Mistletoe lectin A-chain unfolds during the intracellular transport

Igor I. Agapov[a], Alexander G. Tonevitsky[b,*], Natalya V. Maluchenko[c], Mikhail M. Moisenovich[c], Yuliya S. Bulah[a], Mikhail P. Kirpichnikov[d]

[a] *State Research Institute of Genetics and Selection of Industrial Microorganisms, 1st Dorozhnyi pr. 1, 113545 Moscow, Russia*
[b] *Institute of Transplantology and Artificial Organs, Shchukinskaya st. 1, 123182 Moscow, Russia*
[c] *Moscow State University, Faculty of Biology, Vorobjovi Gori, 119899 Moscow, Russia*
[d] *Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya 16/10, 117871 GSP7 Moscow, Russia*

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Abstract Protein conformation during intracellular routing and translocation of the ribosome-inactivating proteins was investigated on hybridomas producing monoclonal antibodies (monAbs) against mistletoe lectin (ML). Decrease in the toxin activity towards these hybridomas is accounted for by the intracellular interaction of monAbs and the toxin resulting in the interruption of enzymatic subunit translocation into the cytosol. Obtained monAbs interacted with denatured ML A-chain (MLA) and a panel of MLA synthetic octapeptides linked to the surface of polyethylene pins. Enzyme-linked immunosorbent assay (ELISA) shows that monAbs recognize five epitopes in denatured MLA. Treatment of MLA by 3 M of guanidine hydrochloride leads to appearance of the epitopes. Hybridoma TA7 has been shown to be insensitive to cytotoxic action of ML. TA7 monAb as we have shown recognizes epitope 101–105, FTGTT, and inhibits the liposome aggregation induced by MLA. A study of the cytotoxicity of ML and ricin for the hybridomas revealed that the unfolding of A-chain is probably required for intracellular transport and cytotoxic activity of ML.

Key words: Mistletoe lectin; Ribosome-inactivating protein; Monoclonal antibody; Protein unfolding

1. Introduction

Mistletoe lectin (ML) is a plant toxin isolated from *Viscum album* leaves. It belongs to the family of ribosome-inactivating proteins type II (RIP II) containing two subunits [1]. The A-chain (MLA) inhibits protein synthesis by enzymatic depurination of an adenosine residue in a highly conserved loop of the 28S ribosomal RNA. The B-chain, a lectin, binds to galactose-containing receptors on the cell surface. ML enters the cell by receptor-mediated endocytosis. Toxin is transported in vesicles through the endosomal cell compartments and the Golgi complex and possibly reaches the endoplasmic reticulum (ER) [2]. The mechanism of RIP II transmembrane translocation in ER is still unclear. A hypothesis has been advanced that the toxins utilize the ER translocation apparatus, which normally exports misfolded proteins from ER cisterns 1 [3]. Perhaps, toxins mimic such proteins via specific conformational changes. We previously showed that the disulfide bond between ML subunits is reduced and that the chains dissociate in ER before the MLA is translocated into the cytosol [4], the intramolecular hydrophobic domain of MLA being exposed upon dissociation. In the cytosol, misfolded proteins are modified with ubiquitin and hydrolyzed in proteasomes. MLA can avoid the degradation because of the absence of Lys residues which are targets for ubiquitination.

MLA can closely interact with phospholipid bilayer because MLA has been shown to induce aggregation and fusion of liposomes in vitro [5,6]. Conformational changes of toxins during their intracellular trafficking remain unclear. Here, we focused on MLA unfolding before translocation of the subunit into the cytosol. Hybridomas producing monoclonal antibodies (monAbs) against denatured ML were used as a model. The toxin resistance of hybridomas was earlier shown to be owing to the interaction of the toxin with antibodies before its translocation into the cytosol [7]. We investigated influence of anti-denatured MLA monAbs on A-chain-induced liposome aggregation. The experiments probe the hypothesis that MLA changes the conformation during intracellular trafficking before translocation into the cytosol.

