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Neutralization of lethal potency and inhibition of enzymatic activity of a phospholipase A_2 neurotoxin, crotoxin, by non-precipitating antibodies (Fab)

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Rabbit antibodies were prepared against both purified catalytic (component-B) and purified non-catalytic (component-A) subunits of crotoxin, the major phospholipase A_2 neurotoxin from the South American rattlesnake. They cross-react with crotoxin-like toxins from the venom of several *Crotalus* species as well as with single-chain phospholipase A_2 neurotoxins from Crotalid and Viperid venoms (agkistrodontoxin and ammodytoxin A) but not from Elapid venoms (notexin). Immunological cross-reactions of anti-component-A and anti-component-B sera with crotoxin and with its isolated components A and B showed that component-A exposes determinants of low immunogenicity which are present on component-B, whereas the major antigenic determinants of component-B are not present on component-A. Anti-component-B antibodies, but not anti-component-A antibodies, neutralize the lethal potency of crotoxin and inhibit its enzymatic activity. Furthermore, non-precipitating anti-component-B Fab fragments were as potent as antibodies, indicating that crotoxin neutralization results from the binding of the antibodies to the catalytic subunit, rather than the formation of an immunoprecipitate.

Phospholipase A_2; Snake venom neurotoxin; Lethal potency neutralization; Enzymatic inhibition; Neutralizing antibody; Neutralizing Fab

1. INTRODUCTION

Crotoxin, the major toxic component of the venom of the South American rattlesnake, *Crotalus durissus terrificus* [1], is a potent neurotoxin which possesses a phospholipase A_2 activity and exerts its pathophysiological action by blocking neuromuscular transmission [2]. It acts primarily at the presynaptic level by altering neurotransmitter release [3–6], like other snake neurotoxins that possess a phospholipase A_2 activity: β-bungarotoxin [7–9], notexin [10], taipoxin [11,12], caudoxin [13] and agkistrodontoxin [14,15]. In addition to its presynaptic action, crotoxin also acts postsynaptically, by blocking the acetylcholine response through stabilization of the acetylcholine receptor in an inactive state that resembles the desensitized state [2,16,17].

Crotoxin consists of two non-identical subunits: a basic and weakly toxic phospholipase A_2, component-B, and an acidic component-A which is devoid of enzymatic activity and is non-toxic [18–20]. Components A and B are tightly associated in the crotoxin complex and can be separated in 6 M urea [21,22]. Component-B is a single polypeptide chain of 122 amino acids and its sequence is homologous to that of other phospholipases A_2 from pancreas and snake venoms [23,24]. Component-A consists of three polypeptide chains linked by seven disulfide bridges [25]; their amino acid sequences are homologous to various parts of a phospholipase

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A_2, suggesting that component-A is produced by limited proteolysis of a phospholipase precursor [26]. Further investigations revealed that purified crotoxin is in fact a mixture of several isoforms having similar pharmacological and enzymatic properties, as expected on the basis of their similar polypeptide structure, although they slightly but significantly differ in specific activity and lethal potency [27,28].

The two non-identical subunits of crotoxin act in a synergistic manner: component-B alone is weakly toxic and may block neuromuscular transmission like the whole toxin, although larger doses are required to cause the same effect; component-A is non-toxic by itself, but applied in combination with component-B, it enhances its pharmacological efficacy [4,5,17–19]. Binding experiments showed that the two subunits of crotoxin separate upon interaction with biological membranes: component-B binds while component-A is released in solution. The isolated phospholipase subunit, component-B, adsorbs to membranes in a non-saturable manner, whereas in the presence of component-A, it binds to a limited number of high-affinity binding sites [17,29]. Although the target acceptor site of crotoxin on synaptic membranes has not yet been formally identified, binding experiments carried out with vesicles of various lipid compositions suggested that negatively charged phospholipids are an important component of this target [30].

Crotoxin-like toxins are widely distributed in the venom of *Crotalus* species, and have been purified from *C. s. scutulatus* [31–33], *C. viridis concolor* [34] and *C. vegrandis* [35]. Since antivenoms are widely used to neutralize the lethal effect of snake venoms, it is of interest to examine the mechanism of neutralization by specific antibodies to crotoxin-like toxins, which are the major toxic proteins of *Crotalus* venoms. In this investigation, we raised rabbit antisera against both isolated crotoxin components. We prepared antibodies and Fab fragments from anti-component-B sera and examined their capacity to neutralize the lethal potency of crotoxin and to inhibit its phospholipase A_2 activity.

