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**PRINCIPAL INVESTIGATOR: Ivan I. Kaiser, Ph.D.**

**CONTRACTING ORGANIZATION: University of Wyoming  
Department of Molecular Biology  
Box 3944, University Station  
Laramie, Wyoming 82071-3944**

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Ivan I. Kaiser

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Department of Molecular Biology  
Box 3944, University Station  
Laramie, Wyoming 82071-3944

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13. ABSTRACT (Maximum 200 words)  
We are using multiple approaches employing the tools of biochemistry, immunology, molecular biology, and pharmacology to gain greater insight into presynaptic neurotoxin structure and function. Protein sequencing of phospholipases A<sub>2</sub> (PLA<sub>2</sub>) have enabled us to identify suspected residues of presynaptic neurotoxins that are involved in activities such as neurotoxicity, myotoxicity, dimerization, etc., and nucleic acid sequencing of both cDNAs and genomic DNA have provided us with a better understanding of the genomic structure and possible regulatory elements within these genes. Chemical modifications of presynaptic neurotoxins have suggested whether these or similar modifications may be involved in modifying PLA<sub>2</sub> function *in vivo*. Higher-ordered structural analysis of Mojave toxin using the techniques of NMR and x-ray diffraction should complement our protein sequencing studies and allow predictions about other 'critical' amino acid residues. We can now express both subunits of Mojave toxin in *E. coli* and are working to isolate these products in biologically active forms. Once accomplished, we can conduct site-specific mutagenesis studies coupled with expression to confirm that certain residues are involved in specific functions. Pharmacological experiments have suggested that PLA<sub>2</sub> neurotoxins do not appear to be endocytosed to exert their effects, unlike most potent enzymatic toxins.

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## INTRODUCTION

### Nature of the Problem

Recent reports in the literature suggested that certain covalent modifications of non-toxic phospholipases A<sub>2</sub> (PLA<sub>2</sub>), significantly enhanced their phospholipase activity. We wanted to examine presynaptic neurotoxins for possible covalent modifications (acylation, transglutamination) that might potentiate neurotoxin activity *in vivo*. If such modification were found to enhance neurotoxicity, new therapy approaches would be suggested.

Glycoprotein analysis, cross-linking/cleavage, NMR, and alkylation studies are structural investigations designed to give a better understanding of neurotoxin structure and function. The proposed glycoprotein analyses will provide basic information on carbohydrates known to exist on some presynaptic neurotoxins, including their mode and location of attachment, number of side-chains, and sugar identification.

With PLA<sub>2</sub> activity associated with all presynaptic neurotoxins, they have the potential for inducing platelet-activating factor (PAF) synthesis in endothelial cells. PAF causes blood platelet aggregation and hypotension at low concentrations ( $10^{-11}$  M) and is therefore an endogenous compound capable of causing severe physiological effects at low doses. Its induction by neurotoxins will be examined in cultured endothelial cells.

Snake venoms represent a complex mixture of proteins, many of which are involved in the breakdown and hydrolysis of biological tissues. Yet, venom is seemingly stored for indefinite periods in venom glands without either causing destruction of the venom glands or venom components themselves. This suggests that various types of natural enzyme inhibitors must co-exist in the venom to inactivate these degradative enzymes. We are hopeful that our proposed 5'-nucleotidase studies involving azido nucleotide analogs may provide us with some initial answers to this curious question. Clearly, identification and characterization of various enzyme inhibitors would be of great interest, since they might serve as potential therapeutic agents.

The molecular biology and immunology sections of the original proposal are extensions of our on-going efforts to use molecular approaches in the development of synthetic vaccines against neurotoxins. Using mutagenesis and partial gene deletion, we are hopeful that we will be able to express a non-toxic, antigenic isoform of the basic subunit of Mojave toxin that can be

used to immunize animals against rattlesnake presynaptic neurotoxins. Identification of the region of the basic subunit that promotes toxicity, may enable us to synthesize a mimicking structural peptide that is capable of generating neutralizing antibodies in animals against crotoxin and its related toxins.

### **Background**

Earlier work in my laboratory has had a strong emphasis on rattlesnake presynaptic neurotoxins. We are interested in these toxins from several viewpoints, including their structure, mode of action, immunology and molecular biology. This proposal was an extension of earlier work, particular the molecular biology and immunology sections, but also includes studies with a number of other presynaptic neurotoxins.

**Structure.** We have completed the sequencing of the basic subunit of Mojave toxin (Aird, Kruggel, and Kaiser; 1990a) and the B-chain of crotoxin (Aird, Yates, Martino, Shabanowitz, Hunt, and Kaiser; 1990b). Except for the determination of the disulfide bond arrangements this completes the amino acid sequence studies on these two proteins. Collaborative work is in progress on the determination of the disulfide bond arrangements within each subunit of rattlesnake presynaptic neurotoxins using x-ray crystallography (Scott) and NMR structural analysis (Bieber).

Primary sequences have also been determined for two myotoxins from the venom of *Bothrops asper*. One, a potent PLA<sub>2</sub> with 67% sequence identity with ammodytoxin a, represents the first myotoxin phospholipase sequenced that lacks presynaptic neurotoxicity. Structural regions that may be responsible for neurotoxicity are proposed (Kaiser, Gutierrez, Plummer, Aird, and Odell; 1990). The second myotoxin sequenced was a lysine-49 PLA<sub>2</sub>, which had key amino acid differences from active phospholipases (Francis, Gutierrez, Lomonte, and Kaiser; 1991a). This work demonstrates that phospholipase activity *per se* is not required in phospholipase molecules for either myotoxicity or edema inducing activities.

During our earlier purification of several snake venom presynaptic neurotoxins, we observed two proteins which were less basic than either notexin or notechis II-5, but were as toxic as notexin in the venom of the Australian Tiger snake (*Notechis scutatus scutatus*). These new toxins,

called scutoxin A and B represent isoforms of notexin (Francis, John, Seebart, and Kaiser; 1991b). Sequencing results in collaboration with Dr. Jim Schmidt (USAMRIID), indicates that scutoxin A and B differs from notexin at only two position (Francis, Schmidt, and Kaiser, 1994).

Crotoxin subunits cross-linked using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, were non-toxic, had reduced immunological cross-reactivity toward mono- and polyclonal antibodies raised to the basic subunit, and had lost >95% of their phospholipase activity (Lennon, Plummer, and Kaiser; 1990). Lack of a cleavable cross-linker did not permit a determination of whether the loss of toxicity was due to either subunit cross-linking *per se* or modification of essential residues.

X-ray crystallography of Mojave toxin in Keith Ward's laboratory has not yielded well defined subunit interfaces. Diffractions from the basic subunit side of intact Mojave toxin crystals have been better than those from the acidic subunit side. Poor resolution of the acidic subunit suggests that this portion of the crotoxin heterodimer assumes multiple configurations. Synthesis of useful heavy atom derivatives to improve the phase information have been unsuccessful to date. Additional crystallization and heavy-atom replacements are still ongoing in collaboration with Dr. David L. Scott in Professor Paul Sigler's group at Yale. This group had preliminary results with crotoxin crystals that were promising, but they have apparently had problems in repeating those results and obtaining satisfactory crystals. Hence their interest in Mojave toxin.