2. Materials and methods

2.1. Materials

Cell culture medium RPMI 1640, L-glutamine, kanamycin and fetal calf serum were from Flow Labs (Woodcock Hill, UK); protein A-Sepharose from LKB-Pharmacia (Uppsala, Sweden); 96-well microtiter plates from Nunclon (Roskilde, Denmark); streptavidin labeled with peroxidase and polyclonal rabbit anti-mouse immunoglobulins from Imtek (Moscow, Russia); all other reagents were obtained from Sigma (St. Louis, MO, USA).

2.2. Protein purification and hybridoma production

ML and its subunits were isolated as previously described [5]. The chimerical toxin consisting of MLA and B-subunit of ricin was prepared as previously described [8]. To produce the hybridomas to MLA, spleen cells from Balb/c mice immunized with MLA in complete Freund's adjuvant were hybridized with sp2/0 murine myeloma cells. The hybridomas were tested by enzyme-linked immunosorbent assay (ELISA) with biotinylated ML and MLA. MonAbs from ascitic fluid were purified by affinity chromatography on protein A-Sepharose. Biotinylation was performed with biotinamidocaproate *N*-hydroxysuccinimide ester as recommended by 'Sigma'. Protein purity was estimated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%).

2.3. Binding and cytotoxic assays

The interaction of monAbs with ML and subunits was analyzed in

*Corresponding author. Fax: (7)-95-315 0501.
E-mail: tonevits@genetika.ru

Abbreviations: IC_{50}, toxin concentration causing death of 50% of cells; monAb, monoclonal antibody; ML, mistletoe lectin; MLA, A-chain of ML; RIP, ribosome-inactivating protein

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ELISA; the data were read with a spectrophotometer 'Multiscan' (LabSystems, Finland). Ricin and ML cytotoxicity was estimated by cell survival in the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test as earlier described [5].

2.4. Epitope prediction and peptide synthesis

Epitopes were predicted on the basis of the data on ML tertiary structure [9], using the DSSP program to estimate the exposition of amino acid residues [10]. Data on the temperature B-factor, the ML secondary structure and the amino acid sequence variation in homologous proteins were also analyzed. The highest immunogenicity was assumed for the MLA regions that contained residues with more than 70% surface area exposed, certain secondary structure elements (mostly β-strands), and had maximum mobility and B-factor. We synthesized 65 octapeptides overlapping by seven residues and together covering MLA regions 1–13, 31–52, 72–108, 112–127, 196–206, 210–229 and 248–255. Peptides were synthesized and attached to polyethylene pins with di-β-alanine linkers (Chiron Technologies, Australia) by the active esters coupling procedure using 9-fluorenylmethyloxycarbonyl-amino acid-pentafluorophenyl esters (Cambridge Research Biochemicals, UK) in the presence of an equimolar amount of 1-hydroxybenzotriazole (Merk, Germany) as described earlier [11]. The procedure was drawn up with the PEPMAKER program (Chiron Technologies). Synthesis was manually performed in 96-well plates (Costar, USA). The N-terminal α-amino groups were acetylated with acetic anhydride, and components protecting side functional groups of the amino acid residues were removed with the mixture of trifluoroacetic acid, 1,2-ethanidithiol and anisole (38:1:1).

2.5. Protein unfolding with guanidine hydrochloride (GndHCl)

GndHCl in different concentrations was added to 0.01 mg/ml of biotinylated MLA and incubated for 60 min at room temperature. For ELISA, the protein probes were dissolved before experiment by 100 times in phosphate-buffered saline.

2.6. Liposome preparation and aggregation

Small unilamellar liposomes were produced by drying 1 ml diphytanoylphosphatidylcholine solution in chloroform (1 mM), on a rotor evaporator. Dried lipid film was hydrated by buffer containing 5 mM TES, 140 mM NaCl, 1 mM EDTA, at pH 7.5. The mixture was treated for 5 min with ultrasound in a nitrogen atmosphere (50 W, 22–28 kHz) at +4°C. Liposome aggregation was monitored from optical density measurements at a wavelength of 350 nm after 30 min incubation with proteins at 37°C using an UV-VIS spectrophotometer 'Ultrospec' (LKB-Pharmacia, Sweden). The concentration of lipids in liposome solution was 0.1 mM, MLA 1 μM, monAbs 3 μM.

