THE USE OF AN AFFINITY PURIFIED ANTIBODY TO DEVELOP THE ANTIGEN AND ANTIBODY ELISA, TO MEASURE THE BLOOD DISAPPEARANCE OF ANTIMYOTOXIN AND TO SCREEN VENOMS FOR MYOTOXIN A

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NOMENCLATURE

A_{280}	Absorbance at 280 nm
A_{405}	Absorbance at 405 nm
BSA	Bovine Serum Albumin
DW	Distilled Water
ELISA	Enzyme-linked immunosorbent assay
g	Gram
Hac	Acetic acid
LD_{50}	Lethal dose 50%
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Milliter
NaCl	Sodium chloride
NaN_2	Sodium azide
NaOH	Sodium hydroxide
nm	Nanometer
PBS	Phosphate buffered saline
PSS	Physiological saline solution
SE	standard error
µg	microgram
µl	microliter

CHAPTER I

INTRODUCTION

Snake venom is a complex mixture of proteins, carbohydrates, nucleotides and other components which can cause deleterious effects such as systemic hemorrhage, local tissue damage and death (Tu, 1977). The most common treatment for rattlesnake venom poisoning in the United States and parts of Central and South America is the use of a polyvalent antivenom known as Antivenin (Crotalidae) (Russell et al., 1973).

Treatment of the snakebite victim may involve the intravenous administration of ten or more units of the antivenin, and still there may be the loss or severe crippling of the hand or foot (Lockhart, 1977). Therefore, the effectiveness of polyvalent antivenin in treating local myonecrosis has been questioned (Stanic, 1969; Glenn and Straight, 1977). It has been demonstrated that antivenin has the ability to neutralize both hemorrhage (Ownby et al., 1984) and lethality (Ownby et al., 1983a) caused by Prairie rattlesnake (Crotalus viridis viridis) venom. However it has little ability to neutralize local myonecrosis (Ownby et al., 1983a). Antiserum to myotoxin a, a pure toxin isolated from C. v. viridis venom (Ownby et al.,

1976), is more effective than polyvalent antivenin in neutralizing the myotoxicity produced by both pure myotoxin a and crude C. v. viridis venom (Ownby et al., 1983a). These results correlate well with the very low titer of antibodies to myotoxin a detected in polyvalent antivenin using immunodiffusion or the enzyme-linked immunosorbent assay (ELISA) (Ownby et al., 1983b). Thus, an antiserum containing antibodies to myotoxin a offers the possiblity for improvement in the treatment of snakebite poisoning.

Myotoxin a is the major myotoxic component of C. v. viridis venom (Cameron and Tu, 1977) and produces myonecrosis very similar to the crude venom (Stringer et al., 1972). It has also been detected in the venoms of other rattlesnakes Crotalus durissus terrificus (Cameron and Tu, 1978), Crotalus viridis concolor (Pool et al., 1981) and Crotalus viridis helleri (Maeda et al., 1978). Treatment following poisoning by these rattlesnakes and possibly others should take into consideration the presence of myotoxin a. Currently, however, very little is known regarding the amount of antivenin to administer or how the presence and amount of venom alters the disappearance of the antivenom. Improvement in snakebite treatment, therefore, must involve assays that are able to detect and moniter antiserum levels. In addition, an assay that can detect specific toxic components in venoms could provide extremely useful information to the clinician for proper treatment of snakebite poisoning.

The enzyme-linked immunosorbent assay (ELISA) is a test that is both sensitive and rapid. The ELISA system has been used for antigen detection (Minton, 1977; Ho et al., 1986a), however its sensitivity to a large degree is dependent on the quality of the reagents (Ho et al., 1986a). Ho et al. (1986a) have used a purified IgG fraction obtained from a protein A column to improve the sensitivity of their venom detection assay. A similar approach should also be taken for an antibody detection ELISA system.

The use of an affinity purified antibody, specifically antimyotoxin, could 1) provide a sensitive assay with low non-specific binding, 2) permit a rapid test for the monitoring and quantitation of antiserum in blood samples, 3) possibly be used for the neutralization of its specific antigen and 4) also be helpful in establishing an antigen detection ELISA system useful in the screening of venoms for the presence of myotoxin a.

Therefore, in this study antibodies to myotoxin a, subsequently called myotoxin, were affinity purified and tested for their ability to neutralize local myonecrosis. The affinity purified antimyotoxin protein was used to set up an ELISA system for the detection of antibodies to myotoxin a in blood samples from mice, and to establish an ELISA for antigen detection. The antibody detection assay was used to detect changes *in vivo* in the level of antimyotoxin in the presence and absence of myotoxin a over a

given period of time. In addition, a myotoxin detection ELISA was used *in vitro* to screen numerous crotalid venoms from various geographic regions for the presence of myotoxin.

CHAPTER II

LITERATURE REVIEW

Snakebite Management

The incidence of snakebites in the United States has been estimated for the period 1950-1959 to be 45,000 bites per year (Parrish, 1980), of which 8000 are due to venomous snakes and of these 6800 are reported (Russell, 1969). In the United States alone there are about 120 species of snakes of which approximately 20 species have proven poisonous (Russell et al., 1975). The vast majority of the poisonous snakes in the United States are pit vipers, subfamily Crotalidae. Pit vipers include rattlesnakes, genus Crotalus; cottonmouths and copperheads, genus Agkistrodon; plus the massasauga and pigmy rattlesnakes, genus Sistrurus (Clement, 1979). Although pit viper bites are seldom fatal, fewer than twelve deaths per year (Minton, 1971; Glass, 1976), local tissue damage with the sequelae of loss of function, deformity and even amputation proves a persistent problem in snakebite treatment.

The treatments recommended for snakebite envenomation are still today considerably varied and controversial. The lack of a standardized treatment for snakebite envenomation

is due partially to the fact that the effects following envenomation are related to the species of the snake, the quantity of venom injected, age and condition of the victim, and the length of time which has elapsed before seeking treatment (Klauber, 1956; Parrish and Hayes, 1970; Ho et al., 1986b). The time lapse before the snakebite victim receives professional attention can vary extensively especially with geographical region (Lockhart, 1965: Glenn et al., 1983: Reid and Theakston, 1983).

Snakebite management varies from the very aggressive surgical method of Huang et al. (1973) which includes excisional therapy along with extensive incision and debridement of the bite site including the subcutaneous tissue, to the less invasive treatment of antivenin administration. Prior to 1970 the most frequent treatments used were cryotherapy, incision and suction, steriods, and polyvalent antivenin (Huang et al., 1978).

Excisional Therapy

Excisional therapy is the surgical removal of the envenomated tissue. The rational behind the use of excisional therapy in the case of a snakebite is the physical removal of venom which remains localized at the bite site for a short period of time (Huang, 1974). The systemic effects are hopefully also lessened since the blood levels of the venom are decreased by the tissue removal. However, this somewhat primative treatment of

snakebite by excision of tissue at the bite site has never shown significant benefit (Gennaro and McCullough, 1961). Huang et al. (1974) have reported treatment of 54 pit viper bites using excisional therapy, and although there was no mortality, the patients showed signs of necrosis, sloughing of skin grafts and deformities. Gutierrez et al. (1984) have shown that skeletal muscle regeneration does occur after myonecrosis induced by Bothrops asper venom. Removal of necrotic tissue may eliminate the myogenic cells responsible for muscle regeneration.

Incision and Suction

It has also been reported that treatment of snakebite by incision and suction can reduce the venom dose by fifty percent if administered within three minutes of the snakebite. After longer periods of time the venom binds to the tissue and is no longer available for removal by this method (Gennaro, 1977). Therapy by incision and suction has never been shown to benefit human victims of viper bites, since usually sufficient time has elapsed that makes any attempt to remove the venom by this method useless (Arnold, 1979). In fact prolonged suction may result in additional tissue damage (McCullough and Gennaro, 1968). Incisions can also aggravate bleeding, especially in bites causing non-clotting blood, as well as damaging nerves and tendons, introducing infection and delaying healing (Reid and Theakston, 1983).

Cryotherapy

The use of cryotherapy for snakebite victims was recommended twenty-five years ago as a means to decrease the enzymatic activity of the venom by lowering the temperature of the affected limb (Arnold, 1975), and thus reducing local tissue damage until hospital treatment might become available (Gennaro and Watt, 1977). Cryotherapy is the practice of packing the site of envenomation in ice or immersing it in ice water for hours to days in order to prevent tissue destruction (Lockhart, 1965; Stahnke and McBride, 1966). However, the use of cryotherapy for treatment of snakebite envenomation has been shown to cause additional tissue damage (Bennett et al., 1961; McCullough and Gennaro, 1970). Gennaro et al. (1977) reported the results of a clinical analysis of thirty-six snakebite victims. They found that seventy-five percent of the amputations had received cryotherapy from a period of twelve hours to three weeks. Cryotherapy has also been investigated experimentally by Yap and Perry (1960) and Gill (1970). Their results indictate an increase in local tissue damage and a prolonged disability when cryotherapy is used.

Tourniquets

In the past it has been suggested by investigators

(Trinca, 1963; Stahnke, 1966) that the use of tourniquets could prevent the proximal spread of the venom, and thus keeping the venom in the extremity would reduce both morbidity and mortality. However, tourniquets also decrease normal tissue perfusion increasing ischemia and thereby also increasing tissue damage. It has been recommended that tourniquets be applied only tightly enough to impede the flow in superficial lymphatics and veins. Venom however does not spread by superficial lymphatics and veins (McCullough and Gennaro, 1970), and if edema progresses proximal to the tourniquet, the tourniquet will tighten and perfusion will be decreased (Soloman et al., 1968). Tourniquets not only fail to delay the absorption of venom into the circulation (Ho et al., 1986) but as a consequence of too tightly applied tourniquets amputations have been reported (Synder et al., 1972; Hopkins, 1976).

Corticosteroids

The use of corticosteroids in initial therapy of serious pit viper envenomations has been reported, however the results remain controversial (Minton, 1971; Henderson and Dujun, 1973). Mohamed and Kamel (1963) reported that cortisone with atropine did reduce mortality in dogs and rabbits subjected to the venom from the Egyptian black snake (Walterinnesia aegyptea), and did not appear to affect the neutralizing capability of the antivenin. However, numerous other investigations (Schöttler, 1954b;

Clark, 1971) have shown through clinical and laboratory trials that cortisone offered no benefit to the snakebite patient. Russell (1965) demonstrated that rabbits receiving cortisone plus venom during the venom immunization program fared not as well as the rabbits receiving the venom alone. This finding supports previously reported studies, which showed that corticosteroids fail to protect laboratory animals against the LD_{50} of the venom and in some cases may actually enhance the action of the venom or suppress the animal's immunological defense mechanisms (Schottler, 1954b; Russell and Emery 1961; Gennaro, 1963). In human subjects it has also been found that prednisone failed to lessen either local or systemic effects of Maylayan pit viper bites (Reid et al., 1963). It therfore, seems unlikely that cortisone is of any benefit in the treatment of snakebite victims since it has not been shown to have any effect on the snake venom or on biologically active substances resulting from envenomation (Arnold, 1976).

Antivenin Treatment

The snakebite treatments that have been discussed have obvious disadvantages often leading to complications which can increase morbidity and hinder recovery. Many investigators and physicians realize the need to improve snakebite treatment. Therefore, the production and use of antivenins for snakebite management is an area which has

been studied, and reports discussing its advantages and disadvantages can be found in the literature (Schöttler, 1954a; Loprinzi et al., 1983; Kornalik et al., 1983; Ownby et al., 1986). Treatment alone is not the only factor to influence the patient's recovery, also the toxicity and quantity of venom, depth and location of the bite, the ability of the body's defenses to respond and the time lapse between injury and treatment (Minton, 1954). The efficacy of an antivenin to neutralize the toxicity of the snake venom is due in large part to the length of time that has elasped prior to treatment as well as the antibody titer to a particular snake venom component (Ownby et al., 1983a).

Polyvalent antivenoms. There is the need for potent, safe polyvalent antivenoms in regions where more than one species of snakes exist (Mohamed et al., 1977). A single venom has been reported to produce a more effective species-specific (monovalent) antivenom than an antiserum composed of antibodies against one or more other venoms (polyvalent) antivenom (Russell and Lauritzen, 1966). However it is normally impractical to produce a large number of monovalent species-specific antisera, especially when the snakebite victim is often uncertain about the type of snake which inflicted the bite. Monovalent and polyvalent antivenoms are produced commerically by twenty-eight laboratories in twenty countries (Minton, 1967). In the

United States, Europe, Asia, and other regions of the world polyvalent antivenoms are commerically available (Taub, 1964; Stanic, 1969; Ganthavorn, 1969). However, the efficacy of the antivenom against hemorrhagic, lethal and myotoxic components of the snake venom are variable (Ownby et al., 1983a). The results of Ownby et al. (1983a) indicated that unless the polyvalent (Crotalidae) antivenin is given immediately after the venom from the Prairie rattlesnake (C. viridis viridis), it is not effective in neutralizing local myonecrosis. The polyvalent antivenom was quite effective, however, in neutralizing the lethal effects of the venom (Ownby et al., 1983a). It has also been shown by immunodiffusion and enzyme-linked immunosorbent assay (ELISA) (Ownby et al., 1983b) that the antibody titer of anti-myotoxin in the commerically produced polyvalent antivenin is very low, thus providing a logical reason for the polyvalent antivenin's poor neutralizing capability against local myonecrosis. Russell et al. (1973) observed moderate local tissue damage in rats receiving antivenin ten minutes following a venom injection, if twenty minutes elapsed prior to treatment with antivenin severe localized tissue reactions occured. In Costa Rica the polyvalent antivenin produced against the coral snakes Micrurus nigrocinctus and M. alleni is also effective against the two other coral snake venoms, M. fulvius and M. carinicaudus dumerilii, however the antivenin provides no neutralizing capability against the

venom from M. mipartius (Bolanos et al., 1975). The polyvalent antivenin can be useful in treating snakebite victims but variability in its neutralizing capabilities still exists. Ownby et al. (1986) have shown that Wyeth's polyvalent antivenin can neutralize the local myotoxic activity of Crotalus atrox venom, while having a poor capacity to neutralize the local myonecrosis induced by C. v. viridis (Prairie rattlesnake) venom. In some cases broad paraspecifity of snake antivenin has been found. Venoms from the Australian elapids, such as the tiger snake, death adder, and taipan may contain high concentrations of several important elapid toxins since antisera against them have extraordinarily broad paraspecific activity (Minton, 1967). Also, Sea Snake Antivenene has been shown to provide at least some protective effect against numerous elapid venoms, whereas elapid antivenins fail to neutralize sea snake venom (Minton, 1967). Cobra antivenins are largely genus specific and show considerable variation in their neutralizing capacity. An extensive study conducted by Rosenfeld and Kelen (1966) on the cross neutralization by antivenoms of the coagulant activity of snake venoms known to have a direct coagulant activity showed that paraspecific neutralization was found against venoms of the same genus and also of different genera. In fact the heterologous antivenom was sometimes more effective than the homologous antivenom. It is the hypothesis of Rosenfeld and Kelen (1966) that related

snakes share several common components, and that some snake venoms are more antigenic than others. Therefore, the most antigenic venoms should be used for the preparation of antivenoms since they provide the greatest protection against snakebite envenomation.

Thus, antivenoms can prove to be an extremely beneficial treatment for snakebite patients, providing the antiserum significantly neutralizes the toxic effects of the snake venom, and also providing the patient is not sensitive to horse serum. The polyvalent antivenin commonly used in the United States is produced in the horse. In studies conducted by Alden (1969) and Russell et al. (1970) it was found that some patients requiring antivenin for treatment of bites by venomous snakes were sensitive to horse serum. Russell et al. (1970) reported that with 241 cases of snake venom poisoning, 41 patients were found to have a slightly positive (1 to 2 plus) to markedly positive (3 to 4 plus) skin or eye test when exposed to horse serum. In those with a lesser reaction, Timmerman and Russell (1969) administered the antivenin as advised in the brochure accompanying the antivenin. However, this technique was found to be unsatisfactory since there was still some adverse reactions

Improvement of antivenin therapy is an area that deserves attention since the treatment can produce variable results depending on numerous factors. These variables include quantity and type of venom injected, quality of

antivenin and also the sensitivity of the patient to horse serum. Keeping these facts in mind researchers have attempted to produce an improved antivenom, through purification of the antivenom (Timmerman et al., 1969), production of monoclonal antibodies (Danse et al., 1986; Kfir et al., 1986), and isolation and purification of specific venom components (Tan, 1983; Jeter et al., 1983; Ownby et al., 1986).