**Mechanism of Action.** Crotoxin binding to synaptosomes and their membranes have revealed a high affinity ( $K_d = 2-4$  nM), specific binding site for crotoxin. Crotoxin binding was inhibited by several presynaptic neurotoxins, which were classified according to their inhibitory properties as strong (acidic subunit of crotoxin, Mojave toxin, concolor toxin, taipoxin, and pseudexin), moderate (ammodytoxin A and textilotoxin), weak (notexin and scutoxin A), very weak (notechis II-5) and non-inhibitory (basic subunit of crotoxin,  $\beta$ -bungarotoxin, *Crotalus atrox* and porcine pancreatic phospholipases  $A_2$ , dendrotoxin, and notechis III-4). Purified acidic subunit of crotoxin, the most potent competitor of crotoxin binding, was somewhat more competitive than intact crotoxin and the other strong inhibitors on a molar basis. Our results suggest that crotoxin and several other presynaptic



neurotoxins share common binding sites that may involve a protein on brain synaptosomal membranes (Degn, Seebart, and Kaiser, 1991). Other experiments in collaboration with Dr. Lance Simpson's laboratory (Trivedi, Kaiser, Tanaka, and Simpson, 1990), employing structurally different snake venom presynaptic neurotoxins, were carried out to determine whether neurotoxins undergo antibody escape. Preliminary results suggested that the basic subunit of crotoxin inserts into the cell membrane, but does not cross the membrane to exert its toxicity.

**Immunology.** Overlapping octapeptides, homologous with the complete sequence of the basic subunit of crotoxin were synthesized on polyethylene pins according to the procedure of Geysen *et al.* (1987). These peptides were used to screen our monoclonal antibodies prepared against the basic subunit of crotoxin (Kaiser and Middlebrook, 1988a,b) in an attempt to determine their antigenic determinants. Multiple assays demonstrated that none of the monoclonals recognized our solid-phase, linear peptides. When the same octapeptides were used to screen rabbit polyclonal antibodies raised to crotoxin, four regions of the basic subunit protein sequence repeatedly showed antigenicity. These were sequences 1-9, 31-44, 102-115, and 113-122, suggesting that these four regions within the basic subunit are antigenic. Four peptides corresponding to these antigenic regions were synthesized, conjugated to rabbit serum albumin, and rabbits immunized. Resulting antisera failed to neutralize against crotoxin's lethality in mice, although there was slight extension of survival times (Kaiser, unpublished data). Variations of this experiment are still being examined, because of the reported success of others in using the site-directed polyclonal antibody approach to localize the toxic site in the ammodytoxins from the venom of *Vipera ammodytes* (Curin-Serbec *et al.*, 1991).

**Molecular Biology.** Descriptions of the organization of genomic presynaptic neurotoxin genes is presently in its infancy, as is the expression of presynaptic neurotoxic PLA<sub>2</sub>s *in vitro*. We have been studying the genomic structure of the Mojave toxin genes, and have now completed that work, which will be described. Expression of the subunits of Mojave toxin and generation of their native forms in *E. coli* has proven to be a more difficult experimental problem. SDS-PAGE and western blots of acrylamide gels have indicated that we are clearly expressing both subunits in all of our

expression plasmid constructs; the difficulty is in the solubilization and re-oxidation of the subunits to their native form. Our results to date will be discussed.

### **Purpose of present work**

We are using multiple approaches employing the tools of biochemistry, immunology, and molecular biology to gain greater insight into presynaptic neurotoxin structure and function. Our goals are to learn more about (1) the primary, secondary, and tertiary structure of presynaptic neurotoxins, by amino acid sequencing; screening a variety of purified toxins for the presence of natural covalent modifications such as acylation, transglutamination, and glycosylation; chemical covalent modification such as alkylation and cross-linking of dimeric toxins; and in collaborative studies probing higher ordered structures through the use of high-resolution N.M.R. and x-ray crystallography. (2) Determine whether the PLA<sub>2</sub> activity associated with all presynaptic neurotoxins has the potential for inducing platelet-activating factor synthesis in endothelial cells. (3) Screen endogenous crude venoms for various enzyme inhibitors that might serve as potential therapeutic agents. (4) Use as tools the techniques of molecular biology and immunology to develop molecular approaches for the development of non-toxic synthetic vaccines against neurotoxins and determine critical residues in PLA<sub>2</sub>s that promote neurotoxicity, myotoxicity, and other biological activities.

### **Methods of Approach**

**Biochemistry.** a. Determine whether presynaptic neurotoxins are acylated, the extent and site(s) of acylation, and what the biochemical consequences are. b. Determine whether transglutaminases can form intra- or inter-molecular  $\epsilon$ -( $\gamma$ -glutamyl)-lysine isopeptide bonds in crotoxin and the resulting affects on phospholipase activity, neurotoxicity, and immunoreactivity. c. Determine whether there are any structural or functional differences between crotoxins that have had their His-47 selectively alkylated with p-bromophenylacetyl- or methyl-group. d. Screen a variety of venoms for glycoproteins and analyze (e.g. N- or O-linked, complexity, sugar content) the carbohydrate moieties present in the  $\gamma$ -

subunit of taipoxin, paradoxin, and textilotoxin. e. Prepare an enzymatically cleavable protein cross-linker. Use the linker to determine whether or not crotoxin subunit dissociation is essential for neurotoxicity. f. Provide Dr. Allan Bieber (Arizona State University) with sufficient Mojave toxin acidic subunit for 2-D and 3-D NMR investigations. g. Determine whether platelet-activating factor concentration is increased in endothelial cell cultures when exposed to presynaptic neurotoxins.

**Molecular Biology.** Develop a system in *E. coli* for the expression of Mojave toxin subunit cDNAs. Use this system to generate non-toxic, immunogenic forms of the basic subunit for immunization studies. Also use the expression system to generate site-specific mutant proteins of Mojave toxin for structure-function studies.

**Immunology.** a. Attempt to identify the conformational epitope recognized by our crotoxin neutralizing monoclonal antibody using multiple approaches. b. Initiate studies using murine antibody fragment clones specific for Mojave toxin in a bacteriophage  $\lambda$  immunoexpression library to determine their utility with neurotoxins.

**Exploratory studies.** Determine whether inhibitors for degradative enzymes exist in snake venom and carry out as much characterization as possible.

### Body of Report Methods, Results, and Discussion

#### a. Biochemistry.

1. During our PURIFICATION OF NEUROTOXIC PROTEINS from crude *Notechis scutatus scutatus* venom, we encountered a set of acidic proteins which were toxic when injected i.v. in mice at  $\approx 1 \mu\text{g/g}$ . These proteins promoted severe hemorrhaging when injected either i.v. or i.p. in mice, induced a rapid loss in blood pressure in mice when injected i.v., and displayed phospholipase activity against the small  $\text{PLA}_2$  substrate NOB (4-nitro-3-octanoyloxybenzoic acid) as well as phosphatidylcholine. [Initial studies indicated no phospholipase activity was associated with these acidic

proteins, which is incorrect.] These proteins also exhibited apparent molecular weights in the 18,000-21,000 range, slightly higher than the neurotoxin phospholipases (13,000-15,000). In addition, polyclonal antibodies raised to one of these acidic proteins recognized not only the other acidic proteins, but also notexin, scutoxin and notechis II-5, suggesting that the acidic proteins had antigenic regions similar to those in the neurotoxic phospholipases. Thus, it appeared that the acidic proteins could be PLA<sub>2</sub>-like proteins which were modified in some way to appear to have higher apparent molecular weights. We therefore examined these proteins in some detail for unusual structural modifications.

