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Comparison of properties of mistletoe lectin I A-chain and ricin B-Chain conjugate with native toxins

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Chimeric toxic protein was prepared from the mistletoe lectin I A-chain and ricin B-chain by using the disulfide exchange reaction. Ricin and chimeric protein were indistinguishable in binding to immobilized asialofetuin in ELISA. Chimeric protein was more toxic to Jurkat cells than native mistletoe lectin I, but not so effective as native ricin. In the presence of NH_4Cl, which enhances the toxicity of some toxins and immunotoxins, but does not influence ricin toxicity, both ricin and chimeric toxin had equal cytotoxic activity. The possibility is discussed that the ricin B-chain protects the ricin A-chain (RTA) from degradation during delivering RTA from the cell surface to the place where RTA is translocated into the cytosol.

Ricin; Mistletoe lectin I; Chimeric toxin; Mechanism of action

1. INTRODUCTION

During the last years a lot of effort has been made to prepare hybrid cytotoxic proteins consisting of the enzymatically active A-chains of different toxins like ricin, and antibodies or other polypeptides which selectively bind to cell surface molecules [1,2]. The aim of these investigations is to produce directed cytotoxic agents which are able to selectively kill defined groups of cells, e.g. malignant cells. Although the resulting conjugates have a very high specificity of binding to target cells when compared with natural toxins, most of these conjugates are much less toxic than native toxins. This is most likely due to inefficient transfer of the A-chains of hybrids from the cell surface into the cytosol. The rational design of directed cytotoxins requires a better understanding of the entry mechanism of toxin A-chains and will reveal factors that interfere in this process.

Recently it was shown that immunotoxins (IT) constructed by utilizing the MLI A-chain had the same cytotoxic activity as the native MLI and were more effective when compared to ITs constructed by utilizing the ricin A-chain [3,4]. In attempts to elucidate the role of the binding moiety in the entry of the enzymatically active MLA and RTA into the cell, we have prepared a chimeric protein (MLA/RTB) from the MLI A-chain and ricin B-chain and compared the cytotoxic properties of this chimeric protein with those of the native parent toxins.

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Abbreviations: MLI, mistletoe lectin I; MLA, mistletoe lectin I A-chain; RTA, ricin A-chain; RTB, ricin B-chain; IT, immunotoxin; DTT, dithiotreitol; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

2. MATERIALS AND METHODS

Ricin and its subunits were isolated as described earlier [5]. Mistletoe lectin I was isolated from *Viscum album* and MLA was purified according to [6].

2.1. *Preparation of chimeric toxin*

Chimeric toxic protein was prepared using the disulfide exchange reaction. Isolated RTB was incubated with 5 mM of Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid)) for 30 min at 20°C. Excess reagent was removed by gel filtration. Before conjugation MLA was incubated with 50 mM DTT for 30 min at 20°C followed by removing excess DTT. Collected RTB and MLA were immediately mixed, and incubated overnight at 20°C. The resulting chimeric toxin was purified from unreacted proteins by gel filtration on the prepacked column HR 10 × 30 with Superdex G-75 using an FPLC system (Pharmacia, Sweden) and analyzed by SDS-PAGE. Protein bands were visualized by Coomasie brilliant blue R-250 staining or the proteins were electrophoretically transferred to nitrocellulose membrane. After transfer, the nitrocellulose membranes were blocked with 1% BSA, 0.1% Tween-20, 20 mM lactose and incubated with a biotinylated monoclonal antibody to MLA (10 μg/ml in PBS with 0.1% BSA and 0.1% Tween-20, 20 mM Lactose). After washing, the blots were incubated with streptavidin conjugated to peroxidase and developed with a substrate solution containing 3,3′-diaminobenzidine. The amount of chimeric protein in the sample was then estimated from densitogram of the Coomassie-stained gel.

Cytotoxicity assays were carried out on the T-lymphoblast Jurkat cell line by measuring the inhibition of [^{3}H]thymidine incorporation as described in [7].

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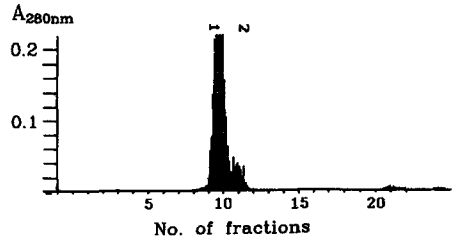

Fig. 1. Purification of chimeric toxin on Superdex G-75 HR 10 × 30. 500 μl of reacted mixture was loaded on the column with flow rate 0.5 ml/min in PBS containing 20 mM D-lactose and 1 ml fractions were collected.

