reduced mannose 6-phosphate-directed cell cytotoxicity implicating an intracellular galactoside lectin function in cell intoxication. In a second report, ricin was cross-linked with two or three dichlorotriazine-activated triantennary glycopeptide affinity ligands [13]. The doubly and triply blocked ricin were coupled to an anti-CD19 monoclonal antibody and tested on receptor-positive cells for toxicity [14]. The cells were 100-fold less sensitive to triply blocked ricin conjugates, again suggesting the residual lectin activity of the doubly blocked conjugates was necessary for a post-binding step in cell intoxication. In an extension of their work, the same investigators added swainsonine to the cells and demonstrated loss of sensitivity to even the doubly blocked ricin conjugate [15]. Since swainsonine is an inhibitor of α-mannosidase II, and its presence prevents formation of intracellular complex galactosyl-terminated N-glycans, the role of intracellular galactosides in RTB enhancement was further implicated. Finally, Newton and colleagues expressed lectin-deficient RTB in *Xenopus* oocytes [16]. The mannosylated products were reassociated with plant RTA and tested for cytotoxicity on mannose receptor bearing mouse peritoneal macrophages and rat bone marrow macrophages. In each case, they reported that the lectin-deficient ricins failed to intoxicate macrophages. They also hypothesized a critical role for intracellular galactoside binding in mannose receptor-directed ricin toxicity.

There are, however, several problems with the hypothesis of an RTB intracellular lectin activity. First, the RTB enhancement function has not been observed for all receptors. RTA immunoconjugates directed to the IL2 receptor, CD22 antigen and CD7 antigen have shown potencies similar to ricin on receptor positive cells [17–19]. Second, the modified ricins used in the experiments mentioned in the previous paragraph (*O*-acetylated ricin, affinity cross-linked ricin and *Xenopus* oocyte ricins) have not been well characterized. Chemical modification of proteins can produce modifications in non-lectin site residues and may alter protein folding. The exact sites of modification of ricin in each case were incompletely described. Further, the *Xenopus* recombinant product was produced in nanogram quantities and was not subjected to any biochemical or immunologic analysis or purification. Significant modification in RTB may have reduced cell intoxication independently of lectin function. Even the swainsonine experiment should be viewed cautiously as alterations in intracellular glycosylation can lead to gross changes in intracellular organelle structure and vesicle transport [20].

We chose to target the mannose receptor with a well characterized recombinant molecule. The triple-site RTB mutant (W37S/Y78H/Y248H) was expressed and purified from Sf9 insect cells [21]. The protein contains mannose-rich oligosaccharides and a three-log reduction in galactoside avidity. We have reassociated the mutant RTB with plant RTA and quantitated heterodimer. Plant RTA (possessing mannose-rich oligosaccharides) and *E. coli*-derived RTA (unglycosylated) were also prepared. In this report, we describe the sensitivity to these molecules of cells possessing or not possessing the D-mannose receptor.

Addition of lactose to the culture medium blocks toxin entry via surface galactoside binding. Further addition of mannan to the medium inhibits intoxication by the D-mannose receptor. The ratio of IC_{50} (concentration of toxin reducing protein synthesis by 50%) in the presence of mannose plus lactose versus lactose alone serves as a measure of mannose-targeted toxicity. We describe, in this study, the activity of wild-type plant ricin, the lectin-deficient insect-derived RTB–RTA hybrid, mannosylated and unglycosylated RTA.

2. Materials and methods

Preparation of toxins.—Plant ricin and RTA were obtained from Inland Laboratories (Austin, TX, USA). Insect-derived triple-site RTB mutant (W37S/Y78H/Y248H) was prepared from recombinant baculovirus infected Sf9 cell supernatants, reassociated with plant RTA and heterodimer concentration determined by sandwich ELISA as previously described [21]. Recombinant RTA was prepared from JM105 *E. coli* transformed with pGEX2T-RTA plasmid as previously described [33].

Glycosylation of mutant RTB.—Glycosylation of triple-site mutant RTB was determined by tunicamycin treatment and metabolic labeling of infected Sf9 cells following the previously reported procedure from our laboratory [22].

