

# Immunotoxins containing A-chain of mistletoe lectin I are more active than immunotoxins with ricin A-chain

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**Abstract** Conjugates of anti-CD25 monoclonal antibodies against cell surface IL-2 receptor with MLIA and RTA were prepared and investigated. Both of the immunotoxins had high specific cytotoxic activity on target cells. The IC<sub>50</sub> value of the anti-CD25/MLIA immunotoxin was 15-fold greater than that of the anti-CD25/RTA. Previous studies of the anti-CD5 immunotoxins with MLIA and RTA showed that the anti-CD5/MLIA IT was 80-fold more active than anti-CD5/RTA IT [Tonevitsky et al. (1991) *Int. J. Immunopharmacol.* 13, 1037–1041]. The surface hydrophobicity of the MLI A-chain was 4-fold higher than that of the ricin A-chain as estimated by binding with ANS. In model experiments with small unilamellar DMPC liposomes, MLIA but not RTA increased the turbidity of liposome suspensions at pH 4.5. Our results indicate that the greater cytotoxic activity of the MLI A-chain immunotoxin probably provided a higher surface hydrophobicity of the protein and the ability to interact with phospholipid membranes.

**Key words:** Immunotoxin; Ricin; Mistletoe lectin I

## 1. Introduction

Immunotoxins are conjugates in which cell-binding ligands are coupled to toxins or their subunits. If the ligand is cell-specific monoclonal antibody immunotoxin should selectively kill target cells in tissue cultures. Plant and bacterial toxins or their enzymatically active parts have been used for preparation of immunotoxins [2]. One of the problems involved in the clinical use of immunotoxins is the lower activity of A-chain immunotoxins as compared to the native toxins.

Ricin from *Ricinus communis* and mistletoe lectin I (MLI) from *Viscum album* are toxic glycoproteins from the RIP with a molecular mass of 63 kDa which consists of two disulfide-linked subunits [3]. The B-chain (34 kDa) is a lectin with two galactose-binding sites [4]. The A-chain (27 kDa) is a highly specific *N*-glycosidase which passes into the cytosol and catalytically inactivates protein synthesis [5]. The structure and mechanism of action of ricin and MLI are similar to those

of other ribosome-inactivating proteins of type II (abrin, modecin and volkensin) [6]. It has previously been shown that immunotoxins with the MLI A-chain are about 50-fold more active than ricin A-chain conjugates [1,7].

In the present paper, we have compared the cytotoxic activity of immunotoxins with the A-chain of ricin or mistletoe lectin I and anti-CD25 monoclonal antibody against IL-2 receptor. We also investigated the ability of A-chains to interact with model phospholipid membranes and the hydrophobic probe ANS.

## 2. Materials and methods

Sepharose CL-6B, Sepharose 4B, *N*-succinimidyl-3-(2-pyridyl-dithio)propionate (SPDP), and dithiothreitol (DTT) were obtained from LKB-Pharmacia (Sweden); cell culture medium RPMI 1640, L-glutamine, kanamycin and fetal calf serum (FCS) were from Flow Labs (UK); all other reagents came from Sigma (USA). Anti-CD25 (IgG1) monAB was kindly provided by J. Kopp and I.J. Korner (Max Delbrück Centrum for Molecular Medicine, Berlin-Buch, Germany).

### 2.1. Isolation of toxins and their subunits

MLI was isolated from *V. album* according to [3]. MLI A-subunit was purified using affinity chromatography. 25 mg of MLI were applied on a 4B lactosyl-Sepharose column (5 ml) and washed using 25 ml PBS. MLIA was eluted with 300 ml of 2% mercaptoethanol in PBS (rate: 0.15 ml/min). MLIA was dialyzed and concentrated on an Amicon concentrating unit.

Ricin was purified from *R. communis* seeds according to [8]. Ricin A-chain was isolated as previously described [8]. The toxin A-subunit preparation was additionally purified from RTB on Sepharose 4B with fixed asialofetuin.

### 2.2. Preparation of immunotoxins and cytotoxic activity determination

The conjugates of monoclonal antibodies and toxin A-chains were prepared by using SPDP as previously described [9]. Briefly, 5 mg antibody (5 mg/ml) was dialyzed against 0.1 M sodium phosphate, pH 7.5, which contained 0.1 M NaCl for a period of 16 h. A 10-fold molar excess of SPDP was added, and the solution was allowed to stand at room temperature for 30 min followed by gel filtration on a PD-10 column (LKB-Pharmacia, Sweden), equilibrated with the same buffer. The number of PDP groups per molecule of monAB was 3.3. 4 mg ricin or MLI A-chain (2 mg/ml) was reduced with 10 mM DTT for 10 min and passed over a Sephadex G-25 column in buffer containing 0.1 M NaCl, 0.1 M sodium phosphate, pH 7.5. Freshly reduced A-chain was immediately mixed with PDP antibody. This mixture was incubated at room temperature for 30 min and concentrated. The prepared conjugate was separated from unreacted A-chain by gel filtration on Sepharose CL-6B. Proteins were analyzed by SDS-PAGE with and without reduction according to [10]. After staining, gels were scanned using an Ultrosan-2202 (LKB-Pharmacia, Sweden). The average number of A-chain molecules per antibody molecule varied from 2.0 to 2.2.

