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Immunological studies of the toxic site in ammodytoxin A

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Two monoclonal antibodies against the native ammodytoxin A and four site-directed polyclonal antibodies against synthetic peptides derived from the primary structure of the toxin were prepared in order to estimate the localization of its toxic site. Some of the antibodies neutralized the lethal toxicity of the toxin, thus indicating an approximate position of the toxic or receptor binding site on the molecule that is different from those predicted by comparison with a number of known sequences.

Phospholipase A_2; Neurotoxin; Toxic site; *Vipera ammodytes*; Monoclonal antibody

1. INTRODUCTION

Ammodytoxin A, formerly designated as the venom fraction 'k2' [1], is the most basic and the most presynaptically neurotoxic phospholipase A_2 from the venom of the long-nosed viper, *Vipera ammodytes ammodytes* (i.v. LD_{50} = 0.021 mg/kg). It is 28 times more lethal than ammodytoxin B (i.v. LD_{50} = 0.58 mg/kg) and 17 times more lethal than ammodytoxin C (i.v. LD_{50} = 0.36 mg/kg) [2]. Besides primary structures of ammodytoxins A, B and C [3–5] and the sequence of homologous protein fraction '1' designated as ammodytin L (Križaj et al., in preparation) on the protein level their cDNAs have also been sequenced [6–9]. All sequenced ammodytoxins are composed of 122 amino acids. Ammodytoxin A and ammodytoxin B differ in only three amino acids at positions 115, 118 and 119, whereas ammodytoxin A and ammodytoxin C differ in two amino acid residues at positions 124 and 128 (numbering according to Renetseder et al. [10]). Differences in the primary structure of ammodytin L are greater and it lacks neurotoxicity. Sequence data have provided some evidence for the location of the toxic site in ammodytoxin which disagrees with the predictions of this site in presynaptically active phospholipases [11–14].

In the present study two monoclonal antibodies were raised against native ammodytoxin A and polyclonal antibodies were raised against four synthetic peptides derived from different exposed regions of the molecule [15]. The enzymatic and lethal activities of complexes of ammodytoxins with these antibodies revealed a so-far unsuspected part of the molecular surface responsible for neurotoxicity.

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2. MATERIALS AND METHODS

Ammodytoxin A was isolated as described earlier [1]. DMEM medium, HAT and HT were from Flow Laboratories (UK). Fetal calf serum was supplied by Hy Clone Laboratories (Utah). Horseradish peroxidase conjugates of goat antimouse IgG and IgG + IgM were obtained from Jackson Immunoresearch Laboratories (Avondale, USA). Freund's complete and incomplete adjuvant and keyhole limpet hemocyanin were purchased from Calbiochem (Behring Corp., CA). PEG 1500 and human serum albumin (HSA) were from Serva (Heidelberg). 2,2'-Azinobis (3-ethylbenzthiazololinesulfonic acid (ABTS), bovine serum albumin (BSA) and *N*-(γ-maleimidobutyryloxy)succinimide (GMBS) were obtained from Sigma Chemical Co. (Missouri). Other reagents were of analytical grade.

2.1. *Preparation of monoclonal antibodies*

Female BALB/c mice were immunized subcutaneously with 0.7 μg of ammodytoxin A in Freund's complete adjuvant (200 μl/mouse) followed with the same dose 14 days later. Two weeks after the second injection, the same quantity of antigen in Freund's incomplete adjuvant was injected intraperitoneally. Two weeks later (3 days before fusion) the mice with the highest titer of antibodies were boosted i.v. with 0.5 μg of ammodytoxin without adjuvant.

Hybridomas were prepared in the usual manner [16,17] using X63-Ag8.653 myeloma cells [18]. The presence of specific antibodies was assessed by ELISA. Positive hybridomas were cloned twice by limiting dilution [19]. Ascites was produced by i.p. injection of 2×10^6 hybridoma cells into pristane-primed BALB/c mice. Ascites fluid was harvested, centrifuged and the supernatant stored at −20°C until use.