Advancements in antisera production

Recently advancements have been made in the production of an improved antiserum to treat local myonecrosis induced by snake venom of the Prairie rattlesnake (C. v.viridis) (Ownby et al., 1979; Ownby et al., 1986). Since the isolation of a pure myotoxin protein (myotoxin a), from the Prairie rattlesnake venom by Ownby et al. (1976) it has been possible to produce an antiserum specific to a pure myotoxic component of the snake venom (Ownby et al., 1979). Myotoxin a is a small basic (isolectric point 9.8) polypeptide, consisting of 39 amino acids having 10 lysines and 1 arginine, and with a molecular weight of 4400 daltons (Ownby et al., 1976). The antimyotoxin serum produced by Ownby et al. (1979) has been shown to react with more than one crotalid venom using the Ouchterlony agar-gel double diffusion technique (Clausen, 1969). Since the titer of antimyotoxin in the commerically produced polyvalent (Crotalidae) antivenin is very low (Ownby et al., 1983b), it is possible to improve the treatment of local myonecro-

sis by the addition of antimyotoxin serum (Ownby et al., 1983a). Ownby et al. (1984) found that the amount of venom neutralized was twice that neutralize by the anti-myotoxin serum alone. Other snake venoms have also been reported to contain basic polypeptides responsible for local myonecrosis (Gonvalves, 1956; Cameron and Tu, 1978; Leonardi et al., 1979). The basic polypeptide (crotamine) isolated from Crotalus durissus terrificus venom resembles myotoxin chemically and also induces a local myonecrosis of skeletal muscle very similar to that caused by myotoxin a (Cameron and Tu, 1978). Interestingly, Ownby et al. (1979) showed that antimyotoxin serum produced against the pure myotoxin protein of C. v. viridis venom can form a preciptitin line with some component found in C. durissus terrificus venom, which is also identical to the line formed between the myotoxin and antimyotoxin serum. Therefore, it can be suggested that an antiserum produced to a pure venom toxin could improve the efficacy of a polyvalent antivenin and provide cross neutralization of specific deleterious effects, such as myonecrosis, due to snake envenomation. Tan (1983), who isolated a low molecular weight toxic fraction from venom of the Malayan cobra (Naja naja sputarix) and found the fraction accounted for almost one hundred percent of the venom's lethal activity. The antiserum produced against this lethal toxin was four to eight times more potent than the commerically available Malayan cobra antivenin produced by immunizing horses with whole Malayan cobra venom. Reid (1964), for example

reported that in severe poisoning by very large doses of antivenom (100 ml) were required. The limited neutralizing potency of the Malayan cobra antivenoms (0.2-0.4 mg venom neutralized per ml of serum) may be due to the absence of antibody formation against one or more lethal components of the venom (Tan, 1983). Lack of antibody formation against specific components of the venom may have various causes, including lack of antigenicity, low concentration of the specific, lethal components and depression of antibody formation by other antigens present in the venom in excessive amounts (Kochwa et al., 1959).

Danse et al., (1986) have recently reported the first study on the neutralization capability of α-Bungarotoxin by monoclonal antibodies. Although there are numerous reports on the production of monoclonal antibodies specific to bacterial toxins (Hayakawa et al., 1983; Galloway et al., 1984) there have been limited reports on the production of monoclonal antibodies specific for snake neurotoxins (Boulain et al., 1982; Strong et al., 1984). The monoclonal antibodies produced against the post synaptic neurotoxin of Bungarus multicinctus (α-bungarotoxin) were found to neutralize the biological activity to varying degrees (Danse et al., 1986). This is in agreement with results from other laboratories that found many monoclonal antibodies, but not all, can have an inhibiting effect on the function of the corresponding antigen (Mochly-Rosen and

Fuchs, 1981; Strong et al., 1984). However, the monoclonal antibodies did protect the mice against the toxic effect of α-bungarotoxin for a few hours to six days, depending on the selected monoclonal population. This is a considerably longer survival time than a period of seventy-nine minutes for the standard toxin dose. Although binding and kinetic studies have not been completed on the α-bungarotoxin and the monoclonal antibody interaction, the authors hypothesize that two factors may be influencing the lack of absolute protection by the monoclonal antibody. First, the affinity of the toxin for its receptor (acetylcholine receptor) may be greater than that of the antibody, or the antibody may be cleared at a faster rate than the toxin, which resists enyzmatic degradation and may be released in a still active state into the bloodstream (Danse et al., 1986). The first hypothesis may be valid for those antibodies which neutralize the toxin for only a few hours, and the second hypothesis is consistent for the monoclonal antibodies which provide the greatest protection. Differences in affinities of the antibody for the toxin or in location of epitopes have also been suggested.

Affinity Chromatography

Affinity chromatography which can be classified as an adsorption chromatography is becoming increasingly popular among researchers for the separation of biological

materials. The technique of biospecific affinity chromatography has been used for the isolation of enzymes, hormones, antibodies and other biologically active compounds (Cuatrecasas and Anfinsen, 1971).

Prior to the use of affinity chromatography the majority of methods for the purification of biological substances were dependent upon gross physical and chemical differences between the substances being separated (Gelb, 1973). Gel filtration permits separation according to molecular size and shape. The principle of ion exchange chromatography is based on the electrical i.e. charge properties of the molecules. Affinity chromatography, however purifies biological substances based upon bio-specific interactions between interacting pairs of substances. The technique is rapid and specific, and permits isolation of a particular protein in a single step (Egorov, 1975). The biological interactions may involve the reaction between antibody and antigen, enzyme and inhibitor or hormone and carrier protein (Teouanne et al., 1980). Regardless of the interacting pairs of substances the basis of separation remains based upon the biospecific interactions of the compounds

The affinity column is prepared by attaching one compound of the interacting pair to an insoluble matrix, which may be a cross-linked agarose gel bead or a polyacylamide gel matrix. A mixture of substances can then be applied to the affinity column. The compound within the

matrix that is capable of binding to the substance attached to the affinity matrix is adsorbed to the column. The forces involved in this binding reaction between the two compounds can include electrostatic, hydrophobic and Van der Waals interactions (Gelb, 1973). The adsorbed compounds or compound depending upon the purity of the attached substance can be removed by changing pH (Sullivan and Russell, 1982) or ionic strength (Bureau and Daussant, 1983). The adsorbed compound is then eluted while the immobilized protein remains attached to the affinity matrix. The choice of eluant or eluants to remove the bound compound from the affinity column depends in many instances upon the nature of the interactions (Morgan et al., 1978). Once the adsorbed material has been removed the affinity column can be regenerated by washing with the initial eluant.

Affinity Purification of Antibodies

The use of affinity chromatography for the study of snake venom components (Gaebert, 1977), and for the isolation of antibodies to specific toxins and snake venoms (Sullivan and Russell, 1983; Russell et al., 1985; Kirchner and Faulstich, 1986) is becoming a growing area of research. Using affinity chromatography Lomonte et al. (1985) isolated antibodies to a myotoxin found in Bothrops

asper venom. The affinity purified myotoxin antibodies were then used experimentally to treat local myonecrosis induced by the crude venom. The investigators were able to determine that the myotoxin of Bothrops asper is a major contributing factor of local myonecrosis, since the affinity purified antibodies neutralized a large amount of the myonecrosis caused by crude venom. Myonecrosis that was not neutralized probably was a consequence of local ischemia induced by the severe alterations of the microvasculatur (Gutierrez et al., 1984). The purified antibodies could also be tested against numerous snake venoms by immunodiffusion. The antimyotoxin was used to test for the presence of immunologically related components in other snake venoms (Lomonte et al., 1985). Of the eleven different venoms tested only one venom, Bothrops schlegelii cross-reacted. Interestingly B. schlegelii has also been shown to induce a type of myonecrosis very similar to that caused by B. asper venom (Gutierrez and Chaves, 1980). Although the antibodies to Bothrops asper myotoxin were very efficient in neutralizing myonecrosis, they were not very effective in neutralizing the lethal effects of the venom. Similar results have been observed by Ownby et al. (1983a) using antimyotoxin serum to neutralize the local myonecrosis induced by C. v. viridis venom. Myonecrosis was significantly reduced by the antimyotoxin serum but lethal effects of the venom were poorly neutralized. Affinity purification has also been used for the isolation

of antibodies specific to neurotoxins. Cobrotoxin, a known neurotoxin from the venom of the Taiwan cobra (Naja naja atra) has been isolated by Yang (1965). It has proven to be the main toxic protein of cobra venom. Immunochemical studies conducted by Chang and Hayaski (1969) on the cobrotoxin and its purified antibody provides evidence that cobrotoxin has three antibody combining sites per molecule. Using affinity chromatography Yang et al. (1977) purified precipitating and non-precipitating antibodies to cobrotoxin. The neutralizing capacities of the affinity purified antibodies were greater than that of the cobrotoxin antisera. Yang et al. (1977) found that the non-precipitating antibodies had the ability to neutralize the toxic effects of the neurotoxin. However, Christian (1970) suggested that the failure of antibodies to precipitate may result from their limited recognition of multiple antigenic determinants on complex antigens. One hypothesis that has been offered regarding the biological differences between precipitating and non-precipitating antibodies is that non-precipitating antibodies are capable of binding with only a small number of antigenic determinants, lattice formation of the antigen-antibody complex will be incomplete and, depending on the degree of its incompletion, the complexes will remain soluble in the precipitin reaction (Kabat and Mayer, 1961). Another hypothesis proposed to explain why antibodies fail to precipitate with the specific antigen, include univalency

of the antibody and low affinity of the antibody for the antigen (Yang et al., 1977). The non-precipitating antibody to cobrotoxin demonstrates, however an ability to neutralize the major toxic component of the venom. Therefore, caution must be taken when generalizing to the presence of non-precipitating antibodies and their effect on an antigen.

Tarng et al. (1986) have used affinity purification to isolate an endogenous antihemorrhagic factor from the serum of the opossum (Didelphis virginiana). Endogenous antihemorrhagic factors from the sera of some snakes and warm blooded animals have been found to neutralize the hemorrhage activity of snake venoms (Ovadia, 1978). Some of these factors have been isolated and characterized (Garcia and Perez, 1983). Affinity chromatography of opossum sera by Tarng et al. (1986) has allowed the purification of antihemorrhagic factors and permits the study of neutralization processes. Their results showed that affinity chromatography isolated the antihemorrhagic factor in a single step with an increase in the specific activity of the factor.

Since affinity purification of biological substances is proving an efficient means to isolate specific desired proteins, the purification method has been experimentally tested to remove the extraneous protein content from commerically available antivenin. The extraneous protein is that portion of the antiserum not important in venom

neutralization, however it is often times responsible for sensitivity reactions, which include anaphylaxis (Sullivan and Russell, 1983). The commerical antivenin that was affinity purified so as to obtain an IgG fraction was Wyeth's (Crotalidae) antivenin. Since this antivenin is commonly used throughout the United States to treat snakebite poisoning the results are of considerable clinical significance. The composition of the commerically produced antivenin used in the experiment by Sullivan and Russell (1983) can best be described by a brief explanation of the process involved in the preparation of the polyvalent (Crotalidae) antivenin. One thousand milliters of crude serum are collected from horses immunized against a mixture of venoms consisting of C. adamanteus (eastern diamondback rattlesnake), C. atrox (westernback diamond rattlesnake), C. durissus terrificus (South American rattlesnake) and Bothrops atrox (Fer-de-Lance). The crude serum then is diluted in 2000 milliters of distilled water and 156 grams of ammonium sulfate are added per liter of serum. A precipitate which forms overnight and consists of residual fibrin is then removed. One hundred and forty-four grams of additional ammonium sulfate are added per liter of serum, and a precipitate containing the active globulins is then collected. The precipitate is dialyzed to remove salts and later clarified using centrifugation Finally a 0.85 percent sodium chloride solution is added and the antivenin is concentrated (Timmerman and Russell, 1969).

Using affinity chromatography Russell et al. (1985) purified and concentrated the IgG fraction of the polyvalent antivenin and enhanced its efficacy. Affinity chromatography of the polyvalent antivenin eliminates the ammonium sulfate precipitation step, that proves to be costly and can result in the loss of neutralizing IgG. Also, ammonium sulfate co-precipitates many other serum proteins besides the desired IgG molecules, thus increasing the possibility of an allergic reaction to the horse serum.

The experimental protocol used by Sullivan and Russell (1982) and Russell et al. (1985) to purify the polyvalent antivenin involved the use of four different affinity columns. Coupled to each column was a single venom. The venoms chosen were C. adamanteus, C. atrox, C. viridis helleri (Southern Pacific rattlesnake) and C. scutulatus (Mojave rattlesnake). The latter two venoms are not used in the production of commerically produced antivenin, however the antivenin provides good protection against these venoms. One vial of antivenin (10 milliters) was applied to each column. Each column was then eluted with phosphate buffered saline followed by glycine, pH 2.5. It was shown that the first peak contained the extraneous proteins and the second peak eluted by glycine, pH 2.5 consisted of the purified IgG antibodies. Purified IgG's were then tested for their ability to provide protection against hemorrhage, lethal effects, fibrinogen clotting

activity and platelet aggregation activity induced by the four snake venoms. The results showed that for all parameters measured the affinity purified antibodies were more efficiacious than the commerically produced antivenin. In animals sensitized to horse serum the purified antibodies did not produce anaphylaxis or anaphylactoid reactions. Thus the affinity purified IgG antibodies provides an immunopharmacological agent, which offers protection against deleterious effects induced by crotaline venoms and does not cause a sensitivity reaction.

It is therefore noteworthy that affinity chromatography is being applied to a wide range of toxins in venom research. The methodology offers to many investigators a rapid, sensitive and inexpensive means of purifying the toxin. The selective isolation and purification of biologically important macromolecules by affinity chromatography exploits the unique biological property of these proteins or polypeptides to bind ligands reversibly, therefore the potential usefulness of affinity chromatography, particularly in venom research, is virtually limitless.

Enzyme-Linked Immunosorbent Assay (ELISA)

There has been a trend recently in the area of assay systems to move away from the more expensive radioimmunoassays (RIA) involving isotopes, and therefore necessary precautionary steps, to the equally sensitive but

relatively inexpensive and versatile ELISA. ELISA does not require isotopes that often have relatively short shelf-lives or require special safety measures for handling. The ELISA systems involve the use of antigens, haptens, or antibodies labelled with an enzyme to measure a variety of biological substances including, pit viper venoms (Minton et al., 1984) as well as antigens associated with parasitic diseases from a variety of geographical locations (Voller et al., 1976). The assay systems have been given various names including enzyme-, enzymic-, enzymatic-, and enzymo-immunoassay (Skelley et al., 1973). Enzyme-linked immunoassay, enzyme-labelled immunoassay, enzyme coupled immunoassay, immunoenzymatic assay, and enzyme-linked immunosorbent assay are synonymous (Wisdom, 1976).

Types of ELISA

Enzyme-immunoassay systems can be divided initially into two main categories, the heterogenous and homogenous assay systems. The majority of enzyme immunoassay systems reported in the literature fall into the heterogenous assay systems, since they prove particularly suitable for the measurement of biological substances having a molecular weight of 10,000 daltons or more (Voller et al., 1979).

The homogenous enzyme-immunoassay uses a hapten linked to an enzyme, such that when the enzyme combines with the antibody the enzyme activity is altered. The test sample is allowed to react with enzyme-linked hapten followed by

the addition of the specific antibody. If the test sample contains any of the hapten it will combine with the antibody displacing the enzyme labeled hapten. Enzyme substrate is added to the mixture and any displaced enzyme labelled hapten can degrade the substrate. The color change due to substrate degradation can then be read spectrophotometrically. The homogenous assay systems do not require any separation steps and can be performed within a matter of minutes. Therefore, the homogenous systems are being widely used to test for drug abuse as well as measuring therapeutic drug levels of digoxin, lidocaine and phenobarbital.