3. Results and discussion

Mice immunization with MLA combined with the complete Freund's adjuvant yielded monAb preferentially recognizing the denatured protein. The adjuvant could change the protein conformation so that the inner hydrophobic fragments became exposed [12]. Hybridomas TA7, TA71, TA72, TA73, TA74, TA75, TA76 and TA77 producing monAb with the highest affinity for MLA were selected and cloned. Cytotoxic activity of ML on hybridoma cells has been determined and hybridomas were divided into three groups with ML resistance: 15 times higher (TA7) (Fig. 1), comparable (TA72, TA73 and Ta74) or 3–5 times lower (TA71, TA75, TA76 and TA77) than of sp2/0 cells. The toxin concentration causing death of 50% of cells (IC_{50}) of ricin on hybridomas was used to correct the above data for antibody-independent toxin resistance. For instance, the ricin resistance of TA7 hybridoma was 1.5 times higher than that of sp2/0. With such correction, TA7 cells proved to have a 10 times higher ML resistance as compared with sp2/0.

The sensitivity of hybridoma cells producing monAbs to ML depends on the intracellular interaction of monAb with

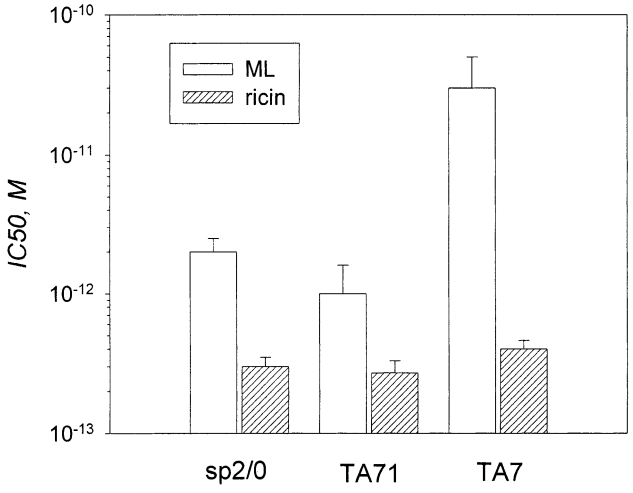

Fig. 1. IC_{50} of ML and ricin on hybridoma cells synthesizing monAbs against MLA.

toxin molecules. Additionally, monAbs in the hybridoma culture media might prevent ML binding to the cells. We excluded this binding by repeated washing of cells with cell culture media without serum. The maximum number of binding sites for ML on specific and control hybridoma cells does not differ (data not shown). Papain treatment of the cells to eliminate external monAb molecules does not change the sensitivity to the toxin [7]. Thus, hybridoma cell resistance is due to intracellular interaction of toxins with newly synthesized antibody molecules.Antibody specificity was assayed with native, denatured and adsorbed on plate MLA, ML, ricin and ricin A-chain (RTA). Ricin and RTA were used as controls, since ricin and ML are similar in intracellular pathway, mechanism of action, amino acid sequence and tertiary structure [9,13]. All monAbs had comparable affinities for MLA adsorbed on a plate. TA71, TA75 and TA77 monAbs did not react with denatured ML, as observed with denatured MLA. These monAbs bound with adsorbed RTA and, less efficiently, with native RTA. The cross-interaction of these monAbs with ricin and RTA is explained by its amino acid homology and structural similarity with ML and MLA. Interestingly, neither of these monAbs conferred ricin resistance to the corresponding hybridomas. Competitive enzyme immunoassay allowed division of hybridomas into five groups differing in monAb specificity: (1) TA72, TA73; (2) TA74; (3) TA71, TA75, TA77; (4) TA76 and (5) TA7. TA5 monAb against native MLA [14] was used as a control. MonAbs from different groups showed a partial cross-reactivity. Thus antigen binding with TA72 and TA73 monAbs decreased binding with TA7 monAb completely and with other monAbs on about 50–60%. The resistance of TA7 hybridoma to ML is possibly determined by the specificity of monAb produced.