Monoclonal antibodies were purified from the ascites by affinity chromatography on ammodytoxin C-Sepharose 4B [20]. The concentration of antibody was estimated by the absorbance at 280 nm assuming $A^{1\%}_{1cm}$ = 14.0 and 11.8 for IgG and IgM, respectively. ELISA was performed in microtiter plates. The wells were coated with the optimal concentration of ammodytoxin A (2.5 μg/ml) or synthetic pep-

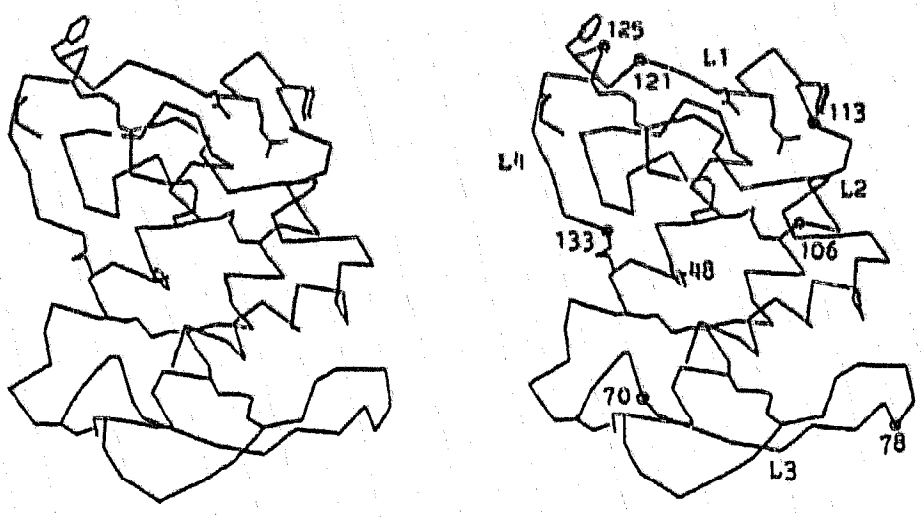

Fig. 1. Stereo view of ammodytoxin A model [5]. Residues Tyr-115, Arg-118, Asn-119, Phe-124 and Lys-128 shown with side chains are exchanged in natural, less toxic mutants. Enzymatic active site His-48 and positions of peptides L1 to L4 are also indicated. Residue numbering is according to [10].

tides (20 μg/ml) in 0.01 M hydrogen carbonate buffer, pH 9.6. After overnight incubation at 4°C the wells were rinsed 3 times with phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20. 100 μl of sample was applied to each well and incubated for 2 h at 37°C. After incubation, the plates were again washed 3 times with PBS Tween 20. 100 μl of peroxidase labelled goat anti-mouse IgM + IgM at a dilution of 1:5000 in 1% BSA was added to each well and incubated for 2 h at 37°C. After washing with PBS/Tween 20, 100 μl of 0.1% ABTS and 0.012% H_2O_2 in 0.01 M citrate phosphate buffer, pH 4.5, were added to the wells. After incubation for 30 min at 37°C the absorbance was measured at 410 nm using a Minireader II (Dynatech, Denkendorf).

Anti-peptide antibodies were raised towards peptides corresponding to the residues 113–121 (L1), 106–113 (L2), 70–78 (L3) and 125–133 (L4) (cf. Figs 1 and 3), synthesized by the solid phase method [21]. In the first three peptides an additional cysteine was added to the C-terminus to allow the peptide to couple to the protein carrier via the –SH group by GMBS [22,23]. Polyclonal antibodies against four peptide conjugates were prepared in rabbits by three subcutaneous injections into the feet at two-week intervals using KLH conjugates emulsified in complete Freund's adjuvant. The titer of specific antibody was determined by ELISA. Antibodies were purified from the serum by affinity chromatography on protein A-Sepharose 4B [24] followed by affinity chromatography on ammodytoxin C-sepharose 4B [20].