2. EXPERIMENTAL

Crotoxin from *C. durissus terrificus* venom (collected in Brazil) and its isolated components A and B were purified as described by Hendon and Fraenkel-Conrat [19]. Component-A and component-B were emulsified with 50% Freund's adjuvant and were administered s.c. at 3-week intervals. Boosts, which were performed when the serum titers were decreasing, were achieved by injecting i.m. the same components, in the presence of incomplete Freund's adjuvant. Two immunization protocols were performed, they differed by the dose of administered antigen (50 or 750 μg per rabbit). Three rabbits were immunized with each protocol.

Microtitration plates (96 wells) were coated in phosphate-buffered saline (PBS) by overnight incubation of antigen (1 μg/ml) and saturation was carried out with 5% milk powder in PBS. Plates were washed with PBS containing 0.1% Tween-20. The solutions to be tested (100 μl/well), diluted in PBS containing 5% milk, were incubated 1 h at 37°C, then washed. β-Galactosidase-labeled goat antibodies anti-rabbit immunoglobulin (Biosys, Compiègne) were added at a 1:3000 dilution, incubated 1 h at 37°C and washed. Substrate for β-galactosidase (100 mM sodium phosphate, pH 7.0, containing 1 mM Mg-Titriplex, 1 mM $MnSO_4$, 1 mM $MgSO_4$, 10 mM *S*-methylcysteine and 2.65 mM *p*-nitrophenyl-β-D-galactoside) were added and the absorbance recorded at 405 nm with a Dynatech microplate reader. Chase experiments were performed by preincubating the sera to be tested with a series of concentrations of the antigen used to coat the titration plates, in PBS containing 5% milk.

Inhibition of phospholipase A_2 activity was carried out by preincubating for 1 h at 37°C a fixed concentration of enzyme (20 μg/ml) with variable amounts of serum, antibodies or Fabs and by testing the residual enzymatic activity by titrimetry [36], using egg lecithin solubilized by sodium cholate, or non-micellar dihexanoyl-L-α-lecithin, as substrates [37,38]. The neutralization potency of serum, antibodies or Fabs was determined by incubation for 1 h at 37°C of a fixed concentration of toxin (usually 20 LD_{50}/ml) with dilutions of the solution to be tested. Aliquots (0.2 ml per 20 g body weight) were then injected i.v. in mice, to estimate the residual lethal potency. The toxin neutralizing capacity (ED_{50}), expressed as μg crotoxin neutralized per mg antibodies, was the dose of antibodies that neutralized 50% of the crotoxin lethal potency.

3. RESULTS AND DISCUSSION

We immunized rabbits with isolated component-A and isolated component-B from crotoxin. Table 1 shows that the antiserum directed against component-B has a much higher ELISA titer than that directed against component-A. This is consistent with previous reports, showing that component-B is much more antigenic than component-A [39]. Immunization protocols differing by the quantity of injected antigen gave very similar results (not shown), indicating that isolated component-A is a weak antigen.

In a first series of experiments, we examined the immunological cross-reactions by ELISA. The

Table 1

ELISA titers of anti-component-A and anti-component-B sera

Antigen	Serum	
	Anti-component-B	Anti-component-A
Component-B	1/11000 (0.13)	1/500 (0.03)
Component-A	>1/1	1/300 (0.2)
Crotoxin	1/10000 (0.5)	1/400 (0.2)
Agkistrodontoxin	1/2500 (0.1)	1/150 (0.1)
Ammodytoxin A	1/40 (0.1)	1/10
Notexin	>1/1	>1/1
β-Bungarotoxin	>1/1	>1/1
C. adamanteus phospholipase	>1/1	>1/1
V. berus phospholipase	>1/2 (n.i.)	>1/1
B. atrox phospholipase	>1/1	>1/1
N. nigricollis phospholipase	>1/2 (n.i.)	>1/1
Porcine pancreas phospholipase	>1/1	>1/1
C. d. terrificus venom (Brazil)	1/4000 (0.2)	1/200 (0.2)
C. d. terrificus venom (Columbia)	1/5000 (0.2)	1/250 (0.3)
C. d. cascavella venom (Brazil)	1/6000 (0.15)	1/100 (0.3)
C. s. scutulatus venom (USA)	1/7000 (0.2)	1/100 (0.2)