One publication (Francis, Williams, Seebart, and Kaiser, 1993) has been published describing this group of toxic acidic proteins called HT<sub>a</sub> to i. A polyclonal antibody raised against one of the proteins in the group HT<sub>g</sub>, binds to other purified proteins suggesting that they are isoforms of the same protein. Many other elapid crude venoms contain proteins which recognize the polyclonal antibody raised against HT<sub>g</sub>. Crotalid and viperid crude venoms do not recognize this antibody although some of their component proteins are known to exhibit hypotensive and hemorrhagic activities. A combination of gel-filtration on Sephacryl S-200, cation-exchange and anion-exchange chromatography allows isolation of the *N. s. scutatus* proteins in high purity. They are the first hypotension inducing proteins to be purified from an Australian elapid.

2. STRUCTURE OF A NEW TYPE OF PLA<sub>2</sub>. A related second manuscript (Francis, Coffield, Simpson, and Kaiser, 1994) has just been submitted for publication. It describes the structure of , HT<sub>e</sub>, one of the above mentioned acidic proteins and clearly has a phospholipase A<sub>2</sub> amino acid sequence. It contains 125 amino acids rather than the 119/120 found in other *N. s. scutatus* PLA<sub>2</sub>s, because it also has the loop of residues (62-66) found in helix D of pancreatic PLA<sub>2</sub>s, the  $\gamma$ -subunit of taipoxin and the D-subunit of textilotoxin. High sequence identity is found between the first 57 and the last 25 amino acids of HT<sub>e</sub> and other *N. s. scutatus* PLA<sub>2</sub>s. In the central section containing the pancreatic loop and the  $\beta$ -wing, sequence similarity with other *N. s. scutatus* PLA<sub>2</sub>s is low. The  $\beta$ -wing amino acids are highly homologous to taipoxin- $\gamma$ . HT<sub>g</sub>, an isoform of HT<sub>e</sub>, has an almost identical

sequence to HT<sub>e</sub> in the central section. Neuropharmacological and neurophysiological measurements show that HT<sub>e</sub> is not neurotoxic like notexin and consequently it exerts its toxicity through a different mechanism from the neurotoxic PLA<sub>2</sub>s. Unlike taipoxin-γ and textilotoxin-D, HT<sub>e</sub> and HT<sub>g</sub> are not glycosylated and are not otherwise modified. HT<sub>e</sub>, HT<sub>g</sub> and the other acidic proteins hydrolyze the synthetic PLA<sub>2</sub> substrate, 3-octanoyloxy-4-nitrobenzoic acid as well as L-α-phosphatidylcholine.

3. N.M.R. ANALYSIS. We prepared about 30 mg of purified acidic subunit of Mojave toxin for Dr. Allan Bieber at Arizona State University for him to initiate NMR structural analysis. Upon receipt, they attempted to remove a minor contaminant that could be separated by reversed phase chromatography and lost a portion of the material as a result of a flask implosion during lyophilization. They have isolated additional acidic subunit, taken initial spectra, and are in the process of initiating their structural analysis.

4. X-RAY STUDIES. After discussions with Dr. Keith Ward at the Naval Research Laboratory and Dr. David Scott, in Professor Paul Sigler's x-ray diffraction group at Yale, we purified about 25 mg of Mojave toxin and sent it to Scott. This is a "last gasp effort" to salvage a lot of work and effort originally carried out in collaboration with Dr. Keith Ward on the x-ray crystallography solution of crotoxin/Mojave toxin structure. To date, Scott has been able to grow small crystals, but nothing large enough to be useful in heavy-atom replacement studies which will be required to solve the structure. Attempts to grow larger crystals are continuing.

5. We have discontinued our work on the enzyme 'DESTABILASE' from leech saliva. Our source of saliva from Accurate Chemical and Scientific Corp. did not prove satisfactory. We found low protein content in the saliva from Accurate (≈60μg of protein/ml) and no esterase activity on a synthetic chromogenic substrate that had been shown by others to be hydrolyzed by 'destabilase'. We were unable to obtain saliva from medicinal leeches (*Hirudo medicinalis*) in our laboratory. We have therefore gone to chemically cleavable cross-linkers. Work with them has just gotten underway. We would like to continue work on 'destabilase' if a satisfactory source of leech saliva becomes available.

6. NEUROTOXIN BINDING SITE LOCALIZATION. In collaboration with Dr. Lance Simpson's laboratory, we have participated in a series of pharmacological experiments that were combined with physical chemistry experiments to identify the site at which phospholipase A<sub>2</sub> neurotoxins localize to produce their poisoning effects. This work has resulted in a novel conclusion about phospholipase A<sub>2</sub> neurotoxin action. Unlike most potent toxins that are enzymes, phospholipase A<sub>2</sub> neurotoxins do not appear to be endocytosed to exert their effects. Instead, these toxins bind to the cell surface and then undergo a molecular rearrangement that gives them access to substrates in the membrane. It is at this site that the toxins act to produce blockade of neuromuscular transmission. Details of this investigation may be found in the recent paper by Simpson, Lautenslager, Kaiser, and Middlebrook (1993).

7. SPIDER VENOM PROTEINS. Several years ago we collaborated with Dr. George Odell at Oklahoma State University on the isolation and characterization of two peptides that were isolated from the venom of the Mexican red knee tarantula (*Brachypelma smithii*). Preliminary results indicated that one of these peptides had sequence similarities to myotoxin a, a small protein present in venom from *Crotalus viridis viridis*. More recent work indicates that any similarities between these sequences are minimal. In spite of this, the work we did appeared significant enough to warrant publication. Dr. Steve Aird took this data with him when he left a postdoctoral position in my laboratory about four years ago with the intent to finish several relatively small experiments and ready the work for publication. Unfortunately he was unable to do this. We have now completed the necessary work and have recently submitted a manuscript describing the results. (see Kaiser *et al.*, 1994).

8. We have conducted experiments on the ACTIVATION OF CROTOXIN and porcine pancreatic phospholipase, in our standard phospholipase assay using phosphatidyl choline and with the synthetic substrate 4-nitro-3-octanoyloxybenzoic acid (NOB). With our standard assay, we see the characteristic activation with intact crotoxin, but not with the basic subunit. Porcine pancreatic PLA<sub>2</sub> shows about a 40-fold stimulation following incubation with the substrate for about 20 min. Using 1-Palmitoyl-2-[1-<sup>14</sup>C]oleoyl-*sn*-glycero-3-phosphocholine as substrate, with

crotoxin, we found at most 1-3% of the molecules monoacylated with the radioactive fatty acid. With porcine pancreatic PLA<sub>2</sub> no label was found to be incorporated. These values are substantially lower than expected, based on earlier work by Dr. Robert Henrikson using [<sup>14</sup>C]NOB. We have been unable to obtain any of this labeled substrate from Henrikson, and are presently attempting to have some synthesized by Dr. Bruce Branchini, from whom we have obtain unlabeled NOB. Once we obtain this labeled substrate, we will repeat our earlier studies and look for label incorporation into crotoxin and porcine pancreatic phospholipase.

9. GLYCOPROTEINS. Using an enzyme immunoassay for the detection of carbohydrate in glycoconjugates (from Boehringer-Mannheim), we screened a number of purified presynaptic neurotoxins available to us. Out of these (ammodytotoxin,  $\beta$ -bungarotoxin, caudotoxin, crotoxin, Mojave toxin, notexin, notexin II-5, pseudotoxin, textilotoxin, and taipoxin), only textilotoxin and taipoxin contained detectable carbohydrate. Higher molecular weight proteins in crude venoms from *Pseudechis porphyriacus* and *Notechis ater occidentalis* also gave strong positive signals for the presence of carbohydrates. Based on treatment of O- and N-glycosidases, these higher molecular weight proteins appear to be N-linked and not associated with presynaptic neurotoxins--our main interest.