Many researchers, however, find the heterogenous assay system the method of choice. Heterogenous assay systems depend on the assumption that an enzyme can be linked to either an antigen or antibody while retaining both immunological and enzymatic activity (Voller et al., 1979). The heterogenous assay system in contrast to the homogenous enzyme-immunoassays all involve a separation step whereby the reacted enzyme-labelled component is separated by washing it from the unreacted enzyme-labelled material.

The types of heterogenous immunoassay systems can be divided into six categories. These categories are 1) the competitive method of ELISA for assaying antigen, 2) the double antibody method of ELISA for antigen detection, 3) the modified double antibody sandwich ELISA for antigen detection, 4) modification of the indirect ELISA for assay

of antigen, 5) the indirect method for antibody detection and 6) solid-phase anti-IgM ELISA.

Antigen Detection Methods

The double antibody antibody sandwich ELISA is commonly used for antigen detection. The immunoglobulins or purified antibody are attached to the solid phase, followed by a washing step. The test solution containing the antigen is added, then the plate is incubated and then washed. Enzyme labelled specific antibody to the antigen is added, washed, and followed by the addition of enzyme substrate. The color change is proportional to the amount of antigen in the test solution.

The Modified Double Antibody ELISA

The modified ELISA for antibody detection varies from the double antibody systems in two important ways. The solid phase is coated with IgG or a purified antibody and test solution is added. Antibody specific to the antigen is again added, however now it is antibody produced in a different species from that coating the solid phase. Next an enzyme labelled anti-globulin is added and incubated, followed by washing. The anti-immunoglobulins must be reactive with the immunoglobulin added in the second step but not react with the immunoglobulin used to coat the plate. The enzyme substrate is added and color change is

proportional to the amount of antigen in the samples.

Indirect Method

The indirect method permits the detection and measurement of antibodies. Specific antigen is attached to the solid phase which is later washed. The test solution is added, incubated and washed. Next the enzyme labelled anti-globulin is added and allowed to react. Finally enzyme substrate is added and a color change occurs in antibody containing solutions. The amount and rate of color change is related to the amount of antibody present.

ELISA and Toxin Research

The application of ELISA to snake venom research has been reviewed by Theakston (1983). Detection of the venom or some component of the venom, and the ability to detect antibodies administered against it are critical for improved treatment of snake bite envenomation. In many parts of the world, particularly the tropical countries of Africa, South America and Southeast Asia snakebite poses a major medical problem. The lack of a rapid, sensitive and inexpensive assay for venom detection often times prevents the proper treatment of snakebite cases. The ELISA procedures most commonly adopted for snakebite studies are the indirect method for antibody detection and the double sandwich method for venom antigen detection.

Recently Ho et al. (1986a) have critically appraised the applications of the ELISA in the study of snakebite.

In tropical countries where snakebite constitutes a major health problem, applying the immunodiagnosis technique of ELISA for the treatment of snakebite envenomation may prove extremely helpful. However, Ho et al. (1986a) indicate the need for an awareness of specific problems unique to ELISA and antigen-antibody detection. The major problems that are encountered with some ELISA systems are non-specific reactivity, cross reactivity of the assay and the quality of reagents that can affect both the assay's sensitivity and specificity. Specificity of venom antigen and antibody detection assays is generally taken as the lack of cross reactivity between closely related species (Theakston, 1983). Non-specific binding can also occur with normal serum samples. Greenwood and Whittle (1981) have reported a particularly high non-specific reactivity associated with sera from people indigenous to the rural tropics. This can perhaps be explained by high levels of immunoglobulins, heterophile antibodies and rheumotoid factor. These and other serum components can attach in a non-specific manner to the solid phase or interact with reagents raised in other mammalian species. The seriousness of the problem of non-specific binding has been shown by Ho et al. (1986a) in an epidemiological study conducted in Thailand. Using the indirect antibody detection assay their results indicated that seventy-five percent of normal, healthy subjects had been bitten by the Thai cobra (Naja kaouthia) and had suffered sufficient envenoming to elict an antibody

response. This figure implies that seventy-five percent of randomly selected normal individuals in eastern Thailand had to have been bitten on more than one occasion and yet had no history of signs or symptoms of envenoming. The percentage might more logically be explained as a non-specific response by an indigenous population of the tropics. Theakston et al. (1981) have reported from epidemiological surveys conducted in Ecuador and Nigeria, that snakebite victims had detectable levels of venom-antibodies for as long as forty years after the bite. Their criteria for this conclusion was a cut-off absorbance value of 0.10, since it represented a visually detectable color change. However, Ho et al. (1986a) know of no other example of circulating antibodies persisting forty years after a single antigenic challenge. The arbitary cut-off value chosen by Theakston et al. (1981) may not be appropriate for such conclusions.

Similar non-specific reactivity have been reported in the venom detection assay. Lwin et al. (1984) have shown the detection of venom in a number of patients in Burma, who had neither systemic or local signs of venom poisoning. In addition a persistant level of venom was found in patients that had received adequate antivenom therapy.

Snake venom has been described as a mosaic of antigens, and common antigens have been shown in related and unrelated species, such as Elapidae and Viperidae (Kuekarni and Rao, 1956). Therefore, it is somewhat sur-

prising that cross reactivity, another factor to influence specificity, has not been a major problem in many snake venom assays (Coulter et al., 1980). One hypothesis for this observation is that shared antigens are of low antigenicity or are present only in minute amounts in whole venoms (Ho et al., 1986a). Partial cross reactivity in antibody detection is seen with snakes within the same subfamily and is complete among snakes of the same genus. The use of single venom components as antigens may reduce cross reactivity among species of the same subfamily, but is unlikely to affect the detection of antibodies to snakes of the same genus, since they probably share many common antigens. Using antibody enzyme conjugates of three individual pit viper venoms Crotalus atrox, Crotalus scutulatus scutulatus and Agkistrodon contortrix, Minton et al. (1984) were able to detect venoms of most North American pit vipers at concentrations of ten micrograms or less. However, species identification was not always possible. The specificity of antigen detection can be improved with the preparation of specific antibodies. Theakston et al. (1977) have recommended the use of the ammonium sulphate precipitated fractions of immune globulins. A comparison made by Ho et al. (1986a) of the sensitivity of a double antibody sandwich ELISA for antigen detection using an ammonium sulphate preparation of immunoglobulins was found to compare very poorly with that of IgG from the same antiserum purified by protein A

affinity chromatography. The increased sensitivity of the antigen detection assay obtained with the affinity purified IgG fraction allows the accurate measurement of small changes in venom levels, which are especially useful for serial measurements of venom levels.

Monoclonal antibodies to purified venom components should be in principle, the preparations of choice, however, they are often not available in field conditions or in rural areas, where snake bite cases are most likely to occur. In addition, monoclonal antibodies may have several disadvantages. Pukrittayakamee et al. (1983) have shown that in a competitive binding radioimmunoassay using a monoclonal antibody to factor X activator found in Thai Vipera russelli venom, that non-specific reactivity is a major problem even in antigen detection assays using monoclonal antibodies. One explanation for this occurence, is that monoclonal antibodies are not as avid as polyclonal antibodies. It may be that monoclonal antibodies do not adsorb well to plastic surfaces and in the case of enzyme labelling, the antibodies may in some way be altered. Whatever the explanation, it appears that an affinity purified polyvalent antibody permits an increase in assay sensitivity without an increase in non-specific binding.

Another method to decrease non-specific binding is by testing body fluids that are low in protein content, for example urine or wound aspirates (Ho et al., 1986b).

Thirty-four patients with systemic envenomation by the Malayan pit viper (Calloselasma rhodostoma) were tested by Ho et al. (1986b) using ELISA for the presence of venom in urine specimens, wound aspirates and blood samples. Urine tests were positive for eighty-seven percent, eighty-eight percent for wound aspirates and ninety-seven percent for blood samples. The presence of venom in urine specimens depends partially on the time elasped between the bite and admission to the hospital, and that in wound aspirates on the speed of dispersion of the venom from the bite site. A positive wound aspirate, however, is no proof that systemic envenoming has occurred, and therefore is less useful as an indicator for therapy (Ho et al., 1986b).

Minton et al. (1984) found in experiments with rats and mice envenomated by injection and natural bites that venoms of the pit vipers tend to stay localized near the site of injection, appearing in the blood and urine only when relatively large amounts of venom have been injected. Copperhead venom was detected at the bite site twenty-two hours after a sublethal bite, and in the urine three hours afterwards. The copperhead venom, however, was not detected in the blood. Minton et al. (1984) tested numerous biological fluids and tissues taken at autopsy for the presence of crotalid venom. Lung, trachael exudate and pleural fluid showed the presence of venom more frequently than peritoneal fluid, kidney and brain.

The success of immunodiagnosis for the detection of snake venoms and antibody titers in various populations has encouraged researchers to develop an ELISA that would be suitable for field conditions. The ELISA procedure used in the laboratory requires the use of a spectrophotometer to determine the absorbance and can require a matter of hours to complete. These requirements which can easily be accomodated under experimental conditions, may however make the ELISA unsuitable for field conditions. Therefore, Chandler and Hurrell (1982) developed an enzyme immunoassay system suitable for field use. The snake venom detection kits were distributed to hospitals and laboratories in Australia. The results indicated that an excellent correlation existed between clinical diagnosis, and detection and identification of snake venom in samples of blood, urine or bite site swab washings.

The venom detection kit consists of six capillary tubes, each coated with rabbit IgG containing antibody against one of the five immunologically distinct Australian snake venoms (Sutherland, 1976). Each tube is joined by silicone rubber tubing and connected to a one milliter syringe. The test kit is the double antibody sandwich type ELISA for antigen detection, which involves the drawing in and explusion of the reagents provided in the kit. Briefly the steps required for venom detection are 1) drawing a sample, whole blood, urine or bite site fluid into the series of capillaries, 2) flush the sample out of the tubes

after ten minutes, wash with buffer, 3) add the mixture of rabbit anti-snake venom urease conjugates, 4) incubate ten minutes, wash the capillary tubes and 5) add enzyme substrate. A positive control capillary tube is included with each kit to indicate the color change to expect when venom is present in the test sample. Chandler and Hurrell (1982) found the kit was consistently capable of correctly detecting and identifying venom in samples containing at least fifteen micrograms per milliliter within a total of forty minutes. The lower limit could be reduced if incubation times were increased. Clinical specimens tested previously by RIA by Coulter et al. (1978) were correctly indentified by the ELISA if the concentration was ten nanograms per milliter or more.

A qualitative enzyme immunoassay for the rapid clinical identification of snake venom is now also available in Australia. Coulter et al. (1980) have developed an ELISA that is capable of detecting 0.5 ng/ml of crude snake venom in approximately ninety minutes or two nanograms crude venom in thirty minutes. Two factors contribute to the improved sensitivity of the clinical ELISA in comparison to the values obtained with the field detection kit. The clinical ELISA uses an incubation time that is slightly more than twice that required for the field kit, and the incubation temperature used is 37 C in comparision to ambient temperature. The combination of the field kit available for venom detection and the clinical

labortory ELISA has helped improve the diagnosis and treatment of snakebite patients in Australia.

The use of ELISA is not limited to venom detection in the field of venom research. Theakston and Reid (1979) used the indirect ELISA method (Theakston et al., 1977) for screening antivenom potency. Antivenom from three geographical regions were chosen to elvaluate for potency. The commerical antivenoms tested were provided by West Germany, South Africa and India. Antivenoms that had expired were also tested for potency. Initially five median lethal doses (LD_{50}) were injected into mice followed by varying amounts of antivenom. The LD_{50} is the dose of venom when injected into mice which will cause death in fifty percent of the population within twenty-four hours. After twenty-four hours the neutralizing median effective dose (ED_{50}) was determined. The ED_{50} is the least amount of antivenom needed to prevent the death in fifty percent of the mice tested. Therefore, the lower the ED_{50} the higher the neutralizing capabilities of the antivenom. The optical densities for each dose of used antivenom were tested using the ELISA system. Thus, a reference standard for each antivenom was obtained and the ED50 determined <u>in vivo</u> was then compared to the optical density of the reference standard. Expired antivenoms had consistently low neutralizing potencies. Compared with the <u>in vivo</u> potency test ELISA is a rapid and simple test, however, at present an ELISA reference standard must be established for

each commerical source of antivenom.

More recently Gopalakrishnakone et al. (1981) have used the ELISA indirect method for the detection of antibodies to the lethal neurotoxin, crotoxin, from Crotalus durissus terrificus venom. Crotoxin is a complex of a basic phospholipase A2 and an acidic protein (Habermann and Breithaupt, 1978).

Gopalakrishnakone et al. (1981) raised antibodies in rabbits against the reconstituted crotoxin complex. An indirect ELISA method and immunodiffusion were used to test for a reaction of homologous and heterologous Crotalus toxins and venoms to the antiserum. The ELISA system proved to be the more sensitive detector of the antigen-antibody reaction than immunodiffusion. Although the results given were qualitiative and not quantitative the indirect ELISA method clearly indicated a reaction of the antiserum to the a) crotoxin complex b) homologous phospholipase A_2, c) crotapotin, d) C. d. terrificus venom, e) Mojave toxin and f) C. horridus atricaudatus venom. The phospholipase A2 from elapid, hydrophiid, bee venoms or porcine pancreas showed no cross reaction with the antiserum used. The reaction of the antiserum between the crude venom (C. d. terrificus) and Mojave toxin from C. s. scutulatus venom were the strongest and markedly similar. Crotoxin and Mojave toxin have both been shown to inhibit transmitter release from motor end terminals (Hawgood and Smith, 1977). The results obtained by

Gopalakrishnakone et al. (1981) suggest a close antigenic relationship between these two lethal toxins.

CHAPTER III

AFFINITY PURIFICATION OF ANTIBODIES TO MYOTOXIN A

Introduction

The use of good quality reagents in the enzyme-linked immunosorbent assay (ELISA) can improve the assay's sensitivity by decreasing non-specific binding (Ho et al., 1986a). Improved assay sensitivity has partially been accomplished by the protein A purification of the IgG fraction (Ho et al., 1986a). In addition, affinity purification of antibodies to tetanus toxins and cobrotoxin yielded antibodies that provided improved neutralization to specific deleterious effects induced by these toxins (Yang et al., 1977; Lazarovici, 1984). Lomonte et al. (1985) have also used affinity purified antibodies to a myotoxin from Bothrops asper snake venom to efficiently neutralize localized myonecrosis.

Since the antibody titer to the major myotoxic protein, myotoxin a, from Crotalus viridis viridis venom has been shown to be in low concentrations in the polyvalent (Crotalidae) Antivenin (Ownby et al., 1983b), the affinity purification of antibodies to myotoxin a could

possibly be useful for the improved neutralization of local myonecrosis induced by myotoxin a, and secondly would be extremely helpful in establishing sensitive antibody and antigen detection assays.

Materials and Methods

Myotoxin a

Twenty-three milligrams of myotoxin a purified by Ownby and Colberg (unpublished results) from Crotalus viridis viridis venom was coupled to an affinity column. The myotoxin a was tested for its biological activity on skeletal muscle in mice and for its ability to interact with its specific antibody in an enzyme linked-immunosorbent assay (ELISA).

Female mice (CD-1, Charles Rivers) weighing between 24-25 grams were injected intramuscularly (i.m.) in the thigh with purified myotoxin a (1.5 µg/g). Twenty-four hours following the injection the mice were killed by cervical dislocation and a sample of skeletal muscle from the injected thigh was taken and processed for histologic study.

Immunodiffusion

The Ouchterlony double-diffusion technique (Clausen, 1969) was used to test for a precipitin band between myotoxin a and antibodies in the crude serum. The tests were run for eight hours at room temperature using 1% agarose gels. Myotoxin a was tested at a conentration of 1 mg/ml against crude serum obtained from three rabbits immunized against the antigen. Affinity purified antibodies eluted with distilled water (DW), acetic acid (Hac) and phosphate buffered saline (PBS) were tested at a concentration of 0.60 mg/ml against the myotoxin. A concentration of 0.60 mg/ml is twice the concentration of antibody used in a later experiment to test for neutralization of myonecrosis. The antibody eluted with DW was tested also at a concentration of 1.23 mg/ml.