The binding of antibodies to isotoxins was determined by ELISA performed as above with the following modifications: 2n dilutions of antibodies in 1% BSA were applied to the wells after overnight incubation with ammodytoxins A, B, C. After incubation for 2 h at 37°C and washing with PBS-Tween, 100 μl of peroxidase labelled goat anti-rabbit IgG or IgG + IgM at dilution 1:5000 in 1% BSA was added to each well and incubated for 2 h at 37°C. The subsequent steps were performed in the same way as above.

Toxicity of immune complexes was assayed after monoclonal antibodies and monospecific polyclonal antibodies were incubated with 3 i.p. LD_{50} of ammodytoxin A in molar ratio 2.5:1 for 30 min at 56°C. The mixture was injected i.p. to groups of 6 mice (25 g). Controls were injected only with ammodytoxin A or antibody. Phospholipase activity of immune complexes and of ammodytoxin A was determined according to De Haas et al. [25].

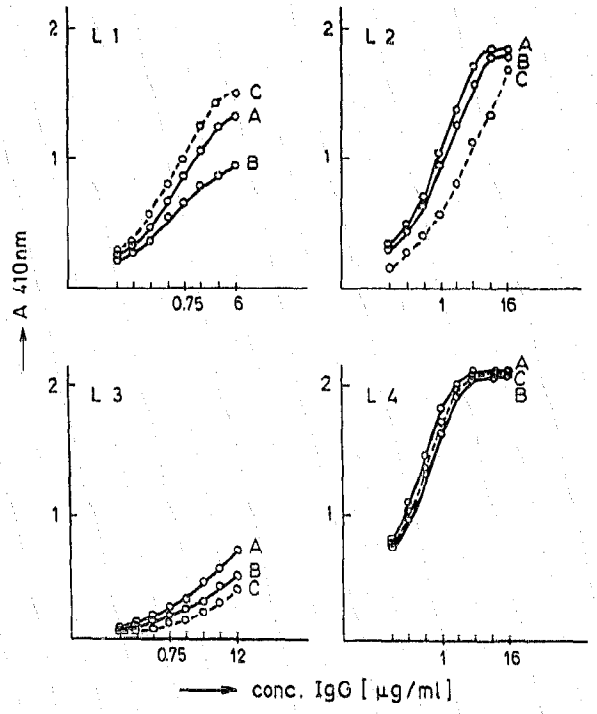

Fig. 2. Binding curves of anti-L1, anti-L2, anti-L3 and anti-L4 polyclonal antibodies to ammodytoxin A, B and C in ELISA test.

3. RESULTS AND DISCUSSION

Because of the high toxicity of ammodytoxin A, the amount of antigen was limited during the immunization procedure which resulted in low titres of specific antibodies before the fusion. Three fusions were performed to obtain monoclonal antibodies against the native ammodytoxin A giving a hybrid growth in 308 out of a total of 696 wells containing selective HAT medium. Seven wells were positive as determined by ELISA, but

Table I

Enzymatic activities and toxicities of ammodytoxin A-antibody complexes

Sample	Relative PLA_2 act.	Toxicity
Control (atx A)	1.00	+ + +
Polyclonal ab		
L1 (IgG)	0.98	+
L2	0.75	-
L3	0.36	+ + +
L4	1.19	+ + +
Monoclonal ab.		
K321	1.19	+ + +
K326	0.78	-

only two cell lines developed suitably and were cloned by the limiting dilution method. The finally chosen clones were designated as K321 and K326 and were grown i.p. in BALB/c mice. Both cell lines produced only small amounts of antibodies which were purified from ascitic fluid by affinity chromatography on protein A-Sepharose 4B and were identified as the IgG_1 and IgG_2 subclass. The pure monoclonal immunoglobulins G showed a family of bands on isoelectric focusing around pH 6.55 (K321) and 5.85 (K326).

The purity of site-specific polyclonal antibodies was determined by SDS-PAGE electrophoresis and the specificity was established by ELISA. The binding of site specific polyclonal antibodies to ammodytoxins A, B and C is shown in Fig. 2. Enzymatic activities and toxicities of the complexes of antibodies with ammodytoxin A are shown in Table I. Monoclonal antibody K321 did not neutralize the toxicity or affect enzymatic activity of ammodytoxin A even when added in 10-fold excess, whereas antibody K326 in molar ratio 2.5:1 completely abolished the toxicity, resulting in the survival of all 6 tested animals.