ELISA titers were defined as the serum dilution that produced half of the maximal response. Numbers in parentheses are the concentrations of antigens (in μg/ml) responsible for half-reduction of the maximal ELISA response in chase experiments. The indicated values are the means of three independent experiments, standard deviations being ± 10%; n.i. indicates that the ELISA titer was not detectably decreased in chase experiments

anti-component-A serum reacts with crotoxin and with its isolated component-B (table 1). This observation, which is in agreement with a previous study of Kaiser et al. [39], may result from the similarity of the protein structure of the two subunits [26]. On the other hand, anti-component-B serum reacts with crotoxin but, at variance with the observation of Kaiser et al. [39], does not react with isolated component-A. This non symmetrical immunological relationship might be due to a contamination of the component-A preparation by component-B. In order to test this hypothesis, we first estimated the presence of component-B in the preparation of component-A by measuring its phospholipase activity and we found that the contamination was less than 0.01%. We also fractionated the anti-component-A serum by affinity chromatography on a component-A column and observed that the protein peak which did not adsorb to the column reacted neither with component-A nor with component-B, whereas the immunoglobulins which adsorbed to the column and were eluted at pH 2.0 (100 mM glycine-HCl) reacted with both components (not shown). Therefore, the non-symmetrical immunological relationship between crotoxin subunits cannot be attributed to a contamination of component-A by component-B. It may be explained by the fact that component-A exposes determinants of low immunogenicity which are also present on component-B, whereas the major immunogenic determinants of component-B are not present on component-A. This may be related to the fact that component-A most probably derives by limited proteolysis from a precursor which is closely homologous to component-B [26].

We further analyzed the immunological cross-reaction of anti-component-A and anti-component-B sera with toxic and non-toxic phospholipases A_2 from snake venom and with porcine pancreas phospholipase A_2. Both sera strongly react with *C. durissus terrificus*, *C.d. cascavella* and *C.s. scutulatus* venoms which are known to contain crotoxin-like toxins (table 1). Both sera also cross-react with agkistrodontoxin and more weakly with ammodytoxin A, which are phospholipase A_2 neurotoxins from the Crotalid or Viperid snakes, *Agkistrodon halys pallas* [14,15] and *Vipera a. ammodytes* [40], constituted of a single polypeptide chain. Neither anti-component-A nor anti-component-B serum shows any cross-reaction with the other toxic and non-toxic phospholipases A_2 listed in table 1, including the single-chain neurotoxin, notexin, from the venom of an Elapid snake, *Notechis s. scutatus*. Immunological similarities and differences between crotoxin, agkistrodontoxin and ammodytoxin A have been confirmed by reciprocal cross-reaction performed with rabbit sera prepared with agkistrodontoxin and ammodytoxin A (not shown). These observations are in agreement with those of Kaiser et al. [39] who reported that neither anti-component-A nor anti-component-B sera cross-react with β-bungarotoxin and phospholipases A_2 from *Naja naja atra* venom. On the other hand, they disagree to some extent with the conclusion of Middlebrook and Kaiser [41]

who reported that an anti-crotoxin serum, which cross-reacts with crotoxin-like toxins and single-chain phospholipase neurotoxins from Viperid venoms, also cross-reacts weakly with β-bungarotoxin and notexin from Elapid venoms. Similarly, Nicholson et al. [42] recently reported that about 50% of mouse sera prepared against notexin cross-react with crotoxin. These differences may be due to the different immunization protocols and/or the sensitivity of the ELISA used to assess immunological cross-reactions.

We observed that anti-component-B serum also neutralized the lethal potency of crotoxin and inhibited its phospholipase activity (table 2). This observation, which is in agreement with a previous study of Da Silva and Bier [43], prompted us to purify specific anti-component-B antibodies. This was achieved by a double ammonium sulfate precipitation at 30% saturation and by an immunoaffinity chromatography on a column of component-B. Table 2 indicates that, as could be expected, the capacity of antibodies to neutralize the lethal potency of crotoxin and to inhibit its phospholipase activity increased in a parallel manner, about 30-fold during purification. It is also of interest that the same amount of anti-component-B antibodies was required to neutralize either the lethal potency or the phospholipase A_2 activity of crotoxin (table 2).