Macrophage cells.—Mouse peritoneal macrophages were prepared using 12-week-old female Balb/c mice house in an IACUC approved facility according to MUSC protocol #1432 (approved 3/7/96) which follows the procedure of H. Ziegler [34]. Cells from peritoneal lavage (2×10^6/mL) were directly seeded into 96-well plates (2×10^4/well in 100 μL of medium) and 35 mm petri dishes (2×10^5/dish in 2 mL of medium). Medium was RPMI1640 with 10% fetal calf serum and 0.01 M HEPES. After 2 h at 37 °C/5% CO_2, wells and dishes were washed three times with warm Hank's balanced salt solution buffered with HEPES prior to assay.

J774E mouse macrophage cell line [23] was cultured in α-MEM with 10% fetal calf serum, 60 μM thioguanine and transferred from flasks to wells and dishes by exposure to trypsin-EDTA (Gibco). Cells were plated at 2×10^4 cells/well in 96 well plates and 2×10^5 cells/35 mm dish, and incubated a further 24 h at 37 °C/5% CO_2 prior to assay.

MMR61 rat fibroblasts transfected with mouse mannose receptor cDNA were grown in Dulbecco's MEM containing 10% fetal calf serum and 400 ug/mL G418 [24]. Cells were split and transferred to wells and dishes again by trypsinization and incubated a further 24 h prior to assay.

KB human epidermoid carcinoma cells obtained from the American Type Culture Collectin (Rockville, MD) were grown in Dulbecco's MEM with 10% fetal calf serum [25]. Cells were removed from flasks by trypsin treatment and seeded for experiments identically to the other cell types.

Cytotoxicity assays.—Washed peritoneal macrophages and KB cells in 96-well plates were resuspended in 100 μL/well of leucine-free RPMI1640 with 10% dialyzed fetal calf serum and different concentrations of toxins. J774E and MMR61 cells were treated identically except leucine-free DMEM medium was used. Twelve different concentrations of toxin were used in each experiment. Sets of wells in each experiment contained 60 mM lactose and/or 1 mg/mL yeast mannan. After 20 h, 50 μL of leucine-free media containing 1 μCi ^{3}H-leucine (Amersham, 300 mCi/mmol) with or without added lactose and/or mannan was added to the wells. The cells were again incubated 4 h at 37 °C/5% CO_2 and then harvested with a Skatron cell harvestor onto glass fiber filter mats. Filters were dried and counted in Econofluor liquid scintillation fluid in a LKB liquid scintillation counter. The IC_{50} was determined for each toxin/cell type/medium condition as the toxin concentration that reduced protein synthesis to 50% of control. Each assay was performed in quadruplicate. The mannose receptor-directed toxicity was quantitated by the ratio of the toxin IC_{50} in the presence of lactose plus mannan to the IC_{50} in the presence of lactose alone. KB cells were assayed identically.

Immunofluorescence assay.—Mouse peritoneal macrophages, J774E, MMR61, and KB cells were attached for 24 h at 37 °C/5% CO_2 to petri dishes, fixed in 3.7% formaldehyde in PBS (15 min), washed with 2 mg/mL BSA in PBS and 0.1% saponin, and incubated in PBS plus BSA with rabbit anti-mouse mannose receptor antibody [23] for 30 min at 4 °C. The cells were rewashed with PBS and reacted with goat anti-(rabbit Ig) conjugated to rhodamine (Jackson Immunoresearch, West Grove, PA) at 25 μg/mL with 0.1% saponin for 30 min at 23 °C. After a final wash with PBS, the cells were fixed in 3.7% formaldehyde in PBS, mounted under a #1 coverslip in 90:10 glycerol–PBS and examined using a Zeiss

Axioplan epifluorescence microscope (63 ×, N.A.1.4 planapochromat objective). Fluorescence images were recorded using Kodak Tri-X film, and negatives were digitized and edited using Abobe Photoshop software and a PowerMac 8500/120 computer. Relative intensities for the brightness of mannose receptor reactions in the different cell types were, respectively, (−) for KB, (+ + +) for J774E cells, and (+) for MMR61 and mouse peritoneal macrophages [scale (−)–(+ + + +)].

3. Results

Triple-site mutant RTB glycosylation.—To determine if the triple-site mutant RTB had similar mannose-rich oligosaccharides to wild-type plant RTB, W37S/Y78H/Y248H RTB was immunoprecipitated from Sf9 infected cell extracts after metabolic labeling with ^{35}S-methionine in the presence and absence of tunicamycin. Autoradiographs of reducing SDS-PAGE showed a molecular weight of 33 kDa in the absence of tunicamycin and 29 kDa in the presence of tunicamycin corresponding to 4 kDa of attached sugars per molecule (Fig. 1).