Antimyotoxin a serum

Antimyotoxin a serum was produced as described by Ownby et al. (1979). Briefly, lyophilized myotoxin was dissolved in 0.85% NaCl and injected at a dose of 0.25 mg/kg into two 3 kg rabbits (New Zealand White). The rabbits were bled for pre-immune serum and subsequently injected with the myotoxin a. The myotoxin solution was emulsified with an equal volume of Freund's complete

adjuvant and 2.0ml of the emulsion was injected intramuscularly between the shoulder blades. One week later a booster injection (2.0ml of an equal mixture of myotoxin and Freund's incomplete adjuvant) was given. Animals were bled at 1-2 week intervals with monthly booster injections. Serum from individual rabbits and bleedings was separated and stored separately at -20 C until used.

Gel Filtration

The IgG fraction of the antimyotoxin serum was isolated and used to develop an ELISA for detecting antibody to myotoxin a.

Dilutions of an IgG fraction containing antibodies to myotoxin were used to coat the ELISA microtiter plates and for a standard curve with the antigen and antibody detection ELISA systems, respectively. Affinity purified antibodies to myotoxin were later used for the ELISA detection systems once a sufficient quantity of antibody was affinity purified.

Thirteen milliters of crude serum containing antibodies to myotoxin a were fractionated using an Ultrogel AcA-34 gel filtration column with PBS, pH 7.4 as the elution buffer. The IgG fraction was tested for antibody activity using the ELISA antibody detection system.

The IgG fraction was then pooled and concentrated to 1.34 mg/ml with the Amicon ultrafiltration concentrating cell using a YM 10 membrane, exclusion limit 10,000 daltons (Amicon Corp. Danvers, MA). Micro-titer plates were coated with 1 ug/ml myotoxin a, and dilutions (1:10, 1:25, 1:50, 1:200, 1:1000 and 1:5000) of the IgG fraction were tested for the presence of antibodies to myotoxin a using the ELISA antibody detection system.

The antimyotoxin obtained by gel filtration was also tested for its ability to bind myotoxin a using an ELISA antigen detection system. Various concentrations of IgG and second antibody were tested for their ability to detect myotoxin a. Initially microtiter plates were coated with either 0.50, 0.10 or 0.01mg/ml of the IgG fraction. A myotoxin a standard curve consisting of 100, 10, 2, 0.2 and 0.1 ng/ml was then measured on the microtiter plates, which were coated with the different amounts of the IgG fraction. The concentration of second antibody (goat anti-rabbit) was kept constant at 1:2000. Secondly sample wells coated with 0.01mg/ml of IgG were tested for their ability to bind myotoxin a when the dilution of the second antibody was only 1:1000.

Affinity Purification of Antimyotoxin

Antiserum against myotoxin a was produced in rabbits and purified using affinity chromatography. Twenty-four grams of myotoxin a were conjugated to an Affi-Gel agarose gel bead support (Bio-Rad, Richmond, CA) for 16 hours at 5 C. The conjugated Affi-Gel was packed in a 1.5 cm diameter, glass column to a height of 8.0 cm. Prior to application of the serum sample the column was equilibrated with phosphate buffered saline (PBS), pH 7.4. Samples of crude antiserum ranging from five to twenty milliters were applied separately to the column at a flow rate of 5 ml per hour, followed by washing with PBS until the absorbance of the effluent returned to baseline. The absorbance of each tube was monitored with the LKB Unicord III recorder. The column was then eluted with distilled water (DW) until an intitial protein peak eluted. The elution was interrupted for 12 to 24 hours after which the elution with DW was resumed until the absorbance returned to baseline. The elution was continued with 25 mM acetic acid (Hac, pH 3.1) until another protein peak eluted. Finally the column was reequilibrated with PBS until the absorbance returned to baseline. All protein peaks were measured at a wavelength of A_{280} nm with a DU-8 spectrophotometer and all fractions

were also tested for the presence of antibodies to myotoxin a using the ELISA antibody detection system.

Enzyme-Linked Immunosorbent Assay (ELISA). The antibody eluted with DW was used to develop an ELISA to detect antimyotoxin and myotoxin a. The antibody detection assay was used to test for the presence of affinity purified antimyotoxin in each fraction. Polystyrene microtiter plates (Flow Laboratories Inc., Mclean, VA) were coated overnight at 25 C with 1 µg/ml with myotoxin a. The plates were washed the following day with washing buffer. Two-hundred microliters of known amounts (serial dilutions) of the IgG fraction containing antibodies to myotoxin a as previously determined, and 200 ul aliquots from each tube obtained by affinity chromatography were added to the ELISA plate. All samples were incubated for two hours at room temperature, then rinsed three times with washing buffer. Two-hundred microliters of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) were added to each well at a concentration of 1:2000 and incubated for two hours at room temperature. The plates were again washed followed by the addition of the phosphatase substrate, p-nitrophenyl phosphate disodium (Sigma Chemical Co., St. Louis, MO). The substrate was allowed to react for thirty minutes. The amount of color formation is proportional to the antibody concentration in

each sample. The absorbance for each well of the microtiter plate was read at a wavelength of 405 nm using an EIA manual reader.

An ELISA double sandwich antigen detection system was used to test the ability of the IgG fraction to bind detectable amounts of myotoxin a. Microtiter plates were coated with 0.50, 0.10 and 0.01ng/ml of the IgG fraction overnight at 4 C. The plates were washed the following day with washing buffer. All incubations of the coated microtiter plates were carried out at 37 C for two hours except for the substrate incubation which was performed at room temperature for thirty minutes. Two-hundred microliters of known amounts of myotoxin a were added to each well. The dilutions of myotoxin a (1:10, 1:100, 1:500, 1:1000, 1:5000 and 1:10,000) were made from a 1mg/ml stock solution of myotoxin. The microtiter plates were incubated and then rinsed three times with washing buffer. Two-hundred microliters of the IgG fraction (0.50, 0.10 and 0.01mg/ml) were added to each well, incubated and then washed. Two-hundred microliters of goat anti-rabbit IgG aklaline phosphatase conjugate (Sigma Chemical Co., St. Louis MO) were then added to each well, incubated and rinsed three times. Finally, two-hundred microliters of phosphatase substrate, p-nitrophenyl phosphate disodium (Sigma Chemical Co., St. Louis, MO) were added. The amount of color formation is proportional to the antigen concentration in each sample. The absorbance for each well

of the microtiter plate was read at a wavelength of 405nm using and EIA manual reader.

Standard Curves. Antibodies obtained from each eluant (DW, Hac and PBS) were pooled separately. The pooled fractions were concentrated using an ultrafiltration concentrating cell with a YM 10 membrane. A comparison of the ability of antimyotoxin eluted with DW, Hac and PBS to bind the purified myotoxin a was determined using ELISA.

Antibody obtained with each eluant was diluted from 10.0 to 0.10 µg/ml and measured using the ELISA antibody detection system. Microtiter plates were coated with purified myotoxin a (1 µg/ml). Two-hunderd microliters of antibody were added to each well except for those wells which received buffer alone. The wells having only buffer tested for non-specific binding.

An antigen detection assay was also performed using the purified antibody. Microtiter plates were coated with 1 ug/ml of affinity purified antibody eluted with either DW, Hac or PBS. Two-hundred microliters of known amounts of myotoxin a (50, 25, 20, 15, 10 and 5 ng/ml) were added to each well except for those wells which received buffer alone.

Neutralization of Myonecrosis by Affinity Purified Antimyotoxin. Affinity purified antibody to myotoxin a eluted with DW, Hac, and PBS was tested for its ability to neutralize local myonecrosis induced by myotoxin a. Three-hundred micrograms of affinity purified antibody obtained with each of the eluants and 750 µg of myotoxin a were incubated together at 37 C for one hour and then centrifuged. An aliquot of supernatant (0.050 ml) was injected intramuscularly (i.m.) into the thighs of individual mice. Female mice weighing 25 ± 0.50 grams, were divided into twelve groups, five mice per group. The twelve groups received the following treatments: 1) antibody from DW fraction + myotoxin, 2) antibody from Hac fraction + myotoxin, 3) antibody from PBS fraction + myotoxin, 4) DW + myotoxin, 5) Hac + myotoxin, 6) PBS + myotoxin, 7) antibody from DW fraction alone, 8) antibody from Hac fraction alone, 9) antibody from PBS fraction alone, 10) DW alone, 11) Hac alone and 12) PBS alone. After twenty-four hours the mice were killed by cervical dislocation, a sample of skeletal muscle (primarily sartorius) from the injected thigh was removed and prepared for histology.

Neutralization of Myonecrosis using Crude Antiserum

Neutralization of myonecrosis using crude antisera was

divided into two experiments. The first experiment tested the ability of antisera from two rabbits immunized against myotoxin a to neutralize two concentrations of antigen.

Experiment I. Antisera were incubated overnight at 4 C with either 0.38 or 0.75 mg/ml of myotoxin. Control solutions consisting of a mixture (1:20) of pre-immune rabbit serum and 0.38 or 0.75 mg/ml of toxin were included in the experiment and treated the same as the antibody test solutions. The following day the solutions were centrifuged and an aliquot (0.05 ml) from each supernatant was injected intramuscularly in the thigh of female mice (CD-1 Charles Rivers) weighing 25 $\pm$ 0.50 grams. The doses of myotoxin a injected were 1.5 and 0.75 µg/g. The experiment consisted of five groups, four mice per group. After twenty-four hours the mice were killed by cervical dislocation and a sample of skeletal muscle (primarily sartorius) from the injected thigh was taken and processed for histology. The neutralization of myonecrosis was determined by the myonecrosis index (Ownby et al., 1982).

Experiment II. Three ratios of antigen:antiserum (1:10, 1:20 and 1:40) were tested for their ability to neutralize local myonecrosis induced by 1.5 µg/g of myotoxin. Control groups received of physiological saline (PSS) and toxin; pre-immune rabbit serum and toxin; antiserum alone; and PSS alone. A ratio of 1:20 for pre-

immune serum and PSS to toxin was selected. The experiment consisted of seven groups, five mice per group. Mice weighed twenty-five grams. Mice were killed after twenty-four hours and a muscle sample from the injected thigh was removed. The tissue was immediately processed and fixed for histology.

Histological Preparation and Quantitation of Myonecrosis

Muscle samples from all neutralization experiments were taken from the injected thigh, cut into small pieces and placed in 2.5% glutaraldehyde for two hours. The tissue was washed three times and fixed with osmium tetroxide for one hour. Next, it was dehydrated serially in ethanol, infiltrated with propylene oxide/polybed 812 (1:1), and embedded in polybed 812 resin, which was then polymerized by heating at 60 C for forty-eight hours. Thick sections (1 μm) were obtained from a Sorvall MT 5000 Ultramicrotrome stained with Mallory's trichrome.

Five sections of muscle tissue per mouse were counted using a Zeiss light microscope equipped with a Zeiss MOP 3 Image Analyzer. The number of vacuolated cells per total number of muscle cells was recorded to obtain a vacuolation index.

Results

Myotoxin a

Purified myotoxin a of Ownby and Colberg (unpublished results) produced vacuolation of skeletal muscle cells typical for this toxin within twenty-four hours after injection. No hemorrhage was observed. The purified myotoxin a was later used to establish the antigen and antibody detection assays.

Immunodiffusion

The toxin formed a single precipitin band on the agarose gel plates when tested against crude serum from rabbits immunized against myotoxin a (Figure 1). Affinity purified antibody eluted with DW, Hac and PBS did not form a precipitin band against myotoxin a.

Gel Filtration

Figure 2 shows the IgG fraction obtained with the Ultrogel AcA-34 column and the presence of antibodies to myotoxin a as determined by the ELISA antibody detection system. Dilutions of the IgG fraction were tested using the antibody detection ELISA (Figure 3). Values with an absorbance reading at 280nm above 0.20 were obtained for the dilutions 1:10, 1:25, 1:50 and 1:200. Table I shows

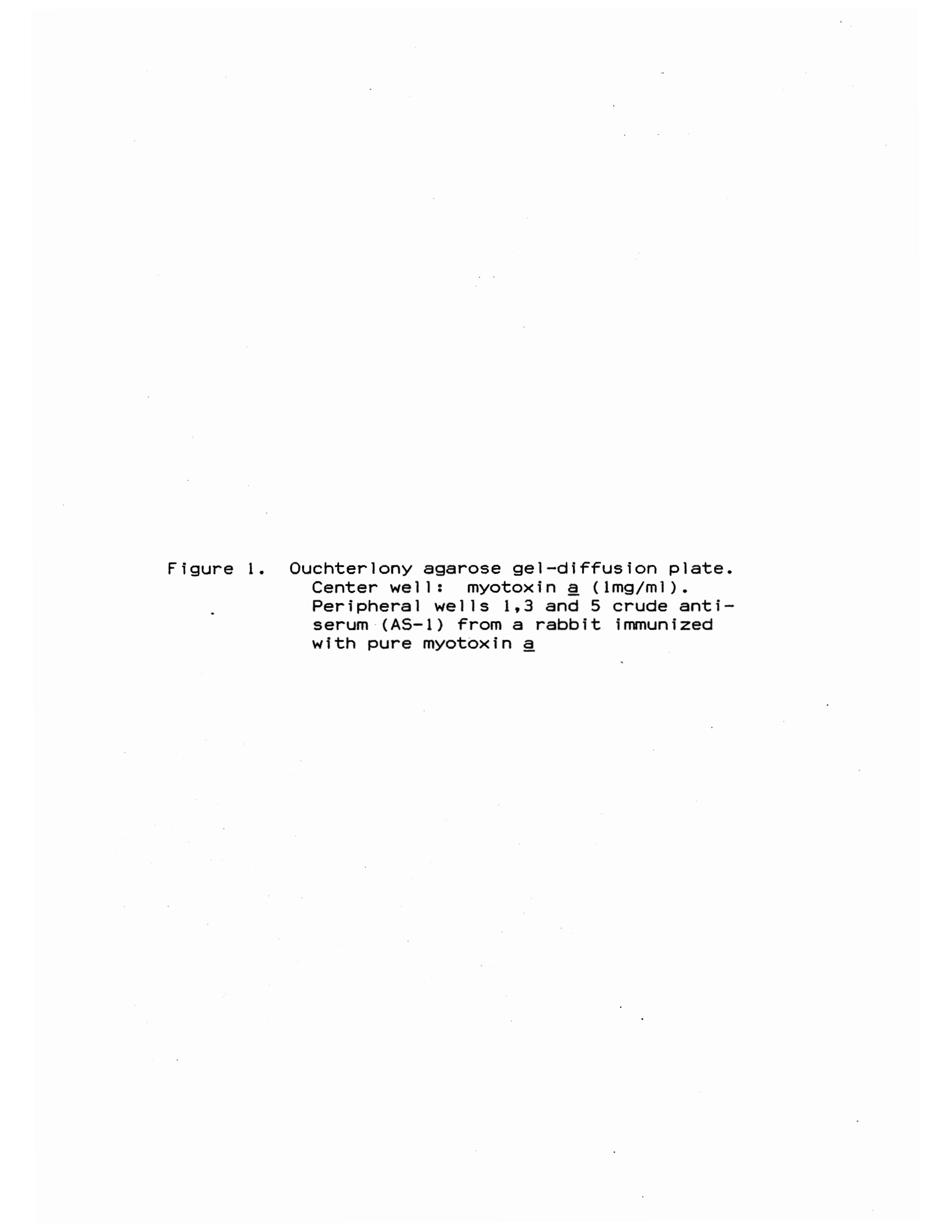


Figure 1. Ouchterlony agarose gel-diffusion plate. Center well: myotoxin a (1mg/ml). Peripheral wells 1,3 and 5 crude antiserum (AS-1) from a rabbit immunized with pure myotoxin a

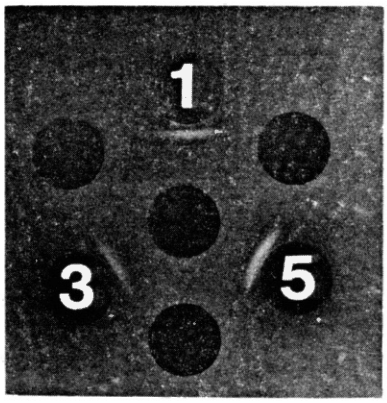

1
3
5

Figure 2. Gel filtration of 13 ml crude antiserum containing antibodies to myotoxin a on an Ultrogel AcA-34 column. The sample was applied to a column that had been equilibrated with PBS, pH 7.4 and eluted with the same buffer. A five ml volume was collected per tube. The peaks were measured at A_{280} for protein content and 405nm for antibody using ELISA.