Cytotoxic activity of immunotoxins was assessed on inhibition of [<sup>3</sup>H]thymidine incorporation into human activated lymphocytes as described [11].

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**Abbreviations:** monAB, monoclonal antibody; IT, immunotoxin; MLI, mistletoe lectin I; MLIA, mistletoe lectin I A-chain; RTB, ricin B-chain; RTA, ricin A-chain; DTT, dithiothreitol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMPC, dimyristoyl-phosphatidylcholine; ANS, 1-anilinonaphthalene-8-sulfonate; IC<sub>50</sub>, toxin concentration causing 50% inhibition of [<sup>3</sup>H]thymidine incorporation into cells

### 2.3. Determination of protein interaction with liposomes and ANS

Small unilamellar liposomes were produced by drying of dimyristoylphosphatidylcholine (DMPC) on a rotor evaporator. Subsequently, the mixture was subjected to ultrasonic treatment for 5 min in a buffer containing 5 mM TES, 140 mM NaCl, 1 mM EDTA, at pH 7.5 under a nitrogen atmosphere (50 W, 22–28 kHz) at +4°C and incubated for 2 h at +28°C. Phase transition and liposome aggregation were monitored via optical density measurements at a wavelength of 350 nm using a UV-Vis spectrophotometer (Perkin & Elmer, Germany) as previously described [14]. The concentration of lipids in the liposome solution was 5 mM in phase transition experiments and 250 mM in aggregation experiments. All liposome aggregation experiments were carried out at 28°C.

Binding of 1-anilinonaphthalene-8-sulfonate (ANS) to toxin subunits was determined as described in [12].

### 3. Results and discussion

The cytotoxic activity of the immunotoxins containing anti-CD25 monAB and MLIA or RTA is shown in Fig. 1. The  $IC_{50}$  for MLIA-IT is  $2.0 \times 10^{-10}$  M and  $4.0 \times 10^{-9}$  M for RTA-IT. Previously, it was shown that the  $IC_{50}$  of isolated RTA and MLIA was more than  $10^{-8}$  M [1]. Therefore, ITs have high specific cytotoxic activity.

The  $IC_{50}$  of IT consisting of anti-CD5 monAB and MLIA was more than 80 times that of IT with the same monAB and RTA [1]. The enzymatic activity of these subunits does not differ in cell-free systems [13]. Both chains have identical carbohydrate components [14,15], therefore differences in IT activity cannot be accounted for by interaction with non-galactose-specific receptors. MLIA probably has structural peculiarities that are important in the intracellular pathway including subunit translocation to the cytosol.

The data on ANS binding with enzymatic subunits showed that the association constant of ANS with MLIA and RTA is  $3.6 \times 10^7$  M<sup>-1</sup>. The high value of the association constant means that A-chains of the plant toxins have highly hydrophobic domains [12]. The maximum number of binding sites for ANS in the case of RTA was 1, and 4 for MLIA. MLIA can bind more effectively with hydrophobic sites of intracellular membranes before translocation into the cytosol.

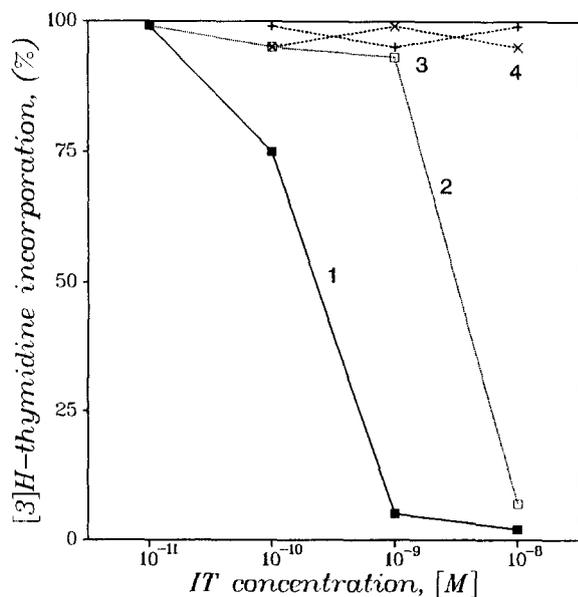


Fig. 1. Cytotoxic activity of anti-CD25/RTA immunotoxin (1), anti-CD25/MLIA (2), anti-CD25 monAB (3), RTA (4).