Assuming that the ML epitope(s) recognized by monAbs play the crucial role in cytotoxicity, we attempted to locate them. The epitope prediction algorithms allowed us to select the most immunogenic regions of MLA. In total, they comprised 31% of the MLA amino acid sequence. The corresponding octapeptides were synthesized (Fig. 2, at the top). Peptides numbered 1–6 include residues 1–13; no. 7, 31–38; nos. 8–12, 41–52; nos. 13–42, 72–108; nos. 43–51, 112–127; no. 52, 161–168; nos. 53 and 54, 174–182; nos. 55–58, 196–

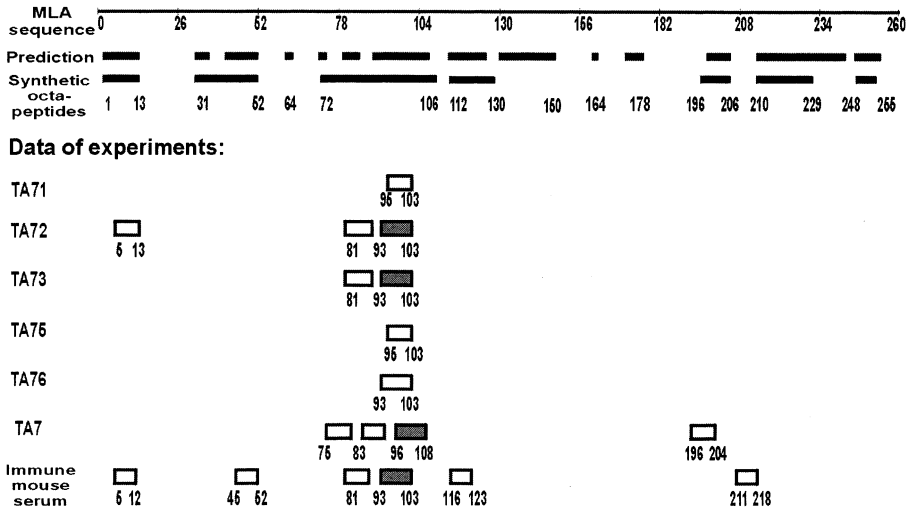

Fig. 2. Epitope mapping in MLA. The results of peptide scanning with monAbs are schematically represented. Optical density at 405 nm: (□) 0.05–1; and (■) 1–3.

206; nos. 59–61, 210–219; no. 62, 222–229; nos. 63 and 64, 231–239; and no. 65, 248–255. The serum of MLA-immunized mice whose spleen cells had been taken for fusion with sp2/0 cells to produce our hybridomas was used as positive control. Serum polyclonal antibodies interacted with peptides containing residues 5–12, 45–52, 81–90, 93–103, 116–123 and 211–218 from the MLA sequence (Fig. 2). The most efficient interaction was with region 93–103 also recognized by TA72 and TA73 monAbs. These monAbs less efficiently bound with peptides containing residues 81–90. TA7 monAb interacted with peptides of the neighboring region 98–108 and had a markedly lower affinity for those of regions 75–83, 87–94 and 196–204 (Fig. 2). TA71, TA75 and TA76 monAbs showed a low affinity for octapeptides from region 93–103 or 95–103. These results were consistent with the above data on monAb cross-reactivity. Octapeptides from region 93–103 recognized by TA72 and TA73 monAbs are a loop formed by a β-strand and partly exposed on the protein surface [9]. The sequence of the epitope is AETHL, 96–100. Comparison of the RTA and MLA sequences showed that a similar epitope is presented in RTA sequence: 102–106, AITHL. The protein loop 93–108 is highly immunogenic and is involved in various epitopes. This loop is not hidden by B-subunit in holotoxin [9]. The region overlaps with the octapeptides binding TA7 monAb (98–108) and weakly binds with TA71, TA76 and TA75 monAbs. This explains the competitive ELISA data that the high-affinity binding with TA72 and TA73 monAbs prevented binding with other monAbs but could not be more than 35% suppressed by any of them. TA7 epitope is 101–105, FTGTT, and also partly exposed on the protein surface [9]. This epitope is not present in RTA sequence. GndHCl denatured MLA and unfolded subunit reacted with TA7 monAb with middle point 4 M of GndHCl (Fig. 3). MonAb MNA5 against native MLA [4] does not bind subunit after GndHCl treatment.