Taipoxin was shown to be a moderately acidic sialo-glycoprotein (pI 5), heterotrimeric protein with a molecular weight of ca. 46 kD. The  $\gamma$ -subunit contains all of the carbohydrate which includes 4-5 residues of sialic acid, 4-5 residues each of N-acetyl-D-glucosamine and galactose, two residues of mannose, and one residue of fucose (Fohlman, Eaker, Karlsson, and Thesleff, 1976). Carbohydrate is N-linked through the Asn-78 in the  $\gamma$ -subunit (Fohlman, Lind, and Eaker, 1977). We will probably not characterize this carbohydrate moiety further. We are however interested in determining whether the carbohydrate moiety plays a functional role in either taipoxin or textilotoxin toxicity. Textilotoxin consists of five subunits, with two identical covalently-linked subunits of subunit D, which is the glycosylated moiety. Our intent is to determine whether it plays a functional role in textilotoxin lethality.

10. PLATELET ACTIVATING FACTOR. We have initiated studies to determine whether platelet-activating factor (PAF) is increased in cultured

endothelial cells in the presence of presynaptic neurotoxins. BvVe endothelial cells (passages 5-9) have been utilized for a series of PAF stimulation experiments with thrombin, ionophore A23187, crotoxin and other factors. Procedures described by Prescott et al. (1984) have been adapted for use with bovine rather than human cell lines. BvVe cells have been cultured with endothelial growth media in 25cm<sup>2</sup> flasks to average yields of  $7.6 \times 10^6$  confluent cells/cm<sup>2</sup> surface area. Modifications have been made in the cell stimulation and chloroform extraction procedures; including washing cells with Hank's buffered salt solution before treatments with thrombin or ionophore, increasing incubation times, increasing concentrations of thrombin or other factors, and pre-warming all solutions. Methodology for the cell culturing, chloroform extraction, thin-layer chromatography, and [<sup>14</sup>C]acetate incorporation have been worked out. We have observed no stimulation of PAF production in BvVe cells treated with crotoxin. However, our positive controls have not been particularly responsive. Thus, we have decided to examine PAF production in primary cultures of bovine umbilical cords. We are waiting for calving season this spring to obtain the required tissue, and will re-initiate the project when tissue becomes available.

11. An undergraduate in my laboratory has isolated an ANTIPROTEOLYTIC, ANTIHEMORRHAGIC PROTEINS from the sera of the the Western Diamondback rattlesnake (*Crotalus atrox*). Using a combination of separation techniques, he has isolated a single chain, 58 kD glycoprotein with an estimated pI-value of about 5.4. He followed the purification of this protein by developing a hide-powder assay system in lieu of a live animal assay, to follow the antiproteolytic activity. The first 21 amino-terminal amino acids were determined and shown to be FQLAG NMDVN TKGTK DWADI G... Data base searches indicate that this sequence has no identity with albumins,  $\alpha$ -globulins, or macroglobulins. Whether this protein has a potential for use in snake bite treatment remains to be determined. A manuscript describing the isolation and partial characterization of this protein is in preparation.

12. To screen for TOXIC SITES WITHIN NEUROTOXINS, we are using a biochemical approach in addition to the tools of immunology and molecular biology. We are presently doing an extensive comparison of the primary



sequences of toxic and non-toxic PLA<sub>2</sub>s by computer, and attempting to identify conserved regions in molecules with selected activities as well as by selected sequencing. We are just beginning this approach and have some preliminary indications that certain residues do appear to be conserved in myotoxic PLA<sub>2</sub>s when compared with non-myotoxic PLA<sub>2</sub>s. Further, sequencing studies of heterodimeric complexes from both crotalids and viperids suggest that selected residues are conserved in PLA<sub>2</sub>s that exist as aggregates (e.g. crotoxin, Mojave toxin, vipoxin from *Vipera ammodytes*, the complex from *Pseudocerastes fieldi*, myotoxin II from *Bothrops asper*, PLA<sub>2</sub> from *C. atrox*) as opposed to monomeric PLA<sub>2</sub>s. Once our MacQuadra 800 computer becomes available, we anticipate the increased computing capacity will greatly facilitate our sequence comparison studies. Approaches complementing this one are the mutagenesis studies using molecular biology techniques and the attempts at preparing antisera to peptides.

#### **b. Molecular Biology.**

1. GENOMIC CLONES. There have been a number of reports in the literature of PLA<sub>2</sub> cDNA clone isolation, but few reports describing the isolation of their genomic clones and none describing the isolation of genomic PLA<sub>2</sub> clones from snakes. As a first step in better understanding the evolution of presynaptic neurotoxins and gaining some insight into genomic control regions, we initiated the isolation and nucleotide sequencing of the genomic clones encoding both the non-neurotoxic, non-enzymatic acidic subunit and the toxic, PLA<sub>2</sub>-active basic subunit of Mojave toxin.

Mojave toxin is a heterodimeric, neurotoxic PLA<sub>2</sub> found in the venom of the Mojave rattlesnake, *Crotalus scutulatus scutulatus*, and is characteristic of all rattlesnake presynaptic neurotoxins. Both subunit genes share virtually identical overall organization, with four exons separated by three introns, which are inserted in the same relative positions of the genes' coding regions. The exon/intron structure is similar to that reported for mammalian PLA<sub>2</sub> genes. Most remarkable is the high degree of nucleotide sequence identity between the two subunit genes. While the exons share about 70% identity, the introns are greater than 90% identical and the 5' and 3' untranslated and flanking regions are greater than 95% identical. These findings support our earlier suggestion (Aird et al., 1985) that the genes

coding for the two subunits arose from a common ancestor. There has clearly been a strong selection on the nucleotide sequence of the non-coding regions during this evolutionary process. This is the first report of genomic sequences of PLA<sub>2</sub>-like proteins from snakes and is currently *in press* in Gene (John, Smith, and Kaiser, 1994). Plasmid subclones of the two subunits have been prepared which contain only those sequences which were reported in the Gene manuscript. Results are summarized in Figure 1.

## 2. EXPRESSION OF THE BASIC AND ACIDIC SUBUNITS OF MOJAVE TOXIN.

One of the technical difficulties of expressing eukaryotic proteins in a prokaryotic system, is proper folding of the protein and proper reoxidation of disulfide bonds. To this end, we have conducted a number of model studies on the reduction and re-oxidation of the basic subunit of Mojave toxin. In a buffer consisting of 50mM Tris-HCl (pH 8-8.5), 10-15mM cysteine, 10mM CaCl<sub>2</sub>, and 0.2-0.25% Triton X-100, we can reconstitute 40-45% of the PLA<sub>2</sub> activity of the reduced basic subunit over a 48-72 hour period. In addition, we have demonstrated that Factor X<sub>a</sub> activity is preserved in the presence of 0.2-0.25% Triton X-100 as long as 100mM NaCl is present.

In 1991 we constructed an *E. coli* expression vector (pTM-N) which contained the ompA signal peptide (21 amino acids) and a linker peptide (6 amino acids) linked to the amino-terminal end of the basic subunit protein, giving a primary product 149 amino acids long. The linker peptide contained the tetra peptide Ile-Glu-Gly-Arg, immediately ahead of the basic subunit protein's N-terminal His, which is recognized by the proteinase Factor X<sub>a</sub>. The vector is transcribed by T7 polymerase, which is present in the host bacterial chromosome under *lac* UV5 control and inducible by addition of IPTG. SDS-PAGE gels and western blots of cell extracts from induced cultures, indicated that 3-4% of the total cellular protein was represented by the basic subunit protein (122 amino acids), containing the 27 amino acid fusion peptide at the amino-terminal end, for a product of 149 amino acids in length.