Proteins were radiolabeled using Iodo-Gen (1,3,4,6-tetra-chloro-3a,6a-diphenylglycouril; Sigma, USA) according to [8]. The binding assay of [^{125}I]proteins was prepared as described in [7].

3. RESULTS

3.1. *Preparation of chimeric toxin*

To prepare the chimeric protein MLA/RTB consisting of the ricin B-chain and the MLI A-chain, the isolated ricin B-chain was treated with Ellman's reagent to generate the activated disulfide. Freshly reduced MLI A-chain was mixed with derivatized RTB to form (via thiol disulfide interchange) a disulfide-linked conjugate, like native toxins. The resulting material (MW 60 kDa) was purified from unreacted toxin subunits and analyzed by electrophoresis and blotting. Gel filtration chromatography on Superdex G-75 showed that approximately 90% of the mixed proteins had formed high molecular weight material (Fig. 1). The material after purification only contained chimeric protein consisting of ricin B-chain and MLI A-chain (Fig. 2A and B). Purified chimeric toxin inhibits the binding of biotinylated ricin to asialofetuin to the same extent as native ricin (results not shown).

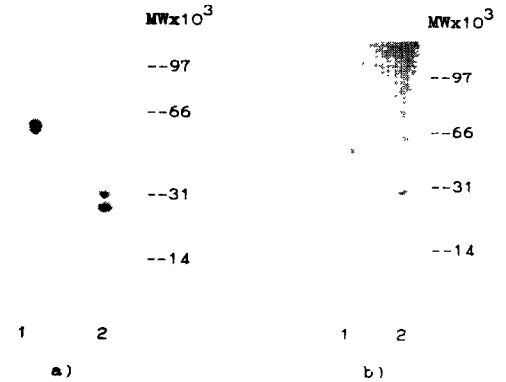

Fig. 2. Electrophoresis and blotting purified chimeric toxin. Fraction 10 designated on Fig. 1 was analyzed by electrophoresis on a 7.5–22% gradient polyacrylamide gel. Lane 1, sample prepared without 2-mercaptoethanol; lane 2, sample prepared with 2-mercaptoethanol. Fraction 10 designated on Fig. 1 after electrophoresis was transferred to a nitrocellulose membrane and treated as described in section 2. Lanes 1 and 2, as in Fig. 2A.

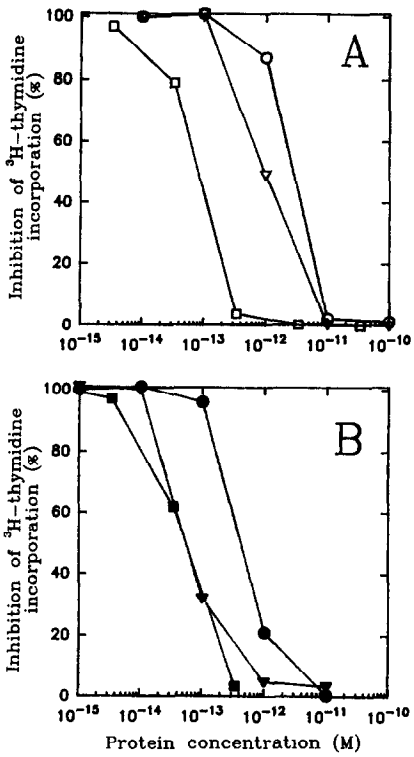

Fig. 3. Cytotoxic effect of native toxins and the chimeric toxin on Jurkat cells. The results are expressed as percentage of [^{3}H]thymidine incorporation of untreated cells (about 100,000 cpm) corrected for background value. (○—○, ●—●), MLI; (△—△, ▲—▲), MLA/RTB; (□—□, ■—■), ricin. Cells were incubated without NH_4Cl; Cells were incubated in the presence of 10 mM NH_4Cl.

3.2. *Toxicity of the chimeric toxin*

The toxicity of the chimeric toxin to Jurkat cells was compared with that of native toxins which had different cytotoxic activity to these cells. The native MLI was about 25 times less toxic than native ricin (Fig. 3A). In order to elucidate the role of toxin binding to cell targets as opposed to cytotoxic activity we quantified the number of binding sites on Jurkat cells for both toxins (Fig. 4). It was found that the maximal number of binding sites on these cells was 5.43×10^6 for MLI and 8.8×10^6 for ricin, respectively. Because MLI and ricin have approximately the same K_a (0.7×10^8 M^{-1} and 0.8×10^8 M^{-1}, respectively), this slight difference in number of binding sites cannot explain the higher activity of ricin when compared to MLI. Chimeric toxic protein, which exhibits the same binding ability as native ricin, was less toxic for Jurkat cells than ricin and only slightly more active than native MLI (Fig. 3A).