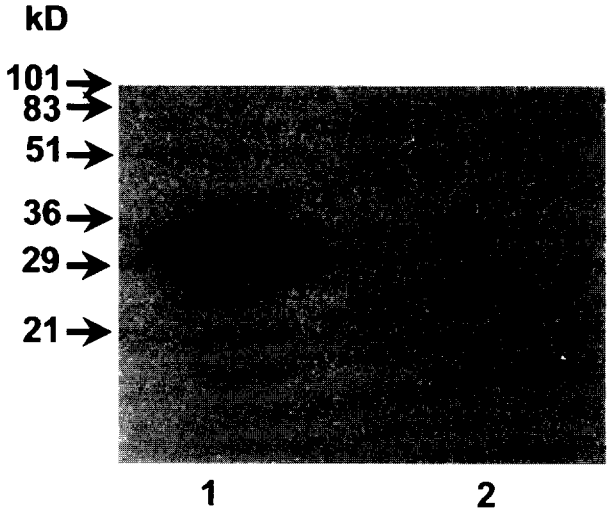

Fig. 1. Immunoprecipitation of lectin-deficient rRTB in the presence and absence of tunicamycin. Autoradiogram of 15% reducing SDS/PAGE of 35S-methionine-labeled cell extracts immunoprecipitated with rabbit anti-ricin (Sigma) and Pansorbin (Calbiochem). Arrows, low-molecular-weight BioRad protein standards: lane 1, non-tunicamycin-treated infected Sf9 cell extract immunoprecipitate; lane 2, tunicamycin (10 μg/mL) treated recombinant baculovirus-infected Sf9 cell-extract immunoprecipitate.

Triple-site mutant RTB–RTA heterodimer.—Triple-site mutant RTB was assayed for trypsin sensitivity, CD spectrum, and immunologic reactivity with a panel of antibodies to RTB [21]. In each case, the triple-site mutant showed similar properties to wild-type RTB suggesting similar protein folding. Two preparations of W37S/Y78H/Y248H RTB–RTA each at 40 μg/mL concentration were assayed for sugar binding on immobilized asialofetuin and live cells. Each showed 0.1% asialofetuin binding avidity relative to plant ricin. The triple-site mutant ricin failed to bind cells displaying galactose residues [21]. Together, these data indicate that triple-site mutant RTB–RTA represents a lectin-deficient ricin that may be compared with wild-type ricin for evaluation of cell intoxication.

Mannose receptor - targeted ricin toxicity.—We then undertook experiments to show that ricin can only enter cells by either galactoside binding or mannose receptor binding. Ricin-related proteins in the presence of excess lactose bind and intoxicate mannose receptor-deficient cells only weakly. The IC_{50} of ricin on KB cells in the presence of lactose was 3×10^{-9} M (Table 1). The IC_{50} of the triple-site

Table 1
Cytotoxicity of ricin proteins on cell lines [a]

Protein	Condition	IC_{50} (M)			
		KB	J774E	MMR61	MPM
Ricin	–	9×10^{-12}	1×10^{-11}	2×10^{-11}	1×10^{-12}
	Lactose	3×10^{-9}	1×10^{-10}	8×10^{-10}	8×10^{-11}
	Mannan	9×10^{-12}	3×10^{-11}	2×10^{-11}	3×10^{-12}
	Lact/Mannan	3×10^{-9}	3×10^{-9}	3×10^{-9}	2×10^{-10}
Triple mutant	–	1×10^{-8}	7×10^{-10}	3×10^{-9}	5×10^{-10}
	Lactose	3×10^{-8}	3×10^{-10}	7×10^{-9}	1×10^{-9}
	Mannan	1×10^{-8}	1×10^{-8}	1×10^{-8}	1×10^{-9}
	Lact/Mannan	3×10^{-8}	2×10^{-8}	3×10^{-8}	5×10^{-9}

[a] MPM = mouse peritoneal macrophages; Lact/Mannan = addition of both lactose and mannan to culture media. Experiments carried out in quadruplicate as described in text. Mean values shown.