As indicated, model studies on the reduction and re-oxidation of the basic subunit of Mojave toxin, indicated that we could reconstitute 40-45% of the PLA<sub>2</sub> activity of the reduced basic subunit. Using protein product recovered from our expression system and the same reduction and re-oxidation conditions, we were unable to generate any significant phospholipase activity. We attributed this to (i) the hydrophobic *ompA*

fusion peptide, which promotes solubility problems, and (ii) our inability to easily purify the expressed product, to eliminate contaminating and possibly interfering proteins. Because of this we have now designed and constructed four additional expression plasmids; two each for each subunit. They have all been designed to promote solubility of our product and facilitate its isolation and purification. I will describe the construction for one of these in some detail. The others were constructed in a similar manner. All are based on the pET-vector series.

We selected expression vector pET 19b (Novagen, Madison, WI), which contains a leader peptide coding for a polyhistidine region (Met-Gly-His<sub>10</sub>) followed by the underlined enterokinase cleavage site (Ser-Ser-Gly-His-Ile-Asp-Asp-Asp-Lys), immediately on the amino-side of the N-terminal His of the basic subunit protein. The polyhistidine and enterokinase regions should provide a hydrophilic tail, improving the solubility of our expressed fusion protein product. In addition, the polyhistidine region can be used as a handle to affinity purify the product on a Ni-column. Construction of the vector and cDNA are outlined in Fig. 2. As with the pTM vector, the pET-vector series is also transcribed by T7 RNA polymerase which is constructed into the host cell chromosome under *lacUV* control and can be induced by IPTG.

Transformed BL21 (no T7 pol gene, consequently a non-expressing host strain) and BL21(DE3) (contains T7 pol gene) cells, containing our newly constructed expression plasmid grew without a problem in L-broth. When IPTG was added to both cultures at mid-log phase, there was no evidence of a newly synthesized protein in the 18 kD molecular weight region on SDS-PAGE in BL21 cells, but a prominent band was present in the BL21(DE3) cells. Western blots using polyclonal antibodies raised to Mojave toxin basic subunit also gave a strong signal in the 18 KD region from extracts from BL21(DE3) cells (see Fig. 3). Sequencing of the expression vector containing the cDNA insert, identified as pTJ7-133#a16, gave the expected sequence with no deletions at the ligation junction sites that would alter the reading frame of the cloned cDNA.

A construct similar to this was prepared using the cDNA for the pro-acidic subunit of Mojave toxin. When expressed in BL21(DE3) cells, a new protein of the appropriate molecular weight was found in the cell extract as determined by SDS-PAGE. Western blots using rabbit polyclonal raised to the acidic subunit, but not the basic subunit, gave a strong signal at the expected

position. We are now in the process of working on the isolation and re-oxidation of the expressed protein.

We have also just completed the construction of two additional expression vectors (using Novagen's pET-17xb), one with the proacidic subunit cDNA and one with the basic subunit cDNA. In both constructs, an N-terminal leader of the first 265 amino acids of the T7 phage gene 10 protein, is followed by the five amino acids which make up the enterokinase proteolytic site, is followed by the 122 amino acids of the appropriate subunit protein. We are hopeful that the long leader in the fusion product will help solubilize the expressed subunit proteins. Induction in host strain BL21(DE3)pLysE shows good expression when the extract is run on SDS-PAGE and western blots using the homologous polyclonal antibody raised to either the basic or acidic subunit gives a strong signal. Thus, we are overexpressing both subunits. Studies on the isolation and reoxidation of the expressed protein are in progress.

3. MUTAGENESIS STUDIES. Even though we have not yet been able to recover biological active basic subunit of Mojave toxin from expressed protein in *E. Coli*, we are initiating mutagenesis studies. This will enable us to move ahead quickly once we are able to reconstitute activity in our expressed subunits. Mutagenesis will allow us to make single amino acid changes or more extensive changes in a region believed involved in a specific biological activity. We are using the mutagenesis scheme described by Deng and Nickoloff (1992) called 'mutagenesis by unique site elimination'. Enzymes and reagents for this method are offered by Clontech (Palo Alto, CA).

### c. Immunology.

1. ANTIGENICITY OF POST-SYNAPTIC NEUROTOXINS. During our purification of proteins from *Notechis scutatus scutatus* using cation-exchange chromatography, we identified several basic proteins with apparent molecular weights of  $\approx 11$  kD. We have demonstrated that one of these proteins is notechis III-4, a "long" postsynaptic neurotoxin containing 73 amino acids (Halpert et al., 1979). Polyclonal antibodies raised against notechis III-4 has been used to investigate the presence of similar postsynaptic toxins in *N. s. scutatus* venom and the venoms of other snakes.

Our recent paper (Francis, Tanaka, and Kaiser, 1993) demonstrates that polyclonal antibodies raised against purified notechis III-4, a postsynaptic neurotoxin from the Australian tiger snake, *N. s. scutatus*, recognize conformational and linear epitopes in notechis III-4 but only conformational epitopes in other *N. s. scutatus* venom proteins including notexin and notechis II-5. Notechis III-4 is markedly deficient in venom from *N. s. scutatus* collected near Lake Alexandrina in Australia, and absent from venoms of *Notechis ater* subspecies, crotalids, and viperids. Of six other elapid venoms screened, only *Bungarus multicinctus* and *Pseudonaja textilis* showed weak cross-reactivity. Reactive protein species in these venoms include the long post-synaptic neurotoxins  $\alpha$ -bungarotoxin and pseudonajatoxin b. Surprisingly the polyclonal antibodies were only weakly neutralizing against notechis III-4 lethality. These studies indicate that the most antigenic regions of the long postsynaptic toxins are the variable or non-conserved regions. A new method for purification of notechis III-4 is reported.

2. TOXIC SITES OF RATTLESNAKE PRESYNAPTIC NEUROTOXINS. Based in-part on some of our earlier unpublished work involving the Geysen procedure and studies reported on ammodytoxin by F. Gubensek, we had five peptides synthesized corresponding to different regions of crotoxin/Mojave toxin. These peptides corresponded to different regions of the basic subunit and are identified as follows: K1 = amino acids 1-9; K2 = 31-44; K3 = 90-103; K4 = 102-114, and K5 = 113-122. Each peptide was attached to the new multiple antigenic peptide system (MAPS), which utilize the  $\alpha$ - and  $\epsilon$ -amino functional groups of lysine to form a backbone to which multiple peptide chains can be bound. We used an eight peptide branch core to which identical peptides were attached, giving a synthetic system purported to be highly antigenic. Two rabbits were immunized with each peptide using Titermax adjuvant, followed by four boosts. Our results have been disappointing and are briefly summarized below.

i) When reacted with homologous antigen, anti-K3 reacts strongly (1000-fold dilution); anti-K2 and K5 reacts moderately (10-50-fold dilution); anti-K1 and K4 were very weak (<10-fold dilution).

ii) Antisera generated against intact crotoxin as well as the individual subunits of crotoxin did not react with any bound K1-K4 peptides. Peptide K5 was recognized moderately by antisera against intact crotoxin as well as

monoclonal antibodies 2, 1, and 5. However, when peptide K5 was pre-incubated with either the polyclonal or monoclonal antibodies prior to mixing with crotoxin, their ability to inhibit the toxin's PLA<sub>2</sub> activity or lethality was not affected.

iii) Antisera raised in only one of two rabbits immunized with K5 reacted with either basic subunit or intact crotoxin (250-fold dilution). This antisera had no affect on crotoxin's PLA<sub>2</sub> activity or lethality. Antisera from none of the other 9 rabbits reacted with intact crotoxin.