Polyclonal antibodies against the four synthetic peptides (L1, L2, L3, L4) (Fig. 3) had different effects on the toxicity of ammodytoxin A (Table I). Antibodies against L2 totally and against L1 partially blocked the toxicity. Antibodies against L3 and L4 had no influence on the toxicity of ammodytoxin A. From these results and from the fact that monoclonal antibodies K326 bind to synthetic peptide L1 and K321 to peptide L4 in ELISA it follows that both monoclonal antibodies apparently bind to the corresponding part of the surface of toxin.

Polyclonal antibodies against peptides L2 and L3 lowered the phospholipase activity by 25 and 64%, respectively (Table I) although the binding of later antibodies is rather weak. Antibodies against L1 and L4 had no effect on the phospholipase activity of immune complexes. The binding of polyclonal anti-peptide (L1 to L4) antibodies to the three ammodytoxins essentially corresponds to differences in the primary structures of these proteins (Fig. 3). Exceptional is the slightly better ammodytoxin C binding of anti-peptide L1 and weaker binding of anti-peptide L2 antibodies. This could be explained by steric effects in ELISA where the orientation of wall-bound ammodytoxin C allows different exposures of binding site.

According to our results, it seems that in ammodytoxins the toxic site comprises a part of the primary structure corresponding to peptides L1 and L2 where several basic amino acids are located. Several authors have studied the structure of different toxins by an immunological approach, but only some were successful in obtaining monoclonal antibodies having neutralization potential against intact toxins and known binding characteristics. Boulain and co-workers prepared monoclonal antibodies against *Naja nigricollis* toxin-α [26] but the epitope was topographically distinct from the 'toxic' site of toxin. Monoclonal antibodies against β-bungarotoxin partially inhibited its phospholipase activity and did not protect against the lethal effects of the toxin, although they doubled the time to death with respect to control animals [27]. Rael et al. prepared monoclonal antibodies against Mojave toxin [28] specific for the basic subunit, but they neutralized neither the toxicity nor the phospholipase A_2 activity of Mojave toxin. Kaiser and Middlebrook, however, reported on crotoxin neutralizing monoclonal antibody raised against the basic subunit of crotoxin [29].

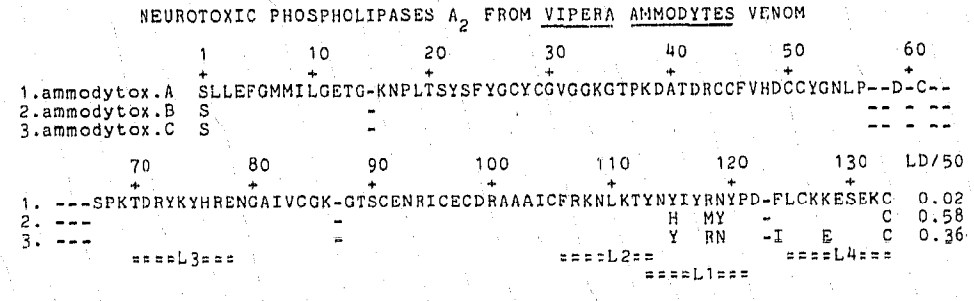

Fig. 3. The primary structures of ammodytoxin A, B and C. Position of four synthetic peptides used for raising the antibodies is indicated. i.v. LD_{50} for white mice is in mg/kg.

The use of antibodies against peptides derived from the primary structure have proved to be a useful tool in the study of structure function relationship of neurotoxins. According to our results, the surface region of ammodytoxin A which binds to neutralizing antibodies is part of a continuous epitope corresponding to peptide L2 (106–113). It would thus be possible to use this epitope as a vaccine. It was also shown that at least part of the ammodytoxin A toxic (or receptor binding) site is located in or near the sequence 106–113 (Fig. 2), which is a highly exposed part of the molecule located outside the areas where toxic sites in presynaptic neurotoxins were predicted by comparison of known primary structures.

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