TA7 monAb suppressed MLA-induced liposome aggregation (Fig. 4), which is well consistent with the ML resistance of hybridoma TA7. Hence, TA7 monAb recognizes MLA in complex with the phospholipid bilayer and suppresses this interaction. As noted above, the resistance of hybridoma cells depends on the intracellular interaction of their monAb with toxin molecules and, therefore, on the monAb specificity [7,14]. MonAb to denatured ML may not recognize its epitope in the cell for many reasons. Epitopes hidden inside the globular molecule may not become exposed during the ML transport. If such conformational changes occur, the life time of the unfolded molecule may be too short for its interaction with monAb. This may be the case when toxin penetrates the lipid bilayer or when monAb and toxin molecules are distant from each other in a intracellular compartment, and the toxin unfolded for translocation has a chance to escape binding with monAb. The resistance of TA7 hybridoma to ML shows that TA7 molecules bind toxin molecules in intracellular compartments. This requires ML to be unfolded during its intracellular transport, because TA7 monAb reacts only with partly denatured MLA. The toxin sensitivity of the other hybridomas producing monAbs to denatured MLA suggests that the unfolded MLA molecule exposes only the epitope for TA7 monAb. This explanation is quite

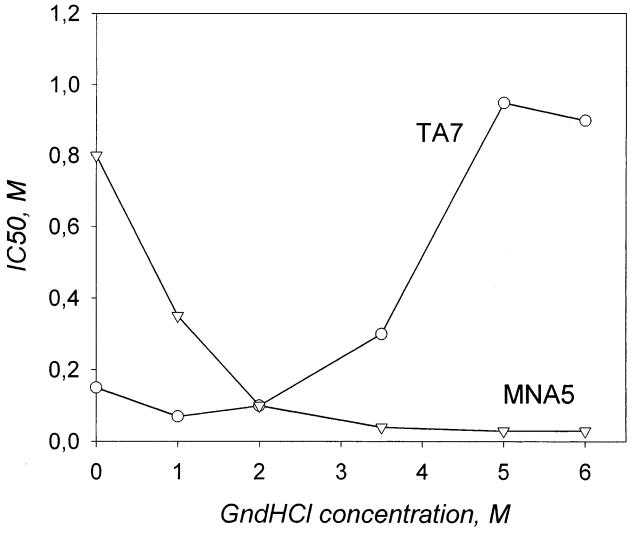

Fig. 3. Effect of monAbs on liposome aggregation induced by MLA.

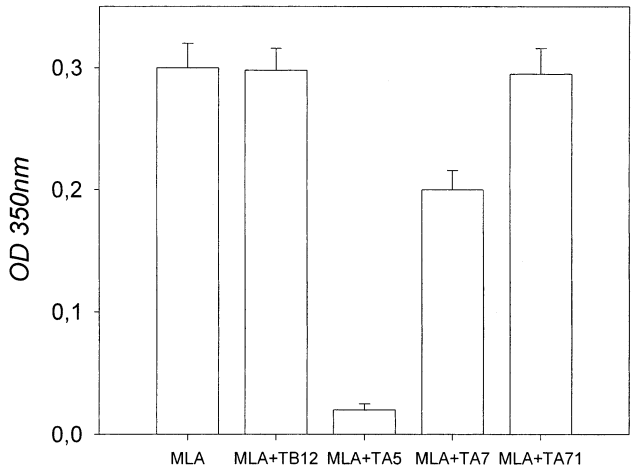

Fig. 4. Interaction of monAbs with MLA treated by GndHCl.

in line with the data on suppression of the MLA interaction with liposomes by TA7 monAb.

The ML resistance of TA7 is far lower than that of hybridomas MNA8 and MNA9 producing monAbs against the native toxin: compared with control cells, they are 600 times less sensitive to ML [4]. Possibly, a transition state of the toxin with the TA7 epitope exposed is a short-lived one.

Thus, our studies have revealed that the MLA molecule is partly unfolded before the toxin is translocated into the cytosol. Introduction of a single disulfide bond in RTA (i.e. fixation of its conformation) has been shown to reduce dramatically its translocation [15]. The unfolding of the toxin in a cell compartment may result from its interaction with cell transport proteins or from its direct contact with the membrane.

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