We prepared monovalent fragments (Fab) by limited proteolysis of anti-component-B antibodies with papain. Fabs were further purified by ion-exchange chromatography on CM-cellulose and chromatography on a protein A column. When tested for their ability to neutralize the lethal potency of crotoxin and to inhibit its phospholipase activity, the anti-component-B Fabs were slightly but significantly more efficient than the intact antibodies (table 2). This indicates that crotoxin neutralization results from the binding of the antibodies to the catalytic subunit, rather than the formation of an immunoprecipitate. Fig.1 further shows that the anti-component-B Fab fragments inhibit more efficiently the phospholipase activity of crotoxin component-B when measured with monodispersed substrate than when determined with mixed micelles of phospholipids and detergent. This indicates that the inhibition of the phospholipase activity does not result from a blockade of the binding of component-B to micelles or biological membranes but is the consequence of a direct or allosteric inhibition of enzymatic catalysis.

Because of the synergistic action of the two crotoxin subunits, neutralization of the lethality of crotoxin might result from the dissociation of the toxin complex. We incubated crotoxin with neutralizing doses of anti-component-B Fabs and checked, by polyacrylamide gel electrophoresis (under non-denaturing conditions), the formation

Table 2

Neutralization of crotoxin lethal potency and inhibition of its phospholipase activity by anti-component-B or anti-component-A antibodies and Fabs

Antibody	Protein		ELISA titer	Crotoxin	
	mg	Yield	(μg/ml)	PLA_2 (μg/mg)	ED_{50} (μg/mg)
Anti-component-B					
Serum	36800	100	9.5	0.8	0.5
Ammonium sulfate precipitate	9200	25	5.6	1.8	1.9
Immunoaffinity column	900	2.4	0.56	14	14
Fab	200	0.5	14.5	22	20
Anti-component-A					
Serum	36000	100	300	<0.005	<0.004
Ammonium sulfate	10125	25	150	<0.01	<0.01
Immunoaffinity column	932	2.5	ND	<0.02	<0.02

Protein concentrations were estimated by the method of Folin. Neutralization and inhibition capacities determined with micellar substrate are expressed in μg crotoxin neutralized or inhibited per mg protein. ND, not done

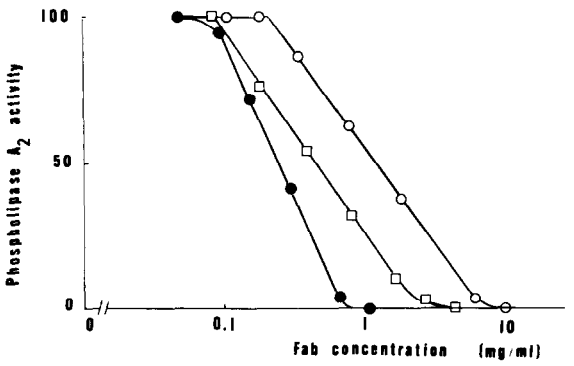

Fig.1. Inhibition of phospholipase A_2 activity by anti-component-B Fabs. Crotoxin (squares) or its isolated component-B (circles) were incubated at a concentration of 20 μg/ml with the indicated concentrations of anti-component-B Fabs. The residual phospholipase A_2 activity was measured by titrimetry, using mixed micelles of egg lecithin and sodium cholate (open symbols) or dihexanoyl-L-α-lecithin in a monodispersed form, i.e. at a concentration below their critical micellar concentration (closed symbols).