Both native toxins differ in their sensitivity to potentiating with NH_4Cl (Fig. 3B). Native MLI was about 10 times more toxic for cells when 10 mM NH_4Cl was included in the incubation mixture than in the absence of NH_4Cl. In contrast, the activity of native ricin could not be potentiated with NH_4Cl and ricin had the same toxicity in the presence of NH_4Cl as in its absence. Chimeric protein MLA/RTB was significantly more ac-

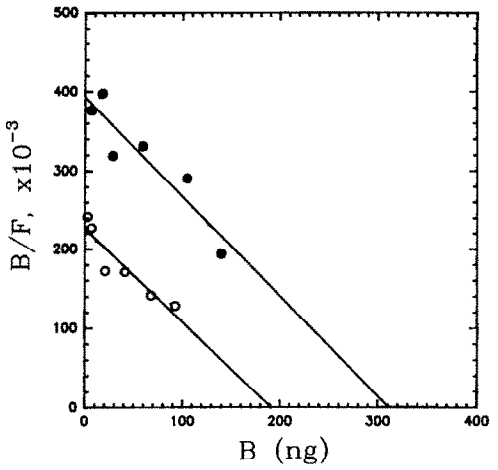

Fig. 4. Binding of ^{125}I-labeled ricin and MLI in Scatchard coordinates. Specific binding was calculated for each point by subtracting the amount of iodinated proteins bound in the presence of 50 mM D-lactose (less that 10% of total counts bound); (●-●), ricin; (○-○), MLI. B, bound protein (ng); F, free protein (ng).

tive in the presence of NH_4Cl and exposed the same cytotoxic activity as native ricin (Fig. 3B).

4. DISCUSSION

The structure and lectin properties of MLI and ricin were described in a number of reviews [1,2]. Both toxins have similar structures and mechanisms of action. They have galactose-binding sites on their B-chains, but both toxins differ in their specificity for galactose-terminated complex oligosaccharides exposed on the cell surface [9]. Therefore they will occupy a different quantity and quality of binding sites on the cell surface, which partly explains the difference in cytotoxic activity of these toxins because their A-subunits have approximately the same enzymatic activity [10].

Enzymatically active A-subunits of both toxins were used for preparing antibody-containing immunotoxins. The immunotoxins containing MLA were more toxic than IT-RTA and were as toxic as native MLI [3,4], whereas the immunotoxins prepared from the ricin A-chain were much less active than the parent native toxin. Cytotoxicity of the ricin A-chain containing immunotoxins could be greatly potentiated by a number of agents, e.g. monensin and NH_4Cl. Furthermore, it is more important that the toxicity of these RTA-immunotoxins could be potentiated by treatment of the cells with ricin B-chain alone [11] or with ricin B-chain coupled to directing antibodies [12]. In addition, immunotoxins prepared by coupling native ricin to monoclonal antibodies are generally more toxic to target cells than immunotoxins prepared from the isolated ricin A-chain. One hypothesis explaining these findings is that the ricin B-chain can disrupt the membrane structure of an intracellular vesicle and form a pore that allows the A-chain to pass into the cytosol. However, although the interaction of the ricin B-chain with the liposomal membrane has been demonstrated in a number of investigations [13,14], the evidence that this interaction is involved in the translocation of RTA is still lacking.

Our findings suggest that the facilitating role of the ricin B-chain is mainly to prevent the ricin A-chain from degradation during their processing in the cells. The ricin A-chain uses this route with maximal efficacy which could be achieved by MLA coupled to the ricin B-chain only in the presence of NH_4Cl slowing down acidification in the phagosome.

Earlier we demonstrated that the ricin B-chain interacts with the ricin A-chain with high affinity and that this interaction is pH-dependent: K_a at pH 5.0 is 10-fold higher than at pH 7.5 [15]. In contrast, interaction between RTB and MLA was much weaker and pH-independent [16]. Just this high affinity interaction between the ricin subunits can provide the decreasing degradation of RTA in the acid intracellular vesicles and explain the facilitating moiety of the ricin B-chain.

The protective capability of the ricin B-chain revealed in the present work might be useful for the constructing new effective recombinant immunotoxins.

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