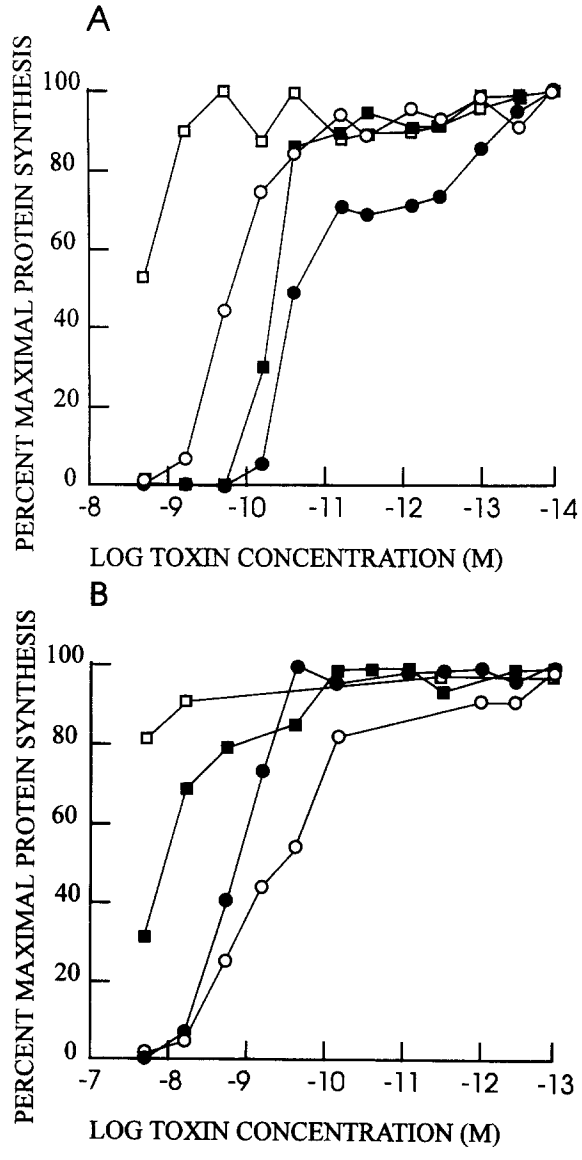

Fig. 2. J774E cell cytotoxicity. Assay as described in text: (A) plant wild-type ricin toxicity; (B) W37S/Y78H/Y248H RTB-plant RTA toxicity. In both, ● medium without additives; ○ medium with 60 mM lactose; ■ medium with 1 mg/mL mannan; □ medium with both lactose and mannan.

mutant RTB–RTA with lactose was 3×10^{-8} M. This experiment shows that in the absence of mannose receptor, ricin enters cells after binding cell-surface galactosides.

In contrast, cells with mannose receptor are sensitive to ricin proteins even in the presence of excess lactose due to mannose-terminated oligosaccharides on plant and insect-derived ricin (Fig. 2 and Table 1). The IC_{50}'s of ricin with lactose on mouse peritoneal macrophages, J774E cells and MMR61 rat fibroblasts transfected with mouse mannose receptor were 8×10^{-11} M, 1×10^{-10} M, and 8×10^{-10} M, respectively. Similarly, the triple-site mutant RTB–RTA with lactose showed IC_{50}'s on mouse peritoneal macrophages, J774E cells and MMR61 cells of 1×10^{-9} M, 3×10^{-10} M, and 7×10^{-9} M, respectively. Thus, the receptor-positive cells were 3–38 fold more sensitive to ricin and 4–100 fold more sensitive to triple-site mutant RTB–RTA heterodimer than receptor-negative cells. Together, the above experiments show mannose receptor targeted toxicity for both ricin proteins.

Lower efficacy of cell intoxication by mannose receptor pathway.—Our next analysis documents the greater cytotoxic potency of the galactoside pathway for ricin. Ricin binds cell-surface galactosides in the absence of lactose and intoxicates cells with an IC_{50} of 1×10^{-12} M, 1×10^{-11} M and 2×10^{-11} M on mouse peritoneal macrophages, J774E cells and MMR61 cells, respectively. Thus, ricin was 1–80 fold more potent in the absence of lactose. As expected, the lectin-deficient mutant ricin had minimal residual galactoside mediated cytotoxicity with IC_{50}'s of 5×10^{-10} M, 7×10^{-10} M, and 3×10^{-9} M on mouse peritoneal macrophages, J774 E cells and MMR61 cells, respectively. This was a 0.4–2 fold relative potency to mutant heterodimer in the presence of lactose. Little intoxication occurs by the galactoside pathway for the lectin-deficient ricin as expected.