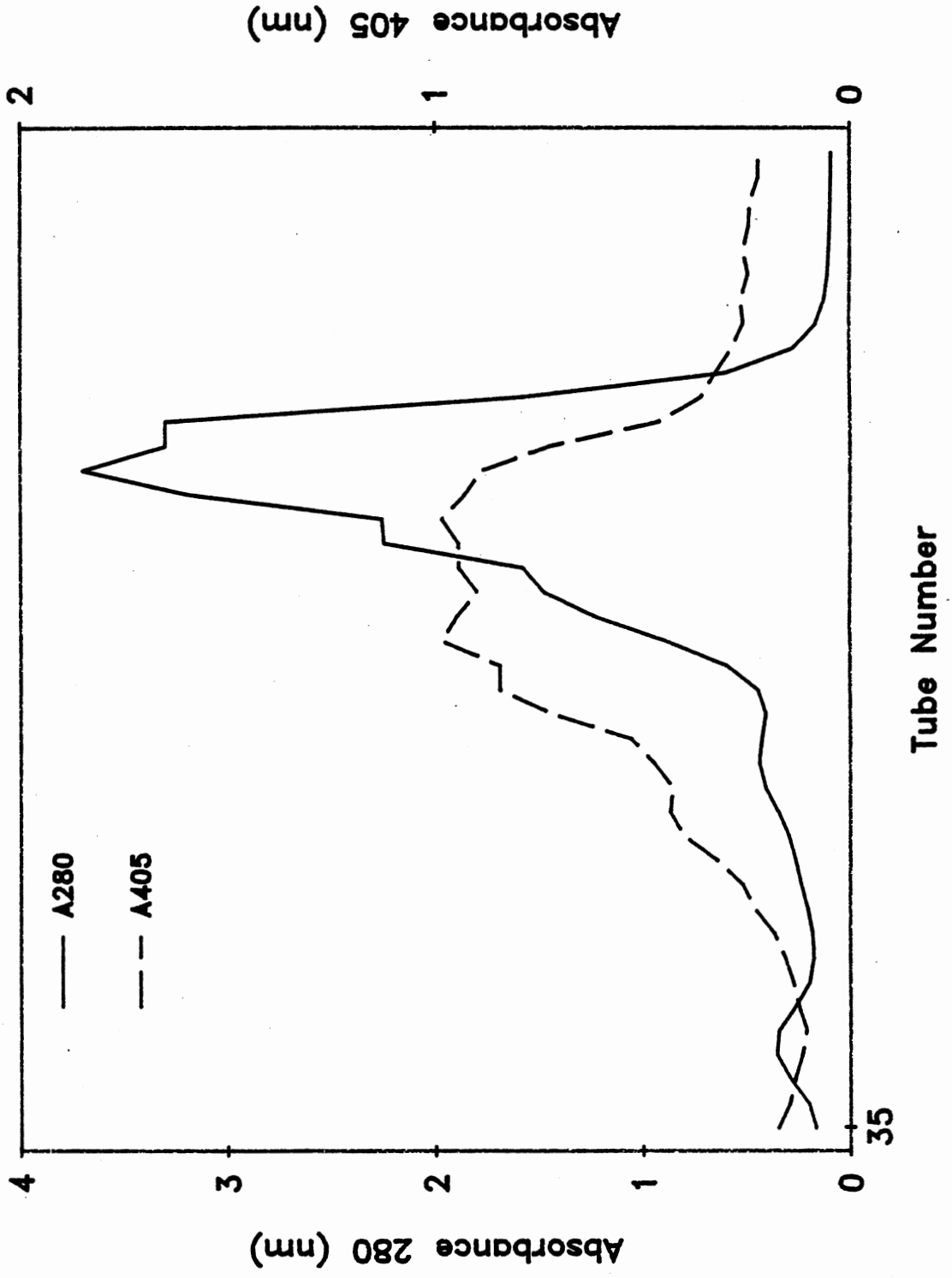

Absorbance 280 (nm)
Absorbance 405 (nm)
Tube Number
A280
A405
0
1
2
3
4
35

Figure 3. Dilutions of an IgG fraction containing antibodies to myotoxin were tested with an ELISA antibody detection system. Microtiter plates were coated with 1µg/ml myotoxin. Standard error bars are not present when they were too small to be visible.

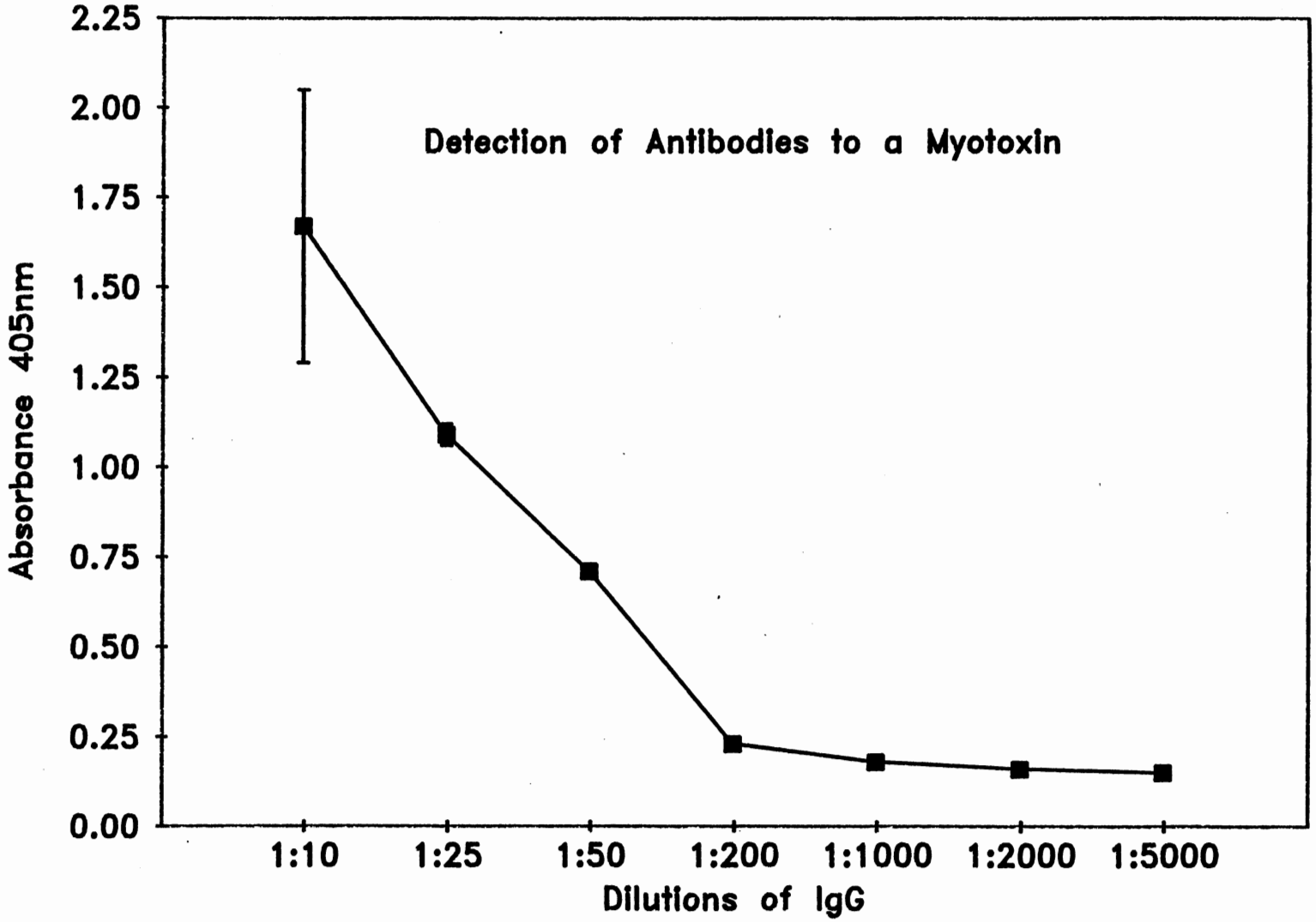

Detection of Antibodies to a Myotoxin
Absorbance 405nm
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1:10
1:25
1:50
1:200
1:1000
1:2000
1:5000
Dilutions of IgG

TABLE I

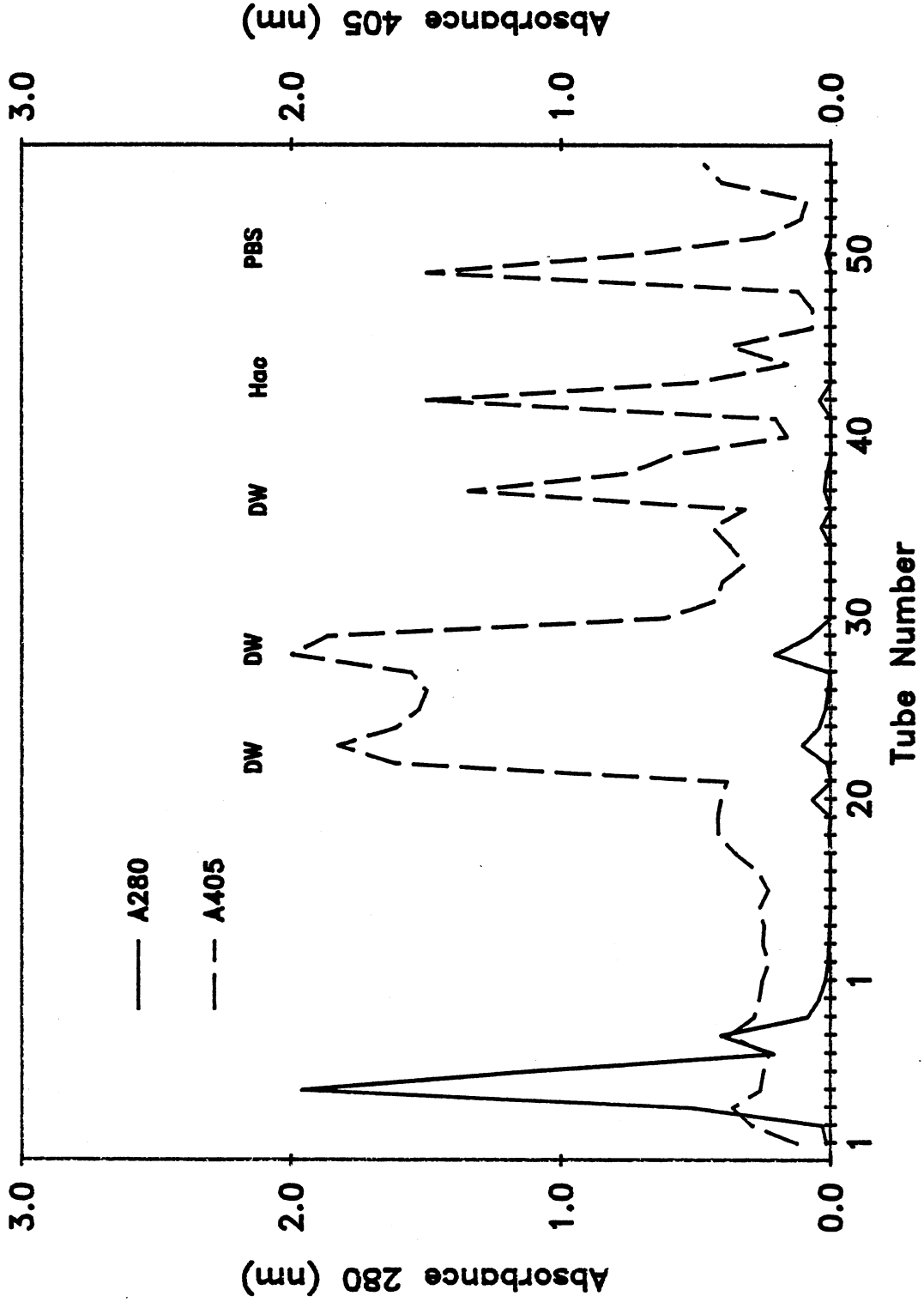
DETECTION OF A MYOTOXIN BY AN IgG FRACTION USING ELISA

Dilutions of Myotoxin*	Absorbance 405 (nm)
1:10	2>
1:100	1.54 ± 0.01
1:500	1.14 ± 0.02
1:1000	1.03 ± 0.02
1:5000	0.38 ± 0.02
1:10000	0.24 ± 0.02
1:1000000	nd
IB**	0.17 ± 0.01

* Dilutions of myotoxin were made from a 1.0mg/ml solution in incubation buffer containing 1% BSA.

** IB=incubation buffer, 1% BSA, represents the background absorbance and it was subtracted from all other absorbance values. Expressed as mean ± standard error. nd= non detectable.

Figure 4. The affinity purification of 5 ml crude serum containing antibody to myotoxin *a* on an Affi-Gel 10 myotoxin *a* coupled column. Crude serum was applied to the column which had been equilibrated with phosphate buffered saline (PBS), pH 7.4. The column was eluted initially with PBS followed by distilled water (DW). After a 12 h interruption the column was eluted again with DW, when baseline was reached the column was again washed with DW followed by acetic acid (Hac), pH 3.1. The final protein peak was obtained with PBS. The column was rinsed with PBS till baseline was again reached. A volume of 5ml was collected per tube. All peaks were measured for protein content at A_{280} and antibody using ELISA at 405nm.


Absorbance 405 (nm)
3.0
2.0
1.0
0.0
PBS
Hac
DW
DW
DW
A280
A405
1
20
30
40
50
Tube Number
1
Absorbance 280 (nm)
3.0
2.0
1.0
0.0

the ability of the IgG fraction (0.01mg/ml) to bind the myotoxin when a 1:1000 dilution of second antibody was used. Sample wells that were coated with 0.01mg/ml of IgG and received a 1:1000 dilution of goat anti-rabbit IgG (second antibody) were able to detect myotoxin a at a dilution of 1:10,000. However, regardless of the amount of IgG used (0.50, 0.10 and 0.01mg/ml) all absorbance readings were at or near background absorbance values if goat anti-rabbit IgG was maintained at a 1:2000 dilution. Thus, different amounts of the IgG fraction used to coat the microtiter plates did not increase the sensitivity of the assay to detect myotoxin a. However, the sensitivity of the antigen detection assay could be increased by using a more concentrated solution of second antibody.

Affinity Purification of Antibodies to Myotoxin a

The affinity purification of five milliters of crude antiserum from a rabbit immunized with pure myotoxin a is shown in Figure 4. The initial peak or void volume had a large amount of protein as determined by A_{280} but only a small amount of antimyotoxin was detected when the fractions were measured with the antibody detection ELISA at a wavelength of 405 nm. The fractions of the void volume had an absorbance between 0.20 and 0.36 at 405nm, but an absorbance of almost 2.0 at $_{280}$nm. Antibody to myotoxin a began to elute with the first washing with

distilled water (DW). The interrupted phase of twelve hours followed by a second washing with DW yielded more antimyotoxin. A third washing with DW was tried but yielded virtually no antibody. This step was subsequently removed from the protocol. The final two elutions with acetic acid (Hac, pH 3.1) and phosphate buffered saline (PBS, pH 7.4) eluted a small amount of antimyotoxin.

Affinity purification of ten milliters of crude serum from a rabbit immunized with pure myotoxin a is shown in Figure 5. The larger sample size yielded more antibody to myotoxin a in the DW, Hac and PBS peaks. However, the void volume also contained antibodies to the myotoxin as determined by the antibody detection ELISA. This finding suggest that the myotoxin coupled to the affinity column was saturated with its specific antibody, and the excess antimyotoxin enters the void volume with other serum proteins. Therefore, to efficiently use the affinity column the smaller sample size (5 ml) is desirable.