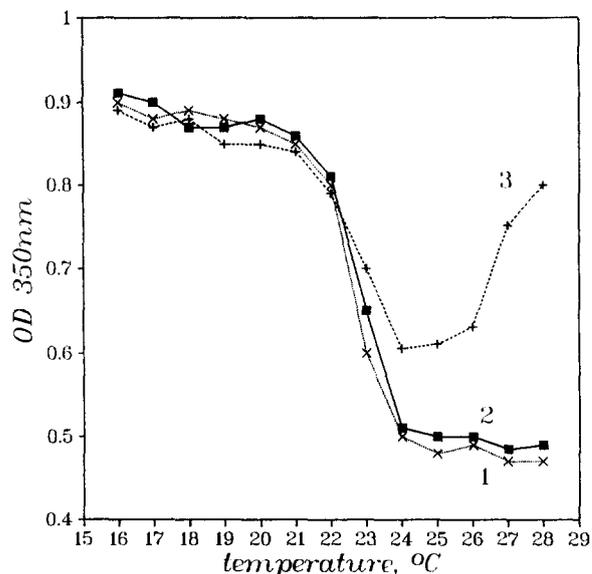
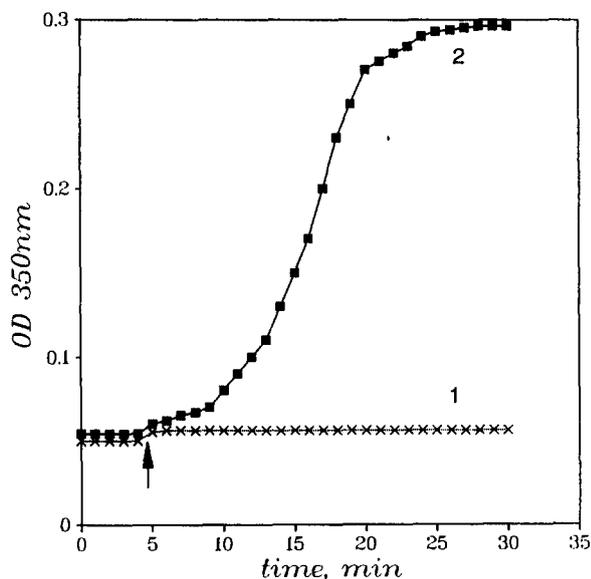


Fig. 2. Phase transition of DMPC-liposomes at pH 4.5. 1, without proteins; 2, in the presence of RTA; 3, in the presence of MLIA.

ITs and holotoxins are internalized via a receptor-mediated pathway [16]. After binding with receptors, the conjugates progress to intracellular compartments with different pH (endosomes, lysosomes, *cis*- and *trans*-Golgi). Ricin reaches the endoplasmic reticulum before undergoing translocation to the cytoplasm [17]. Apparently, MLI also reach the Golgi apparatus before translocation of A-chain to the cytoplasm [18]. Therefore, the A-subunit reaches ribosomes via retrograde transport. This pathway depends on protein-protein and protein-lipid interactions. We performed model experiments for investigation of protein interactions with phospholipid membranes at different pH values. Holotoxins and their subunits did not influence the phase transition of DMPC liposomes (data not shown). Fig. 2 shows the effect of RTA and MLIA on the phase transition at pH 4.5. In the presence of MLIA at acid pH, increasing  $D_{350}$  of the liposome suspension was observed at the temperature of the phase transition. It is possible that the MLIA molecule can undergo a conformational change at pH 4.5 and at the moment of phase transition interact with the disordered phospholipid bilayer. The influence of the proteins on liposome size was investigated at a temperature above the phase transition (28°C, liquid crystal for DMPC). At acid pH, MLIA increased the  $D_{350}$  of the liposome suspension, i.e. the size of phospholipid particles was increased (Fig. 3). Apparently, at pH 4.5 in the MLIA molecule, two hydrophobic sites are exposed as the minimum number that can interact with liposomes.

Protein-lipid interactions depend on protein structure and bilayer composition. Obviously, the effectiveness of the A-chain interaction with the bilayer is very important for protein translocation. Our data suggest that MLIA more effectively interacts with phospholipid bilayers than RTA. It was previously demonstrated using an intrinsic fluorescence method that the structure and stability of these subunits are practically identical [19,20]. IT with two molecules of ricin A-chain had more cytotoxic activity than IT with one RTA [21]. An increase in the hydrophobicity of the toxic part can increase the cytotoxicity of a conjugate. The high activity of ITs con-



taining MLIA is accounted for by their highly hydrophobic nature and ability to interact with model phospholipid membranes. We suggest that the A-chain of MLI is a very promising candidate for the design of immunotoxins.

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