Competition of mannose receptor-mediated toxin uptake by mannan.—The D-mannose receptor negative KB cells showed no inhibition of ricin cytotoxicity by mannan versus control (IC_{50}'s of 9×10^{-12} M for both) or mannan plus lactose versus lactose alone (IC_{50}'s of 3×10^{-9} M for both). Similarly, KB cells showed no effects of mannan on lectin-deficient ricin toxicity in the absence of lactose (IC_{50}'s of 1×10^{-8} M for both) or presence of lactose (IC_{50}'s of 3×10^{-8} M for both).

In contrast, ricin toxicity to all three mannose receptor positive cell lines in the presence of lactose was inhibited by mannan. Mannan increased the IC_{50} to 2×10^{-10} M, 3×10^{-9} M, and 3×10^{-9} M for mouse peritoneal macrophages, J774 E cells, and MMR61 cells, respectively. This represented a 2.5–30 fold reduction in toxicity. Lectin-deficient ricin behaved like ricin with significant mannan inhibition of toxicity on these cell lines. Mannan increased the IC_{50} to 5×10^{-9} M, 2×10^{-8} M, and 3×10^{-8} M for mouse peritoneal macrophages, J774E cells and MMR61 cells, respectively. This yielded a 4.2–66 fold reduction in toxicity. In summary, mannose re-

ceptor directed ricin toxicity was inhibitable by mannan.

Sensitivity of cells to plant RTA and E. coli-derived RTA.—KB cells were insensitive to both plant and *E.coli* RTA (IC_{50}'s were $> 3 \times 10^{-7}$ M). In contrast, J774E cells were sensitive to plant RTA ($IC_{50} = 2 \times 10^{-9}$ M) but not *E.coli* RTA ($IC_{50} > 3 \times 10^{-7}$ M). Further, the plant RTA-J774E cell cytotoxicity was blocked with mannan ($IC_{50} > 3 \times 10^{-7}$ M). Thus, for the first time recombinant RTA has been tested simultaneously with mannosylated RTA to confirm mannose receptor-directed toxicity. The RTA toxicity was 15-fold less potent than lectin-deficient ricin under identical conditions. These experiments confirm the ability to kill cells without cell-surface or intracellular galactoside binding function, but also suggest RTB may have additional intoxication enhancement properties.

Immunologic detection of D-*mannose receptor.*—Finally, we wanted to correlate mannose receptor targeting efficiency of cells with the cell density of receptor. Rabbit antibody to mouse mannose receptor reacted strongly with both surface and intracellular sites in J774E and MMR61 cells lines and many of the adherent cells from thioglycollate treated mouse peritoneal fluid cells (Fig. 3). No binding was observed with the KB human epidermoid carcinoma cells. Localization was seen not only in an intracellular granular organelle pattern, but also in association with the ruffled border of cells, consistent with a cell-surface distribution. The relative density of mannose receptor on different cell lines based on immunofluorescence intensity from (−) to (+ + + +) was KB (−), mouse peritoneal macrophages (+), MMR61 (+ +), and J774E (+ + +).

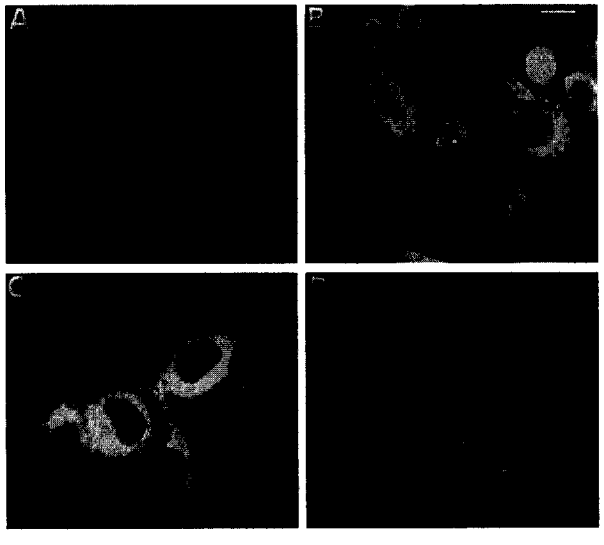

Fig. 3. Immunofluorescence assay of mouse macrophage receptor on mammalian cells. Experiment performed as described in text. Fixation and processing involved formaldehyde followed by saponin so that total cell receptor was observed (inside the cell and cell surface). (A) KB; (B) J774E; (C) MMR61; (D) mouse peritoneal macrophages. Arrowheads = ruffles (indicative of surface receptor distribution). Fluorescence represents binding of rabbit anti-mannose receptor antibody. Mags = ×400; Bar = 16 μm.