Standard Curves. A comparison was made of the ability of antibodies to myotoxin eluted with DW, acetic acid and PBS to bind myotoxin-coated microtiter plates. It was of interest to know if antibodies obtained with the different eluants interacted in a similiar manner with a known amount of toxin (1µg/ml), ie. provide similiar absorbance values. Table II shows the absorbance values at 405nm from an antibody detection ELISA using affinity purified antibody

Figure 5. The affinity purification of 10ml crude serum containing antibody to myotoxin *a* on an Affi-Gel 10 myotoxin *a* coupled agarose column. The column was eluted initially with PBS, pH 7.4 followed by DW. After an interruption of 12h the column was again washed with DW, then Hac, pH 3.1. A volume of five ml was collected per tube. When baseline was reached the column was eluted with PBS followed by Hac and a final PBS elution. All peaks were measured for protein content at A_{280} nm and antibody at 405 nm using ELISA.

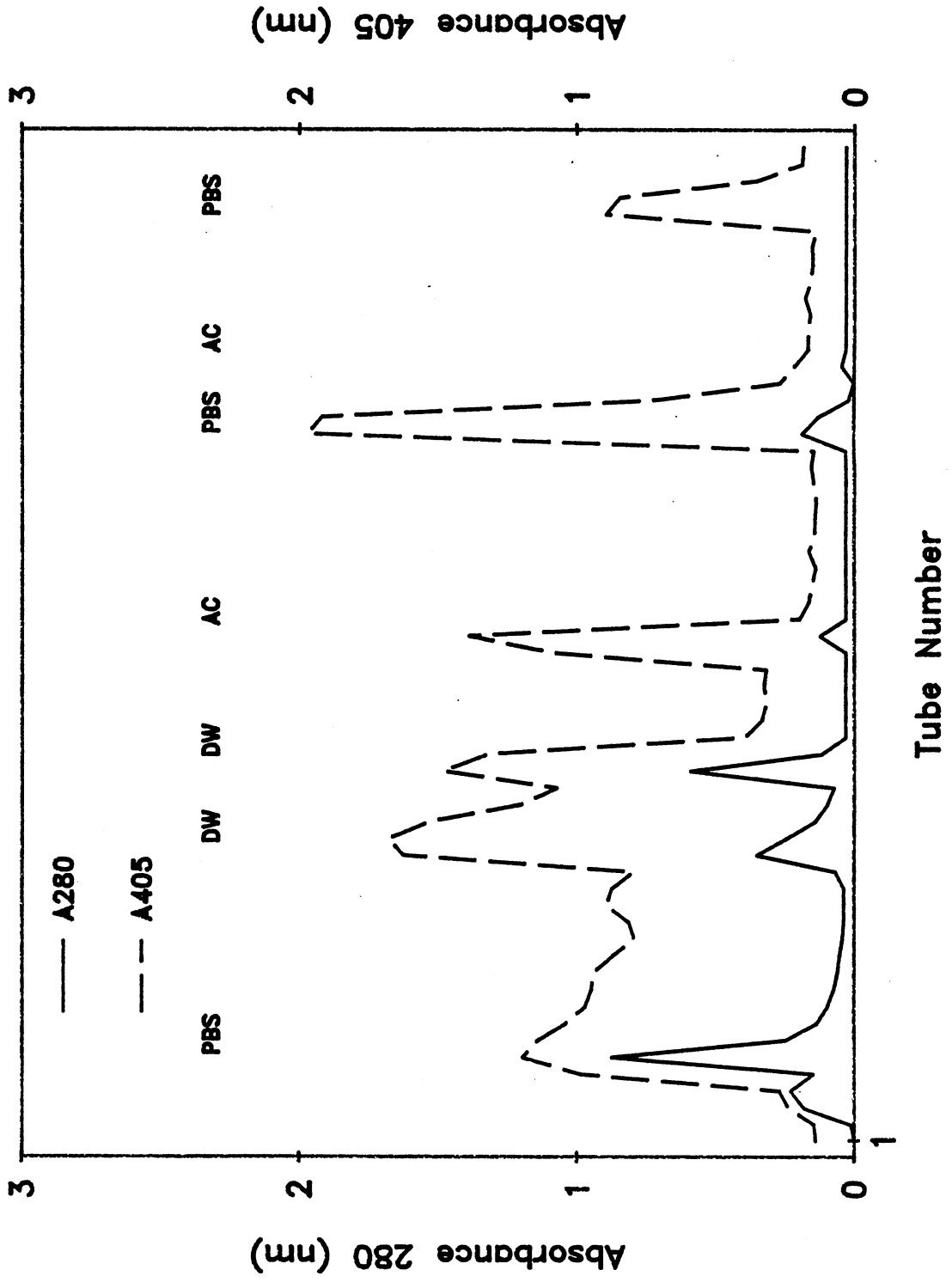

Absorbance 280 (nm)
Absorbance 405 (nm)
Tube Number
A280
A405
PBS
DW
DW
AC
PBS
AC
PBS
0
1
2
3

TABLE II

DETECTION OF AFFINITY PURIFIED ANTIBODY ELUTED WITH DW, ACETIC ACID AND PBS USING ELISA

Ab (ug/ml)*	DW	Acetic Acid	PBS
		Absorbance 405 (nm)	
10.00	0.77	0.80	0.93
7.00	0.79	0.63	0.90
5.00	0.73	0.52	0.76
3.00	0.55	0.48	0.54
1.50	0.48	0.34	0.30
1.00	0.22	0.13	0.10
0.50	0.11	0.04	0.05
0.10	0.06	0.03	0.02
IB**	0.12	0.12	0.12

* Ab = affinity purified antibody eluted with DW, acetic acid, pH 3.1, or PBS, pH 7.4.

** IB = incubation buffer, 1% BSA represents background absorbance subtracted from all other absorbance values.

eluted with DW, Hac and PBS. The correlation coefficients for the DW, Hac and PBS standard curves are 0.87, 0.94 and 0.94, respectively. The antibody eluted with DW was detected at lower concentrations compared to antibody eluted with Hac and PBS. The absorbance of 1 µg/ml of DW eluted antibody was 0.224 compared to 0.130 and 0.098 for antibody eluted with Hac and PBS, respectively. However, antibody eluted with Hac and PBS had higher absorbances at the higher concentrations of antibody when compared to antibody eluted with DW.

Table III shows the absorbance values for myotoxin a using the affinity purified antibody with the antigen detection ELISA. Antibodies to myotoxin a eluted with DW, Hac and PBS were able to detect 50 and 25 ng/ml of myotoxin a. Affinity purified antibody eluted with DW and Hac both had an absorbance value above 0.20 at 405 nm for 20 ng/ml myotoxin. The correlation coefficients for the myotoxin a standard curves using affinity purified antimyotoxin eluted with DW, and Hac are 0.99 and 0.97, respectively.

Neutralization of Myonecrosis by Affinity Purified Antimyotoxin. Table IV shows the myonecrosis indices obtained from mice injected with a mixture of toxin and affinity purified antibody. Myonecrosis was reduced in the presence of antibody eluted with DW, Hac and PBS. The decrease in myonecrosis was greatest for mice treated with antibody eluted with DW. The decrease of myonecrosis for

TABLE III

MYOTOXIN A DETECTION (ELISA) WITH ANTIMYOTOXIN ELUTED WITH DW, ACETIC ACID AND PBS

Solution*	DW	Acetic Acid	PBS
		Absorbance (405 nm)	
IB	0.124	0.144	0.160
M-50 (ng/ml)	0.700	0.548	0.800
M-25 (ng/ml)	0.312	0.265	0.475
M-20 (ng/ml)	0.236	0.304	nd**
M-15 (ng/ml)	0.132	0.153	nd
M-10 (ng/ml)	0.088	0.152	nd
M-05 (ng/ml)	0.047	0.098	nd

* IB = incubation buffer, 1% BSA, represents background absorbance. Background was subtracted from all other absorbance values. M = myotoxin a (ng/ml).

** nd = not determined. PBS eluted a very small amount of protein. This peak was not used in later experiments.

TABLE IV

NEUTRALIZATION OF MYONECROSIS BY AFFINITY PURIFIED ANTIBODY

Treatment*	Myonecrosis index**
AbDW + Myo	7.7 ± 1.8
AbHac + Myo	3.7 ± 2.4
AbPBS + Myo	3.3 ± 1.3
Myo + DW	12.0 ± 4.7
Myo + Hac	7.6 ± 1.7
Myo + PBS	4.5 ± 2.0
AbDW + DW	0.0
AbHac + Hac	1.9 ± 1.7
AbPBS + PBS	0.0
DW	0.0
Hac	3.7 ± 2.4
PBS	0.0

* Toxin and antiserum were incubated together at 37 C for one hour. 0.05 ml of each solution was injected im into mice (n=5). Muscle samples were taken twenty-four hours after the injection. Ab=antimyotoxin, DW= distilled eluant, Hac=acetic acid eluant, PBS=PBS eluant and Myo=myotoxin a. The ratio of antibody to toxin is 300 µg:750 µg.

** Myonecrosis index= number of necrotic cells/number of total cells expressed as mean ± standard error.

mice injected with toxin and antibody eluted with DW, Hac or PBS when compared to toxin alone provided indices of 1.9, 3.9 and 2.3, respectively. Antimyotoxin eluted with DW, acetic acid and PBS reduced myotoxin a-induced myonecrosis, however it was not significant ($p<0.05$).

Control groups did not show any vacuolation of skeletal muscle. Mice treated with acetic acid alone did have some myonecrosis, however these muscle cells did not show the typical vacuolation induced by myotoxin a.

Neutralization of Myonecrosis Using Crude Antisera

Experiment I. Table V shows the amount of myonecrosis expressed as the myonecrosis index induced by two concentrations of myotoxin in the presence or absence of antisera. Myonecrosis induced by 1.5µg/g myotoxin a was reduced by the antiserum, however it was not significantly different ($p<0.05$) when compared to the control group which received the toxin alone. The table also shows that antisera collected from two different rabbits both immunized with purified myotoxin a had different abilities to neutralize local myonecrosis caused by 0.75 µg/g myotoxin. Antiserum (AS-1) did not decrease the percent of myonecrosis induced by 0.75 µg/g myotoxin, however AS-2 slightly reduced the local myonecrosis induced by the same amount of toxin, but this was not significant ($p<0.05$).

TABLE V

NEUTRALIZATION OF MYONECROSIS BY TWO CRUDE ANTISERA WITH TWO AMOUNTS OF TOXIN

Treatment*	Toxin (μg/g)**	Myonecrosis index***
AS-1	1.50	3.3 ± 1.6
AS-1	0.75	4.9 ± 1.8
AS-2	0.75	2.6 ± 1.3
PrI	1.50	10.6 ± 3.4
PrI	0.75	2.9 ± 0.7

* Antisera (AS) was combined with the toxin at a 1:20 dilution. An amount of 0.05 ml was injected into 25 ± 0.50g mice. PrI= pre-immune serum.

** Toxin = myotoxin a

*** Myonecrosis index = number of necrotic cells/number of total cells. Expressed as mean ± standard error (n=4).

Experiment II. Table VI shows the amount of local myonecrosis induced by myotoxin a (1.5 μg/g) in the presence of 1:10, 1:20 or 1:40 crude serum containing antibodies to myotoxin a. Myonecrosis between groups receiving different amounts of antisera was not significantly different ($p<0.05$). Control mice receiving pre-immune serum and toxin had a higher myonecrosis index than mice receiving PSS and toxin, however it was not statistically significant ($p<0.05$). All groups receiving antiserum had less myonecrosis when compared to the control group which received pre-immune serum and toxin. The decreases in myotoxin a-induced myonecrosis were not significant ($p<0.05$).

Discussion

Affinity purified antisera to myotoxin a did not significantly decrease ($p<0.05$) myotoxin a-induced myonecrosis. Crude antisera contained precipitating antibodies that are capable of decreasing the percent myonecrosis induced by 1.5 μg/g, however in the presence of 0.75 ug/g toxin did not significantly ($p<0.05$) decrease myonecrosis. This may be due to the fact that at lower concentrations of toxin, detection of myonecrosis at the level of the light microscope is not always possible. Thus, changes in the amount of myonecrosis due to the presence of the antiserum are also more difficult to detect. Interestingly, increasing amounts of crude

TABLE VI

NEUTRALIZATION OF MYONECROSIS BY A CRUDE ANTISERUM AT THREE DILUTIONS

Treatment*	Toxin (ug)**	Myonecrosis index***
AS (1:10)	1.50	8.5 ± 3.1
AS (1:20)	1.50	12.3 ± 4.8
AS (1:40)	1.50	10.6 ± 3.0
PSS	1.50	6.3 ± 1.9
PrI	1.50	11.6 ± 3.8
AS	0.00	0.0
PSS	0.00	0.0

* 0.05 ml of each solution was injected intramuscularly into a group of mice (n=5). Muscle samples were taken twenty-four hours after the injection. PSS = physiological saline, AS = antiserum, and PrI = pre-immune serum.

** Toxin = myotoxin a

*** Myonecrosis index = number of necrotic cells/number of total cells. Expressed as mean ± standard error (n=5).

antisera did not neutralize more myotoxin a-induced myonecrosis. Ownby et al. (1983a) have observed that myotoxin a plus horse serum induces more myonecrosis than toxin and physiological saline (PSS). This is consistent with the results obtained in this study, which shows not only more myonecrosis when toxin and pre-immune serum are injected, but also an increase in myonecrosis with increasing amounts of crude antiserum. The possibility exists that some component in the crude serum acts to enhance the activity of the toxin by increasing its binding to skeletal muscle.

Following affinity purification of the crude antiserum non-precipitating antibodies were obtained. The presence of non-precipitating antibodies in antiserum has been recognized for quite some time, however the exact nature and role of these antibodies are still not completely understood. Christian (1970) has suggested that a limited recognition of multiple antigenic determinants may result in a failure of antibodies to precipitate. It has also been suggested that non-precipitating antibodies may be low affinity antibodies (Yang et al., 1977). Although, Yang et al. (1977) have reported the isolation of a non-precipitating antibody to cobrotoxin using a column of cobrotoxin-Sepharose, a technique requiring antibody of high affinity. It may be that our antibody has a limited recognition of the antigenic epitopes of myotoxin a or is a low affinity antibody.

The largest percent of our antibody was eluted under gentle conditions. Bureau and Daussant (1983) have successfully used desorption of an antigen using distilled water and an interrupted phase followed by distilled water. However, the affinity of the antigen for its antibody was not tested. Using the ELISA detection system it is possible to obtain some idea of the affinity of an antibody for its specific antigen (Lew, 1984). Since the affinity of an antibody for its antigen can be extemely varied Lew (1984) has suggested that ELISA be used as a qualitative or semi-quantitative test. A high affinity antibody can detect antigen of less than one nanogram when an antigen of high epitope density is used, however a low affinity antibody may be sensitive to levels only greater that two micrograms per milliter (Lew, 1984). Our ELISA results also show differences in antibody sensitivity for its antigen depending upon whether the antibody was eluted with DW, Hac or PBS. Antibody eluted with PBS, the final eluant, had the highest absorbance readings for both the antibody and antigen detection assays, however it proved the least effective in neutralizing myonecrosis. Since the PBS peak yielded a very small amount of antibody it was not possible to test the antibody eluted with this buffer extensively. The initial antibody peak which was eluted with DW may be a lower affinity antibody, as suggested by its lower absorbance readings for both the antigen and antibody ELISA's, this however does not preclude the hypothesis that

the antibody recognizes and binds to the antigenic epitope on the myotoxin located in the active site of the molecule.

Antibody to myotoxin a obtained by gel filtration was not tested for its neutralizing capabilities, however the antimyotoxin of the IgG fraction did bind to the purified antigen in the antibody detection ELISA. Our IgG fraction (0.01mg/ml) was quite capable of detecting myotoxin a, however it was critical that the second antibody was used at a dilution of 1:1000. The IgG fraction was able to detect very small amounts of antigen (1:10,000 or 0.1ng/ml myotoxin). Ho et al.(1986a) have also reported an increase in sensitivity for the double antibody sandwich ELISA using an IgG purified by protein A affinity chromatography. The increased sensitivity of the antigen (Calloselasma rhodostoma) detection ELISA allowed for the accurate measurement of small changes in venom levels. It may be correct to assume that Ho et al. (1986a) had a higher affinity antibody than our antimyotoxin, since the amount of IgG used to coat their microtiter plates was as low as 1-2 µg/ml IgG compared to our 0.5-0.01mg/ml IgG.