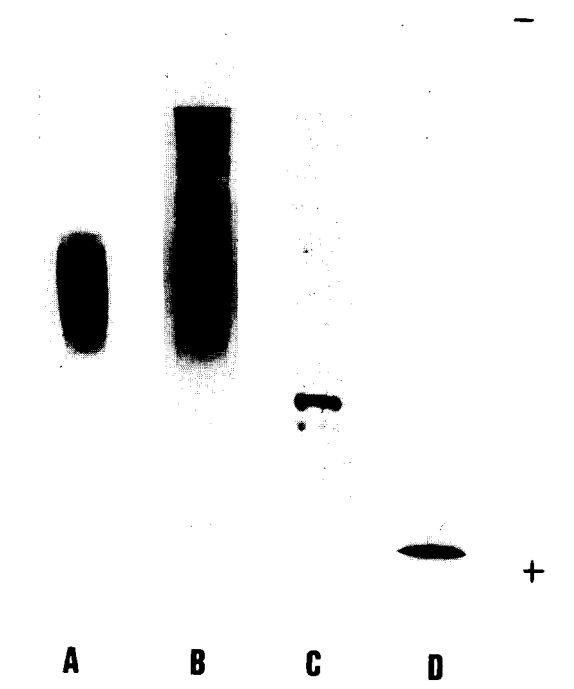

Fig.2. Anti-component-B Fabs do not dissociate the crotoxin complex. Crotoxin (10 μM, 0.25 mg/ml) was incubated for 1 h with anti-component-B Fab (20 μM, 1 mg/ml), then submitted to non-denaturing PAGE. Gels were stained with Coomassie brilliant blue. (A) Anti-component-B Fab; (B) crotoxin and anti-component-B Fab; (C) crotoxin; (D) component-A.

of Fab-antigen complexes and the possible presence of free component-A in the medium. We observed the disappearance of the protein band corresponding to crotoxin, the appearance of protein bands which can correspond to Fab-antigen complexes and did not find any free component-A (fig.2.). Therefore, Fabs do not promote the dissociation of crotoxin, and this cannot account for their protective effect.

We also purified specific anti-component-A antibodies by ammonium sulfate precipitation and immunoaffinity chromatography on a component-A column. Although these antibodies reacted with crotoxin and cross-reacted with isolated component-B in ELISA, they were unable to neutralize its lethal potency and to inhibit its enzymatic activity (table 2).

4. CONCLUSION

Rabbit antibodies raised against both crotoxin subunits were found to react or to cross-react with crotoxin and with crotoxin-like proteins from the venom of several *Crotalus* species. These antibodies do not cross-react with most other toxic and non-toxic phospholipases A_2 from mammalian pancreas and snake venoms, in agreement with previous investigations [39,43]. We observed however that anti-component-B antibodies cross-react with some single-chain phospholipase A_2 neurotoxins from Crotalid and Viperid venoms, agkistrodontoxin and ammodytoxin A, but not with notexin, an Elapid single-chain phospholipase A_2 toxin. The presence of crotoxin-like toxins in the venom of *C. horridus atricaudatus, C. basiliscus* and *Crotalus tigris* has been suggested on the basis of immunological cross-reactions with anti-crotoxin antibodies [44,45]. This appears to be in fact a weak argument because of significant cross-reactions of anti-component-B antibodies with the non-crotoxin-like toxins, agkistrodontoxin and ammodytoxin A. We also observed that anti-component-A antibodies cross-react with component-B whereas anti-component-B do not react with component-A, suggesting that the major antigenic determinants of component-B are not present on component-A.

Antibodies directed to component-B were found to be able to neutralize the lethal potency of native crotoxin, in agreement with the early observations

of Da Silva and Bier [43]. On the other hand, antibodies directed to the non-catalytic subunit, component-A, were found to have no protective effect. The present investigation further shows that non-precipitating antibodies (Fab) are as potent as intact immunoglobulins, proving that neutralization of crotoxin results from the binding of Fabs to the toxin rather than from the formation of an immunoprecipitate. The protective effect of anti-component-B Fabs was further investigated. We found that: (i) anti-component-B Fabs do not dissociate the crotoxin complex; (ii) concentrations of anti-component-B Fabs that neutralize the lethal potency of crotoxin inhibit its phospholipase activity. Taking into account the fact that alkylation of crotoxin by *p*-bromophenacyl bromide simultaneously inactivates its phospholipase activity and abolishes its lethal potency [46,47], this suggests that neutralization of crotoxin by non-precipitating Fabs might result from their binding to epitope(s) belonging at least in part to the enzymatic site of the toxin. Further investigations carried out with monoclonal antibodies directed against component-B are in progress to examine this hypothesis. Preliminary results from this laboratory and from others [48] indicated that some monoclonal antibodies directed against component-B are indeed able to inhibit phospholipase activity of crotoxin and to neutralize its lethal potency.

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