We are continuing this approach by conjugating purified peptides to keyhole limpet hemocyanin and immunizing rabbits using Freund's adjuvant. These two modifications are the major experimental differences between our protocol and that of Gubensek's, who had success with this same basic approach using peptides derived from the sequence of ammodytoxin.

3. We have set up the RECOMBINANT PHAGE ANTIBODY SYSTEM using the methodologies provided in kit form by Pharmacia. BALB/c mice were immunized with the basic subunit of crotoxin, boosted twice, and their spleens removed. Spleen cells were dispersed and incubated with 1 $\mu$ M biotinylated intact crotoxin. Streptavidin-paramagnetic particles were added to the cell suspension to form the biotin-streptavidin complex. Magnetic particles were captured with a magnetic stand and mRNA extraction carried out on the recovered, bound cells. cDNAs were prepared from the isolated mRNAs and amplified to give recombinant antibody DNA. A DNA gel showed the major band to migrate as a 750bp component, which is the size expected for the amplified DNA. No other major contaminating bands were present. Optimized electroporation using commercial high efficiency competence (XL1-blue) cells gave a library containing  $1.5 \times 10^{-6}$  clones. Six clones were selected which bind strongly to intact crotoxin. Acidic subunit of crotoxin is not bound. Cross-reactivity of these clones to other related and unrelated protein was tested. Five unrelated proteins and eight Group I PLA<sub>2</sub>s showed no reaction. Of four Group II PLA<sub>2</sub>s tested, only homologous Mojave toxin and concolor toxin cross-reacted. These findings suggest that the clones are specific to crotoxin and related rattlesnake presynaptic neurotoxins. Reduced and alkylated basic subunit of crotoxin did not react, indicating that the epitopes bound are likely to be conformational.

Epitope comparisons carried out with the six clones indicate that all of them share the same or similar antigenic regions. It is possible that they all

are siblings of each other. For that reason, clone 10G was selected and partially sequenced. Preliminary results indicate that germline DNA Ig V2b-3 gene fragment was used to code for the heavy chain variable region. Additional sequencing is in progress. Attempts to assay inhibition of crotoxin's phospholipase activity and lethality by the clone have been unsuccessful, because phage concentrations of only  $10^{13}$ /ml have been obtained. This concentration is too low for these type of assays. Work is now in progress to express this recombinant phage antibody as a soluble protein, which would give sufficient material for biochemical analyses.

#### d. Exploratory Studies.

1. NATURAL METALLOPROTEINASE INHIBITORS. During our earlier work on the isolation of *Bothrops asper* phospholipase A<sub>2</sub>s, we observed that these venoms also have metal-ion dependent proteases and high concentrations of low molecular weight peptides. *In vitro* experiments demonstrated that two of these peptides, pyroglutamate-glutamine-tryptophan and pyroglutamate-asparagine-tryptophan, are inhibitors of the metalloproteinases with IC<sub>50</sub>-values of about 0.3 mM for pEQW. Based on the concentrations present in the venom glands, the peptides appear to inhibit the venom proteinases while in the venom gland, thereby protecting the snakes from their own proteinases during venom storage. A manuscript describing these results (Francis and Kaiser, 1993) has been published in *Toxicon*.

This paper is of interest for several reasons. First, it demonstrates a function for the relatively high concentrations of peptides found in *B. asper* venom. It also describes another mechanism by which snakes can protect themselves from their own toxic proteins during storage, and suggests how these naturally occurring peptides may be useful in preparing more potent inhibitors of proteinase activity which might be useful for snake bite therapy. Finally, the results described above, may also be applicable in the clinical treatment of tetanus and botulism. If the recent report by Schiavo *et al.* (1992) proves correct and tetanus and botulinum toxins are zinc endoproteinases and this activity is essential for intoxication, then inhibitors based on the tripeptide structures we isolated should be screened for their inhibitory properties of these neurotoxins.

2. MICROSCOPY STUDIES. A postdoctoral associate in my laboratory for the last six months, Dr. Gabriela Canziani, devoted most of her time to light and electron microscopy studies on tissues from crotoxin-treated mice. One project was directed toward the visualization of crotoxin on ultrathin sections of intoxicated neuromuscular junctions from systemically intoxicated mice. We attempted to determine whether there was any evidence of toxin internalization of the neurotoxin. Tissue was removed from animals sacrificed up to two hours following injection of 2 LD<sub>50</sub>'s of crotoxin, embedded and fixed in the absence of osmium tetroxide, and treated with polyclonal antibodies raised against the basic subunit of crotoxin. This was followed by incubation with a second antirabbit-antibody conjugated to gold spheres. Current methodology appears to be adequate to visualize crotoxin. The amount of labeling observed is quite limited on the neuromuscular junctions isolated from the diaphragm. At present we have no evidence of crotoxin localization nor internalization. Dr. Robert Jenkins and Dr. Canziani (who is now at USAMRIID) are continuing these studies in an attempt to get a definitive answer, since the methodology now appears to be satisfactory.

Histological and cytological examination of muscle tissues of mice which have an acquired resistance to crotoxin have been carried out in conjunction with Dr. J. C. Vidal (see Okamoto *et al.*, 1993). These studies indicate a dramatic alteration of diaphragm muscle organization in mice treated with low doses of crotoxin, that are now resistant to up to 12 LD<sub>50</sub>'s of the toxin. Collagen appears to replace 80-90% of the diaphragm muscle tissue. There is evidence of muscle fibre regeneration if crotoxin administration is halted. Dr. Canziani is preparing a manuscript describing these observations. We could find no previous description in the literature of chronic, systemic intoxication by neurotoxins with PLA<sub>2</sub> activity.

### CONCLUSIONS

A group of acidic proteins were isolated from *N. s. scutatus* venom which had i.v. LD<sub>50</sub>-values in the 0.5-1.5 µg/g range in mice. Their apparent molecular weights are in the 18-21 kD range (SDS-PAGE) and they are antigenically related. Neuropharmacological and neurophysiological measurements show that one of these proteins (HT<sub>e</sub>) is not neurotoxic like notexin and consequently exerts its



toxicity through a different mechanism from the neurotoxic PLA<sub>2</sub>s. The acidic proteins promote hypotension and hemorrhage in mice and show PLA<sub>2</sub> activity against both the synthetic substrate 3-octanoyloxy-4-nitrobenzoic acid and phosphatidylcholine. Polyclonal antibodies raised to one of these (HT<sub>g</sub>) also cross-reacts with notexin, scutoxin and notechis II-5, suggesting that these acidic proteins have antigenic regions similar to those in the neurotoxic phospholipases. Sequencing of one of these toxins (HT<sub>e</sub>) reveals it has a PLA<sub>2</sub>-like amino acid sequence, contains 125 amino acids rather than the 119/120 found in other *N. s. scutatus* PLA<sub>2</sub>s, because it also has the loop of residues (62-66) found in helix D of pancreatic PLA<sub>2</sub>s, the  $\gamma$ -subunit of taipoxin and D-subunit of textilotoxin. It is not glycosylated or otherwise modified. It is then, a new type of toxic PLA<sub>2</sub>.

Preliminary results obtained with Dr. A. Bieber on the N.M.R. structural analysis of the acidic subunit of Mojave toxin are promising and are continuing. Attempts by Dr. David Scott to prepare crystals of Mojave toxin suitable for heavy-atom replacement x-ray analysis studies have been unsuccessful to date, but are also continuing.

Work on the enzyme 'destabilase' from leech saliva have been discontinued until a satisfactory source of leech saliva becomes available.