In conclusion, antibody to myotoxin a has been affinity purified and tested for its ability to bind the antigen "in vitro" using the 1) antibody detection ELISA, 2) antigen detection ELISA and "in vivo" 3) by measuring the myonecrosis index of mice injected with a mixture of affinity purified antimyotoxin and myotoxin a. The latter

results have been compared to the ability of crude antisera to neutralize myotoxin a-induced myonecrosis. Affinity purified antibody cannot neutralize myonecrosis, although slight differences in the myonecrosis index were observed between antibodies eluted with DW, acetic acid and PBS. In addition affinity chromatography removes extraneous components of the crude serum which appear to enhance local myonecrosis. Importantly, the affinity purified antibody to myotoxin a was useful in establishing antigen and antibody detection ELISA systems that proved helpful in later investigations.

CHAPTER IV

THE *IN VIVO* DISAPPEARANCE OF ANTIBODY TO MYOTOXIN *A* FROM THE BLOOD

Introduction

ELISA is playing an important role in the development of field tests for the rapid identification of the snake venom in specimens such as blood, urine or bite-site swab washings (Chandler and Hurrell, 1982; Dhaliwal et al., 1983). In addition, ELISA has also been used as an epidemiological tool to detect the presence of venom-antibodies in human snakebite victims from the region of Bambur, Nigeria (Theakston et al., 1981) and to detect circulating antibodies in subjects living in epidemic yellow fever areas (Deubel et al., 1983). Clinical studies suggested that venom-antibody titers too low to be detected by ELISA, may be boosted with further bites, and the homologous venom-antibody may afford significant protection against further poisoning (Theakston et al., 1981). More recently Lwin et al. (1984) and Ho et al. (1986b) have used the ELISA antigen (*Vipera russeli* and *Calloselasma rhodostoma*) detection system in a clinical setting to monitor venom levels. The ELISA has proved particularly useful in these investigations since persistant high blood-

venom levels may indicate the need for more antivenin or suggest that the antivenin being administered is ineffective. However, the use of ELISA to monitor blood levels of administered antibody has not been studied. Currently decisions to administer more antiserum are based in part on the blood level of venom rather than antiserum (Ho et al., 1986b). Although it is extremely useful to detect blood-venom levels, the need to monitor blood-antibody levels is critical.

In the United States numerous crotaline venoms have been shown to share a specific deleterious protein, myotoxin a (Chapter VI) that is known to induce local myonecrosis (Ownby et al., 1984b). An antiserum has since been produced against the myotoxin that is capable of neutralizing the local myonecrosis.

Therefore, an experiment was performed to study the disappearance of antibodies to myotoxin a from blood over a four week period. The difference in rate of disappearance of antibody for different concentrations of antiserum was also studied. An ELISA antibody detection system was used to detect and monitor blood levels of antibody to myotoxin a.

Materials and Methods

Antibody Detection ELISA

Ninety-six well flat bottom microtiter plates (Flow Laboratories, Mclean, VA) were coated with 1 µg/ml of purified myotoxin a. The myotoxin a was diluted with coating buffer, pH 9.6 (1.59g Na_2CO_3, 2.93g $NaHCO_3$, 0.20g NaN_3, 1g bovine serum albumin, BSA dissolved in one liter distilled water). Two-hundred microliters of 1 µg/ml myotoxin a were added to each well and incubated overnight at 4 C. The following day the microtiter plates were rinsed three times with washing buffer (45.0g NaCl, 2.5ml Tween-20 dissolved in 5 liters distilled water). The plates were allowed to soak five minutes between each rinsing. Following the final rinsing, two-hundred microliters of either known concentrations of antibody or blood samples were added to each well. The antibody solutions for the standard curve were diluted with incubation buffer (900ml phosphate buffered saline, PBS pH 7.4, 0.45ml Tween-20, 0.18g NaN_3 and 1g BSA) The antibody standard solutions consisted of the following concentrations 500, 400, 200, 100, 50, 25, 10 and 5 ng/ml. Four wells coated with myotoxin a received two-hundred microliters of incubation buffer alone containing 1% BSA and were assayed for non-specific binding. All samples were incubated for two hours at room temperature. After the incubation period the plates were rinsed three times with washing buffer. Following the final rinsing

two-hundred microliters of goat anti-rabbit IgG (Sigma, St. Louis, MO) at 1:2000 dilution were added to each well. Goat anti-rabbit IgG was diluted in incubation buffer 1% BSA. The plates were again incubated for two hours at room temperature and following the two hour incubation the plates are again rinsed. Two-hundred microliters of phosphatase substrate (p-nitrophenyl phosphate disodium, Sigma, St. Louis, MO) were then added to each well. The phosphatase substrate was diluted to 1 mg/ml in diethanolamine buffer, pH 9.8 (9.7ml diethanolamine, Sigma, St. Louis, MO, 80ml distilled water, 10.0 $MgCl_2$, 0.02g NaN_3). The substrate was allowed to react for thirty minutes at room temperature and the reaction was stopped with 0.050ml 3N NaOH. Absorbance of each well was measured at 405nm with an EIA manual reader. The amount of color formed is proportional to the amount of antibody present in each sample. Absorbance values (405nm) were converted to concentration (ng/ml) of antibody using the standard curve of that plate.

Disappearance of Crude Antiserum

Crude antiserum was injected instead of affinity purified antibody for two important reasons. Ownby et al. (1983a) have shown this crude antiserum capable of decreasiing myotoxin a-induced myonecrosis, thus these are

two-hundred microliters of goat anti-rabbit IgG (Sigma, St. Louis, MO) at 1:2000 dilution were added to each well. Goat anti-rabbit IgG was diluted in incubation buffer 1% BSA. The plates were again incubated for two hours at room temperature and following the two hour incubation the plates are again rinsed. Two-hundred microliters of phosphatase substrate (p-nitrophenyl phosphate disodium, Sigma, St. Louis, MO) were then added to each well. The phosphatase substrate was diluted to 1 mg/ml in diethanolamine buffer, pH 9.8 (9.7ml diethanolamine, Sigma, St. Louis, MO, 80ml distilled water, 10.0 $MgCl_2$, 0.02g NaN_3). The substrate was allowed to react for thirty minutes at room temperature and the reaction was stopped with 0.050ml 3N NaOH. Absorbance of each well was measured at 405nm with an EIA manual reader. The amount of color formed is proportional to the amount of antibody present in each sample. Absorbance values (405nm) were converted to concentration (ng/ml) of antibody using the standard curve of that plate.

Disappearance of Crude Antiserum

Crude antiserum was injected instead of affinity purified antibody for two important reasons. Ownby et al. (1983a) have shown this crude antiserum capable of decreasiing myotoxin a-induced myonecrosis, thus these are antibodies known to neutralize myonecrosis. Secondly, the amount of affinity purified antibody available was not

enough to permit an *in vivo* experiment.

Female mice (CD-1, Charles Rivers, MA) weighing 25 ± 0.5g were divided into six groups, five mice per group. The six groups consisted of mice receiving intravenous injections (i.v.) of 1) 0.10ml crude antiserum, 2) 0.10ml physiological saline (PSS), 3) 0.15ml crude antiserum, 4) 0.15ml PSS, 5) 0.20ml crude antiserum and 6) 0.20ml PSS. Crude antiserum was obtained from a rabbit immunized against myotoxin *a* and tested on a 1% agarose gel diffusion plate for a single precipitin band. All injections were given into the tail vein and blood samples (0.04ml) taken with a capillary tube were diluted with 0.960ml of PBS 1% BSA. Blood samples were taken immediately (zero time), and 0.5, 1, 3, 6, 12, 24, 48, 72, 96h, 2 and 4 weeks following the injections. A two-hundred microliter aliquot from each blood sample was assayed in duplicate using the antibody detection ELISA system. Dilutions of the affinity purified antimyotoxin *a* were used for the standard curve and assayed with each microtiter plate. Also, sample wells coated with antigen and receiving incubation buffer instead of antimyotoxin *a* were always included to test for non-specific binding.

Results

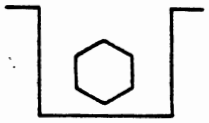
Antibody Detection ELISA

Figure 6 shows a flow chart of the steps involved in the antibody detection assay. Our ELISA plates had

Figure 6. A flow chart illustrating the steps involved in the antimyotoxin detection ELISA.

ELISA FOR ANTI-MYOTOXIN a DETECTION

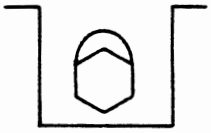
1. MYOTOXIN a ⬡ ADSORBED TO PLATE



WASH

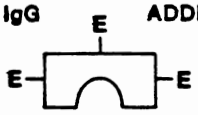
2. SOLUTION CONTAINING ANTI-MYOTOXIN a

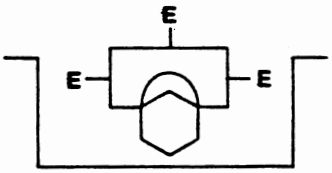
◠ ADDED



WASH

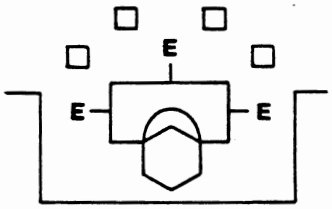
3. ALKALINE PHOSPHATASE LABELLED ANTIBODY TO RABBIT IgG ADDED



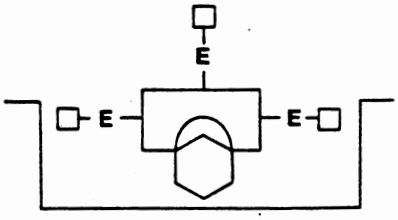


WASH

4. ENZYME SUBSTRATE □ ADDED



5. READ ABSORBANCE OF REACTION PRODUCT □ AT 405nM



extremely low non-specific binding with absorbance readings slightly above 0.10. Table VII shows the absorbance readings for three standard curves using known amounts of affinity purified antibody. The correlation coefficients of the three standard curves are 0.91, 0.97 and 0.96. The small differences between duplicate samples are reflected in the small standard errors. Differences in absorbance readings for the same amount of antibody assayed on different plates are due at least in part to differences between ELISA microtiter plates. Regardless of the plate used the non-specific binding was never above 0.121.

Disappearance of
Crude Antiserum

Figures 7, 8 and 9 show the disappearance of antimyotoxin following the injection of 0.10, 0.15 and 0.20 crude antisera, respectively. The pattern of antimyotoxin disappearance for each group was very similar. Blood levels of antibodies to myotoxin for each group decreased sharply three hours following the injection of antiserum. A slight increase in the level of blood antimyotoxin occured at six hours for each group. However, blood levels of antimyotoxin gradually decreased at 12, 24, 48, 72 and 96h until antibody was no longer detected at two and four weeks. At 24, 48, 72 and 96h blood levels of antibody to myotoxin were very similar among treatment groups. The mice

TABLE VII

DETECTION OF ANTIMYOTOXIN USING ELISA

Ab (ng/ml)*	Plate 1	Plate 2	Plate 3
	Absorbance 405 (nm)		
500	0.439 ± .01	0.687 ± .02	0.568 ± .02
400	0.502 ± .02	0.462 ± .01	0.505 ± .02
200	0.403 ± .01	0.357 ± .01	0.393 ± .03
100	0.270 ± .01	0.229 ± .01	0.225 ± .01
50	0.111 ± .01	0.173 ± .00	0.239 ± .01
25	0.075 ± .01	0.198 ± .01	0.062 ± .00
10	0.031 ± .01	0.028 ± .01	0.027 ± .01
5	0.023 ± .01	0.024 ± .01	0.024 ± .02
IB**	0.116 ± .004	0.121 ± .008	0.119 ± .002

* Ab = affinity purified antibody obtained with the DW eluant. The antibody standard curve was assayed on three different microtiter plates. All samples were assayed in duplicate. Expressed as mean ± standard error.

** IB = incubation buffer 1% BSA. Sample wells were coated with antigen and IB was assayed for non-specific binding. The background values have been subtracted from each absorbance reading.

Figure 7. Graph showing the disappearance of anti-myotoxin a from the blood of mice using the antibody ELISA detection system. Each point represents the mean of five mice. Mice were injected (i.v.) with 0.10ml crude antiserum.

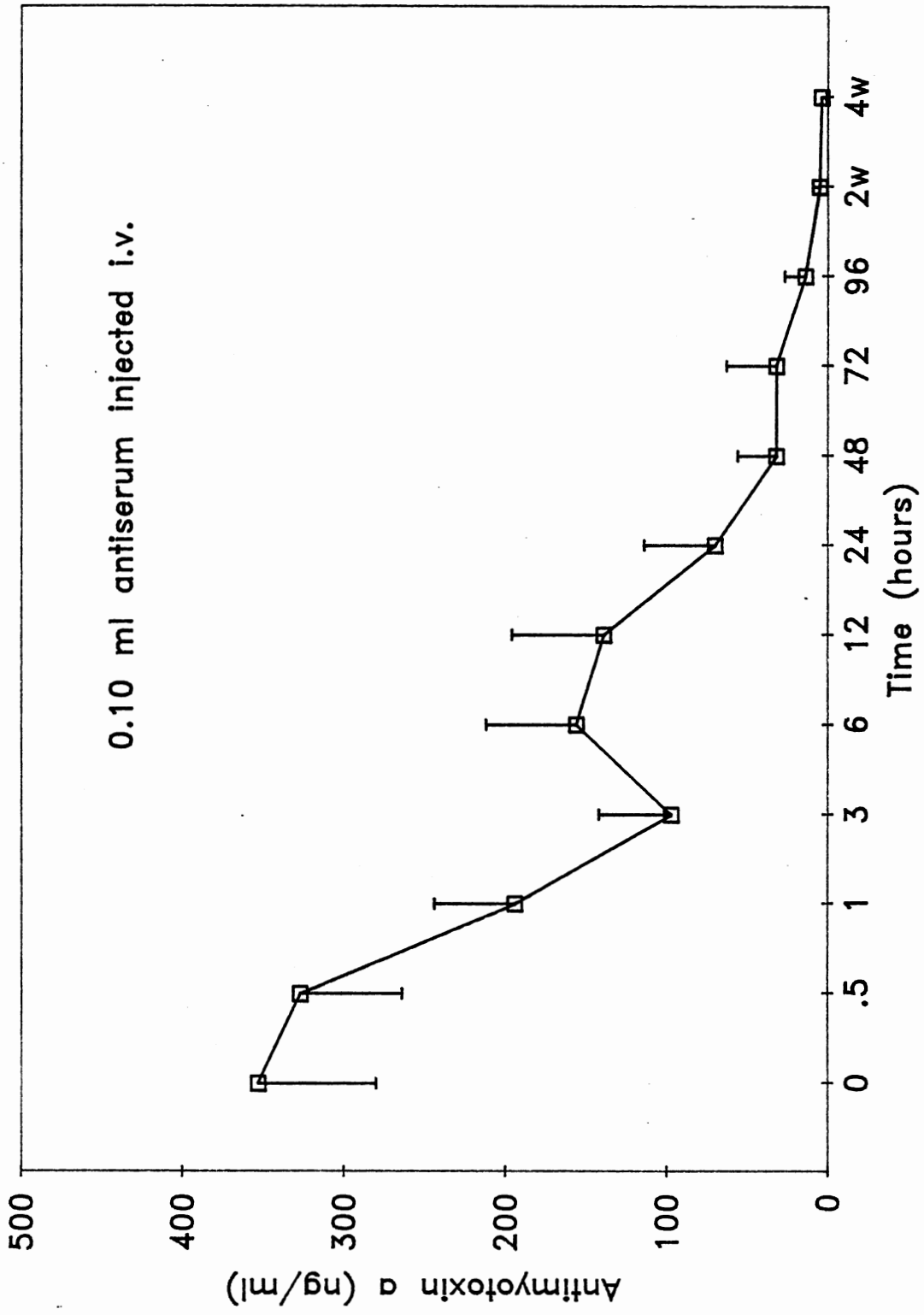

0.10 ml antiserum injected i.v.
Antimyotoxin a (ng/ml)
500
400
300
200
100
0
0
.5
1
3
6
12
24
48
72
96
2w
4w
Time (hours)

Figure 8. Graph showing the disappearance of anti-myotoxin from the blood of mice using the antibody ELISA detection system. Each point represents the mean of five mice. Mice were injected (i.v.) with 0.15ml crude antiserum.