4. Discussion

The mechanism by which internalized ricin toxin is transported to a translocation competent intracellular organelle is unknown. This study was undertaken to evaluate the role of RTB galactoside binding in this critical intoxication step. Plant ricin and insect-derived RTB–RTA heterodimers have mannose-rich N-glycans and thus may be bound and internalized by cells possessing the D-mannose receptor in the presence of excess lactose to block binding to cell-surface galactosides [35–37]. Further, mannose-containing plant RTA but not recombinant unglycosylated RTA showed mannose receptor-specific cytotoxicity similar to previous reports [35,36]. Surprisingly, the lectin-deficient ricin retained cytotoxic potency 15-fold greater than RTA alone, implying either that RTB lectin activity is not required for intracellular transport and enhancement of cell intoxication or that the small amount of residual lectin function (0.1% of normal) is sufficient for critical intracellular routing. If lectin activity is involved, it does not appear to produce measurable surface binding as no lactose effect was observed. The significance of the finding is twofold. We must identify an alternate molecular mechanism for retrograde transport of ricin from the Golgi to the vesicle from which RTA reaches the cytosol. Secondly, ricin fusion toxins and immunoconjugates can be prepared with toxin with markedly reduced to absent normal tissue binding without loss of efficacy.

The lower potency of mannose receptor-mediated toxicity for both lactose blocked ricin and lectin-deficient ricin may be due to reduced surface receptor content or lower avidity of cell-surface binding for the D-mannose receptors and may not reflect altered intracellular processing. The number of galactosyl-terminated cell-surface glycoprotein receptors for ricin has been reported to be about 10^7/mammalian cell [26] versus 10^5/cell for D-mannose receptors [23]. Avidities for each were similar with K_a's of 10^9 M^{-1} [23,26].

The residual cell toxicity of ricin-related molecules in the presence of mannose and lactose was 10^{-9}–10^{-8} M. This toxicity is ten-fold higher than RTA alone and probably reflects incomplete competition at 37 °C by the soluble lactose and yeast mannan.

The greater sensitivity of J774E cells relative to MMR61 or mouse peritoneal macrophages may be due to higher cell-surface mannose receptor density on J774E cells or different intracellular metabolism. The immunofluorescence assay suggests higher receptor content is the cause for the J774E sensitivity difference.

The observation of efficient cell killing in the absence of RTB lectin function for mannose receptor has also been documented for the IL2 receptor using an insect-derived lectin-deficient IL2 ricin fusion molecule [27]. RTB enhancement for other receptors may be due to inefficient internalization by the ligand–receptor complex or misrouting intracellularly away from a translocation competent compartment [28,29]. Further studies of lectin-deficient ricin fusion molecules with alternate receptors will be needed to clarify the functional activity of RTB in other ricin conjugates.

Nevertheless, the cytotoxic activity of mannose receptor-directed lectin-deficient ricin can be explained by any of several hypotheses. Internalized mannose receptor-bound ricin may traffic retrograde to a compartment from which RTA can cross to the cytosol without requiring any additional signals. Cells with the mannose receptor may avoid the need for the galactose-binding function because they use the mannose–mannose receptor interaction, instead, in the Golgi. The mannose-terminated protein transport may be via a default pathway or may be facilitated by membrane address proteins such as ERGIC-53, a mannose-binding ER protein [30]. Alternatively, the mannose receptor may only recycle to a superficial endosomal compartment and, thus, not be a very good mechanism to deliver RTA/RTB to a retrograde pathway in deeper parts of the Golgi. Second, RTB has a putative membrane lipase activity, which may facilitate RTA entry to the cytosol independent of lectin function [31]. Finally, the small amount of residual sugar binding (K_a of 10^5 to 10^6 M^{-1}) may be sufficient to influence intracellular trafficking without effects on cell-surface binding.

Importantly, the potency of several lectin-deficient ricins directed to independent cell-surface receptors now suggests that ricin fusion toxins with large therapeutic windows can be constructed and may be ultimately tested systemically in patients with aberrant target cell populations.

Exploration of the intracellular steps in ricin intoxication remains an important avenue for defining molecular signals for routing of soluble intracellular polypeptides. This study provides a model system for examining other ligand-receptors, and combined with the new molecular tags such as green fluorescent protein [32], may permit better understanding of important chemical reactions that mediate vectorial transport inside cells.

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