Collaborative experiments in conjunction with Dr. Lance Simpson's laboratory, indicate that PLA<sub>2</sub> neurotoxins are not endocytosed to exert their effects. PLA<sub>2</sub> neurotoxins appear to bind to the cell surface and then undergo a molecular rearrangement that gives them access to critical substrates in the membrane which leads to a blockade of neuromuscular transmission.

We find little (1-3% of molecules) acylation of crotoxin following incubation with 1-palmitoyl-2-[1-<sup>14</sup>C]oleoyl-*sn*-glycero-3-phosphocholine substrate. Work with [<sup>14</sup>C]NOB is still in progress.

Of ten PLA<sub>2</sub> presynaptic neurotoxins examined, we found only textilotoxin and taipoxin contained detectable carbohydrate. The  $\gamma$ -subunit of taipoxin and D-subunit of textilotoxin are the glycosylated moieties. Studies on the functional role of the

carbohydrate in textilotoxin lethality are in progress.

A system for examining the effects of various factors on platelet activating factor (PAF) generation in cultured endothelial cells has been established. To date we have not observed stimulation of PAF production in bovine BvVe cells treated with crotoxin. We need to examine PAF production in primary cultured cells rather than cells in later passages (passage 5 to 9).

An antiproteolytic, antihemorrhagic protein (single chain, 58 kD glycoprotein,  $pI \approx 5.4$ ) has been isolated and partially characterized from *C. atrox* sera. It has no identity with albumins,  $\alpha$ -globulins or macroglobulins. It is quite effective in neutralizing protease activity *in vitro* and when pre-incubated with the protease prior to injection into mice.

Based on amino acid sequencing studies and sequence comparison by computer, we have indications that we may be able to identify residues within PLA<sub>2</sub>s that are associated with the activities of neurotoxicity, myotoxicity, and dimerization.

Isolation and sequencing of the genomic clones encoding both the non-neurotoxic, non-enzymatic acidic subunit and the toxic, PLA<sub>2</sub>-active basic subunit of Mojave toxin have revealed virtually identical overall organization. Most remarkable is the high nucleotide sequence identity between the two subunit genes, with the introns showing >90% identity.

We have constructed plasmid expression vectors for both the acidic and basic subunits of Mojave toxin using their cDNAs. SDS-PAGE and western blots indicate good expression of both subunit proteins in *E. coli* following induction. Isolation and reoxidation of expressed proteins are in progress. It appears that we will be able to use the mutagenesis procedure identified as 'mutagenesis by unique site elimination' to generate mutant proteins of both subunits of Mojave toxin.

Immunology studies with the "long" postsynaptic neurotoxin notechis III-4 from *N. s. scutatus* indicate that most antigenic regions of these neurotoxins are the variable or non-conserved regions. Polyclonal antibodies raised to the protein appear to be weakly neutralizing and show some cross-reactivity with conformational epitopes in the presynaptic neurotoxins of notexin

and notechis II-5.

Attempts to raise polyclonal antibodies to peptides from various regions of the basic subunit of Mojave toxin have been disappointing. We have used two different carrier proteins, rabbit serum albumin and a multiple antigenic peptide which utilizes the  $\alpha$ - and  $\epsilon$ -amino groups of lysine. Peptides conjugated to either carrier has not generated useful antibodies. We are repeating this experiment using two major changes in our methodology, namely keyhole limpet hemocyanin as carrier and immunizing rabbits with Freund's adjuvant.

We have successfully generated a synthetic antibody that is reactive against the native, basic subunit of Mojave toxin using the recombinant phage antibody system. We are just completing its sequence determination and are attempting to clone it into an *E. coli* expression plasmid so we can generate sufficient material for biochemical analysis.

*B. asper* venom contains naturally occurring tripeptides (pyroGlu-Gln-Trp and pyroGlu-Asn-Trp) that are inhibitors of metalloproteinases with  $IC_{50}$ -values of about 0.3 mM. These inhibit the proteinases during storage in glands. These peptides may have inhibitory activities toward the toxic bacterial zinc proteinases (tetanus and botulinum toxins).

Microscopy methodology for locating crotoxin at neuromuscular terminals appears to have been satisfactorily developed, and work is continuing with Drs. Jenkins and Canziani. Histological and cytological examination of muscle tissues in mice which have an acquired resistance to crotoxin indicate a dramatic alteration in diaphragm muscle organization. Collagen appears to replace 80-90% of diaphragm muscle tissue. Muscle fibre regeneration occurs if crotoxin administration is halted.

### RECOMMENDATIONS

1. Continue our collaborations with Bieber and Scott on the N.M.R. structural analysis of the acidic subunit of Mojave toxin and the x-ray crystallographic analysis of intact Mojave toxin.
2. Carry on with our microscopy studies in collaboration with

Drs. Jenkins and Canziani. This work complements the neuropharmacology work in Simpson's laboratory. Initiate preparation of some of this work for publication.

3. Attempt to obtain [ $^{14}\text{C}$ ]NOB for acylation experiments involving neurotoxic and non-neurotoxic PLA<sub>2</sub>s.

4. Examine the effects of covalently bound carbohydrate on textilotoxin function by enzymatic deglycosylation of the protein and then subsequently conducting phospholipase assays, toxicity assays in mice, etc.

5. Explore the effects of presynaptic neurotoxins on platelet activating factor production in a primary culture of endothelial cells.

6. Continue our laboratory work and computer analyses of PLA<sub>2</sub> sequences in an effort to identify residues associated with the biological activities displayed by these molecules (e.g. neurotoxicity, myotoxicity, dimerization, etc.).

7. Carry out sequence comparisons of our genomic clones for the subunits of Mojave toxin with other genomic sequences when they become available to search for sequence similarities. These searches may suggest additional control and regulatory regions that may exist within these sequences.

8. Persist in our attempts to raise functional polyclonal antibodies against peptides corresponding to regions of the basic subunit of Mojave toxin, conjugated to keyhole limpet hemocyanin.

9. Continue to work on the isolation and oxidation of expressed subunits of Mojave toxin. This has a high priority. Develop the 'mutagenesis by unique site elimination' system in an effort to be able to express a full-length, non-toxic basic subunit of Mojave toxin that is immunologically cross-reactive with native basic subunit. Test the ability of this non-toxic form of the basic subunit to generate neutralizing antibodies against native Mojave toxin.

10. Further characterize and explore the properties and utility of the 'synthetic antibody' generated with the recombinant phage antibody technique, against the native, basic subunit of Mojave toxin.

11. Determine whether transglutaminases can form inter- or intra-molecular  $\epsilon$ -( $\gamma$ -glutamyl)-lysine isopeptide bonds in crotoxin and the effects of such bonds on different biological functions.

12. Complete the preparation of manuscripts for publication describing (i) the anti-hemorrhagic protein from blood sera of *C. atrox* and (ii) the primary sequence of scutoxin A and B.

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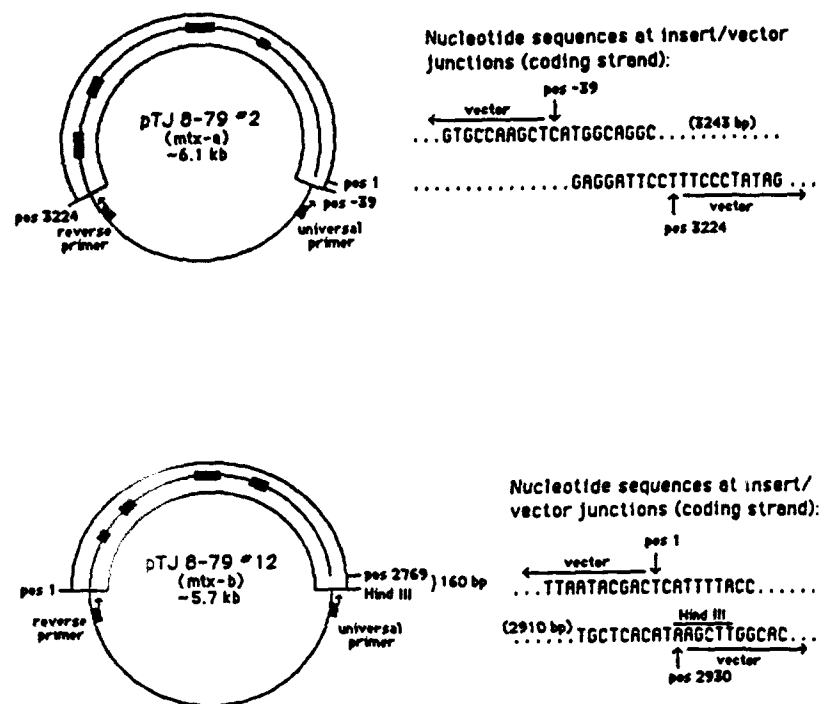


Fig. 1. Plasmid subclones of Mojave toxin acidic and basic subunits have been prepared which contain only those sequences which were reported in the *Gene* manuscript which is currently *in press*.

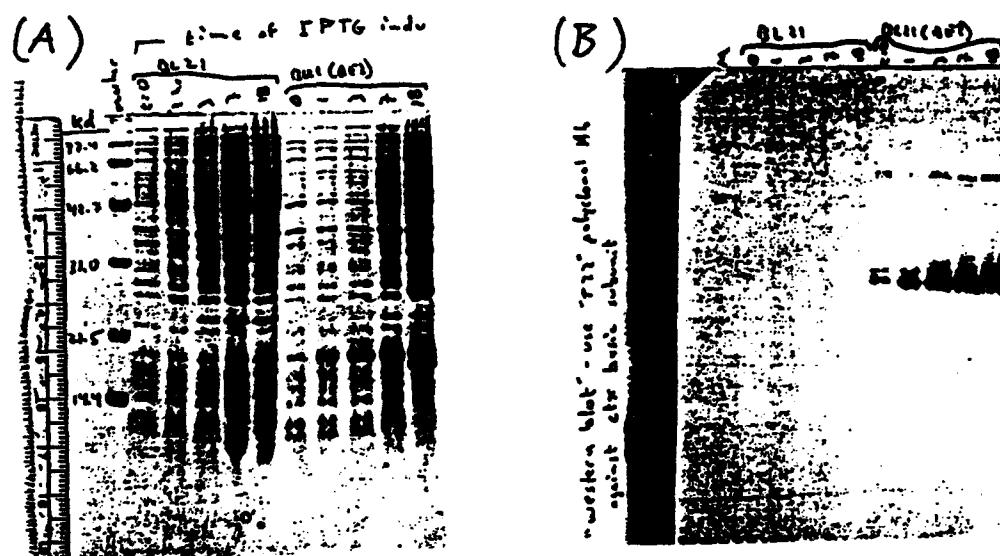
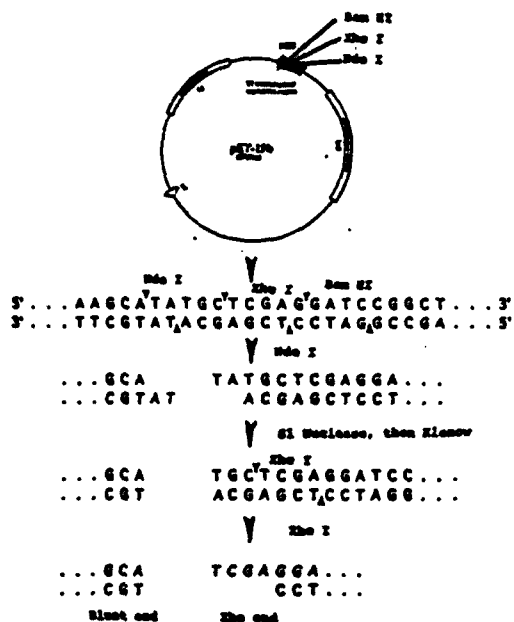


Fig. 3. (A) Coomassie stained SDS-PAGE on 15% gels of *E. coli* BL21 and BL21(DE3) samples containing pTJ7-133#a16 after induction with 1 mM IPTG. Cells were removed at 0, 1, 3, 7, and 18 hours after addition of IPTG, centrifuged, resuspended in protein solubilizing solution and applied to the gel. (B) Western blot of a gel identical to (A). Primary antibody used was raised in rabbits against the basic subunit of Mojave toxin. Amounts of protein added per slot were not constant in this experiment.



## Vector construction



## Insert preparation

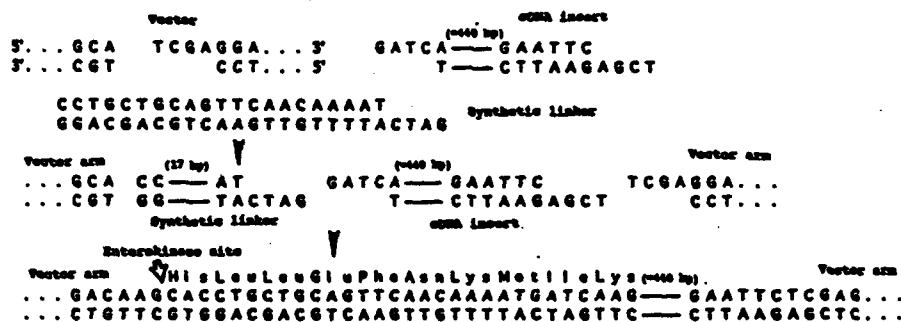
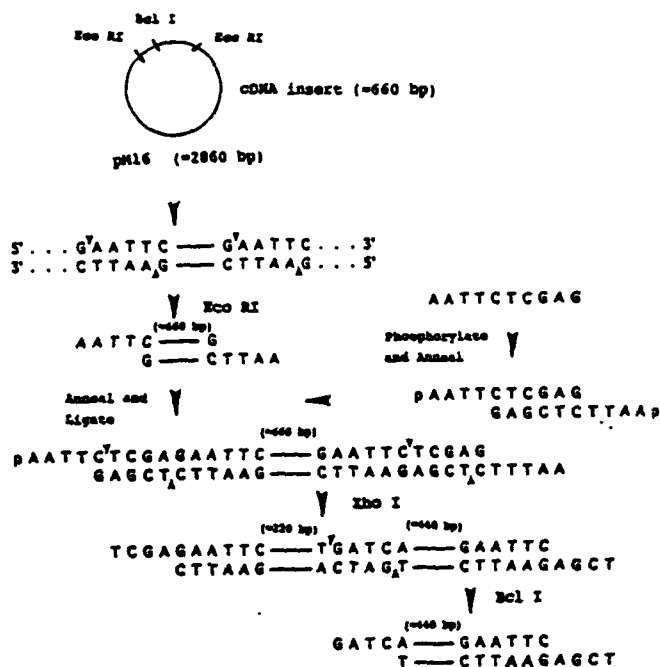


Fig. 2. Construction of pTJ7-133#a16. The insert was prepared as outlined in the above scheme and contained the cDNA fragment containing the coding sequence for the basic subunit of Mojave toxin. It was cloned into expression vector pET-19b as shown. A synthetic linker and adaptor were synthesized to generate the final construct, which included an enterokinase site immediately adjacent to the N-terminal His of the basic subunit protein.