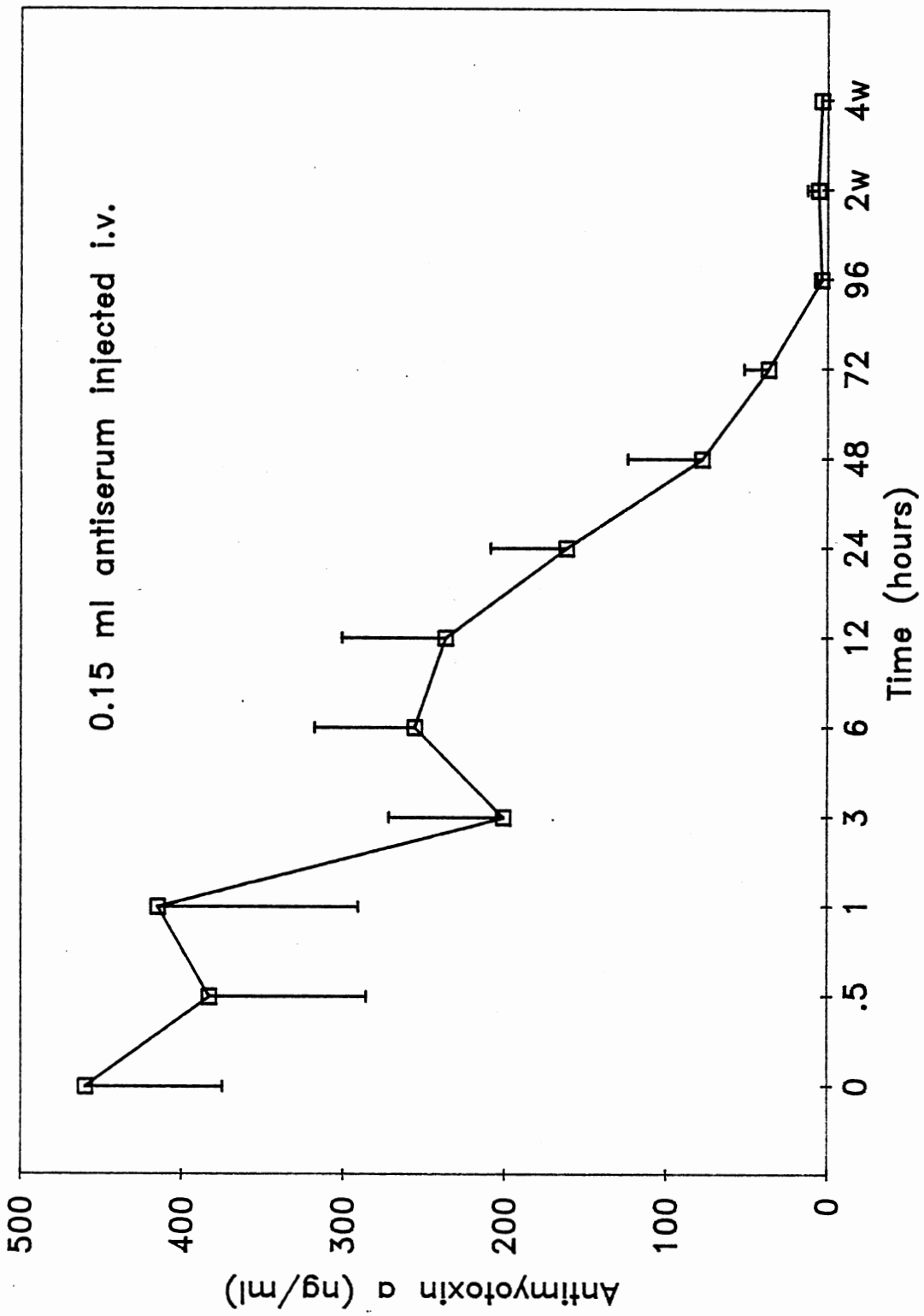
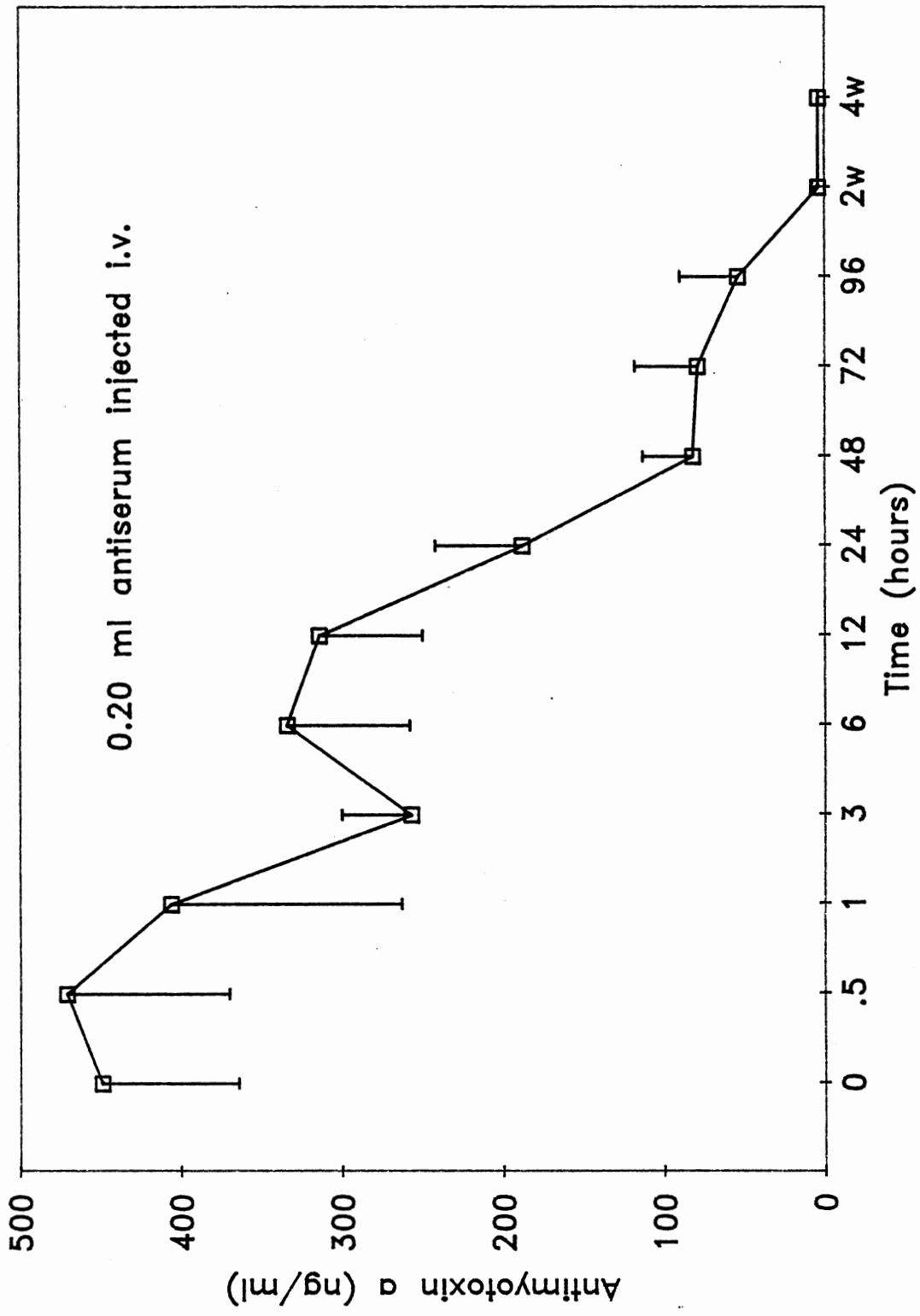

0.15 ml antiserum injected i.v.
Antimyotoxin a (ng/ml)
500
400
300
200
100
0
0
.5
1
3
6
12
24
48
72
96
2w
4w
Time (hours)

Figure 9. Graph showing the disappearance of anti-myotoxin from the blood of mice using the antibody detection system. Each point represents the mean of five mice. Mice were injected (i.v.) with 0.20ml crude antiserum.


0.20 ml antiserum injected i.v.
Antitmyotoxin a (ng/ml)
500
400
300
200
100
0
0
.5
1
3
6
12
24
48
72
96
2w
4w
Time (hours)

injected with 0.10ml antiserum had the lowest blood levels of antimyotoxin, and as might be expected the 0.20ml of crude antiserum had the highest absorbance values during the ninety-six hour sampling period, but decreased to non-detectable levels at two and four weeks identical as in the 0.10 and 0.15ml crude antiserum treated groups. Blood samples taken from the control groups had low background absorbance readings, 0.10-0.20.

Discussion

Blood levels of antibodies to myotoxin a have been monitored over a four week period using ELISA. Although care must be taken when extrapolating results between laboratory animals and human snakebite victims, our results indicate a rapid clearance of antibody from the blood in all groups, particularly within the first three hours. Regardless of the amount of antiserum injected within three hours or less the blood-antiserum levels had decreased by at least fifty percent when compared to zero time. Blood levels of antimyotoxin were very similar between treatment groups at 24, 48, 72 and 96h indicating that the amount of antiserum injected did not significantly alter its disappearance. By two weeks the antiserum had completely cleared from the bloodstream in all groups. Therefore, the effect of time on the rate of disappearance of antimyotoxin from the blood seems to play a more important role than the amount of antiserum injected.

It is particularly noteworthy that after only twenty-four hours the blood levels of antimyotoxin had decreased seventy percent, when compared to zero time. It is also important to keep in mind this rapid decrease in blood levels of antibodies to myotoxin was observed in the absence of toxin. The presence of toxin is certain to increase the rate of disappearance of the antiserum from the blood (Chapter 5). Clinically the low levels of antibodies to myotoxin observed at 24h (as well as 48, 72 and 96 hours) are critical since such low concentrations of the antibody would probably not neutralize the myotoxic effects of the venom.

Ownby et al. (1976) have reported the sequence of degenerative events in skeletal muscle cells following an intramuscular injection of 1.5µg/g of pure myotoxin a. Their results show that partial vacuolation of muscle cells occur at 6, 12 and 24 hours and complete vacuolation and loss of striations at 48 and 72 hours. Thus, the degenerative effects induced by myotoxin occur over a matter of days. It is therefore, logical that a neutralizing level of antiserum must be sustained for perhaps days to properly treat myotoxin a-induced myonecrosis.

Ho et al. (1986b) have used the venom detection ELISA to monitor changes in blood-venom levels. The results of their assays were then used in part to decide upon further treatment. If blood-venom levels remained elevated or showed a tendency to increase, adminstration of more

antivenin was considered. The same rationale can be applied when monitoring blood-antivenom levels. High levels of blood-antivenom would indicate additional antivenom is not required, conversely low or non-detectable blood-antivenom levels indicate the need for more antivenom.

Closer attention must also be given to blood levels of crude antiserum especially since it is known that the administration of antiserum can result in further complications for the snakebite victim, such as serum sickness (Loprinzi et al., 1983; Jeter et al., 1983). Investigators (Russell et al., 1970; Kocholaty et al., 1971) have produced an antivenin in goats rather than horses as a means to avoid the allergic response seen in some patients to horse serum. However, the use of the ELISA to monitor blood levels of the antiserum may help minimize the allergic response seen in some patients by avoiding unnecessary administration of the antivenin and also indicate the time more antiserum should be administered.

In conclusion this current study provides important information regarding the disappearance of antibodies to myotoxin from the bloodstream, which has not previously been available. The level of antibodies to myotoxin decreases rapidly within the first three hours, gradually declining to low concentrations at 24, 48, 72 and 96h. These results are of considerable clinical importance, since it raises the question whether the low levels of

antibodies to myotoxin observed at and after twenty-four hours are adequate to neutralize myotoxin *a*-induced myonecrosis. This is a particularly important question because it is known that degenerative effects due to myotoxin can continue for a matter of days. In addition it is important to keep in mind that these values represent the disappearance of crude antiserum, however not in the presence of myotoxin *a*. The effect of myotoxin *a* on the disappearance of the crude antiserum is addressed in a later study.

CHAPTER V

THE USE OF ELISA TO DETECT CHANGES IN BLOOD LEVELS OF CRUDE ANTISERUM IN THE PRESENCE OF MYOTOXIN A

Introduction

The enzyme-linked immunosorbent assay (ELISA) is a technique which is gaining popularity in the area of venom research. Recently Theakston (1983) has reviewed the advantages and possible applications of ELISA in clinical studies of snake venom poisoning. The ELISA has since been used successfully in field and clinical trials to detect blood-venom levels of snakebite victims. In Australia the ELISA has been developed for the rapid detection of venom in bite site swabings and blood samples (Coulter et al., 1980), and in Thailand ELISA has been used to measure venom levels in the blood of snakebite victims (Ho et al., 1986b). Using ELISA, Ho et al. (1986b) observed that the blood-venom (Calloselasma rhodostoma) levels of snakebite victims can slowly increase following the initial decline of blood-venom levels. Therefore patients who are released may later have a reappearance of symptoms associated with snake venom poisoning.

Although the role and importance of ELISA in venom

detection is considerable, few investigators have used ELISA to monitor blood-antivenom levels. Theakston et al. (1981) have used ELISA to measure blood levels of antibodies produced by previous snakebite victims, however blood levels of experimentally injected antivenom have not been studied.

In the United States rattlesnake venom poisoning often results in local tissue damage. Ownby et al. (1976) isolated a myotoxic component, myotoxin a, from the Pairie rattlesnake (Crotalus viridis viridis) venom and produced an antiserum to the pure myotoxin a (Ownby et al., 1979). The antimyotoxin a serum is more effective in neutralizing local myonecrosis than the commerically produced Wyeth's polyvalent (Crotalidae) antivenin. However, the polyvalent antivenin is more effective in neutralizing lethality (Ownby et al., 1983a).

It would be of considerable interest and help to detect and monitor blood antivenom levels in the presence and absence of the toxin. Decisions to administer more or less antivenom could be made in part on the basis of blood-antivenom levels. Therefore, an experiment was designed that used ELISA to measure the disappearance of blood-antivenom in the presence of known amounts myotoxin a.

Materials and Methods

Myotoxin a

Myotoxin a was purified by Ownby and Colberg

(unpublished results) and the myotoxin was tested for its biological activity activity on skeletal muscle in mice as described in Chapter III.

Crude Antiserum

Serum was obtained from a rabbit immunized with pure myotoxin a as described in Chapter III. The crude antiserum was tested on a 1% agarose gel diffusion plate against myotoxin a for a single precipitin band. Previously this antiserum has been shown to neutralize the myotoxin activity of 0.75ug/g myotoxin a (Ownby et al., 1983a).

Disappearance of Crude Antiserum

Forty-five female mice weighing 25 ± 0.50g (CD-1, Charles Rivers) were divided into nine groups, five mice per group. All intravenous injections (i.v.) were given via the tail vein and all intramuscular injections (i.m.) were given into the dorso-lateral aspect of the right hind thigh of the mice. Crude antiserum was always injected i.v. toxin or PSS was injected i.m. The volume of crude antiserum was 0.20ml and the volume of PSS or toxin was 0.05ml. The nine groups were as follows: 1) crude antiserum 5 min before 1.5µg/g myotoxin a, 2) crude antiserum 5 min before 0.75µg/g myotoxin a, 3) crude antiserum 5 min before PSS, 4) crude antiserum immediately after 1.5µg/g myotoxin a, 5) crude antiserum immediately after 0.75µg/g myotoxin a, 6) crude antiserum immediately

after PSS, 7) crude antiserum 30 min after 1.5µg/g myotoxin a, 8) crude antiserum 30 min after 0.75µg/g myotoxin a and 9) crude antiserum 30 min after PSS. Blood samples (0.04ml) were taken from the tail vein using a capillary tube and immediately diluted in 0.960ml incubation buffer 1% BSA. Blood samples were taken immediately (zero time) and, 0.5, 1, 3, 6, 24, 48, 72 and 96h after the antiserum injection.

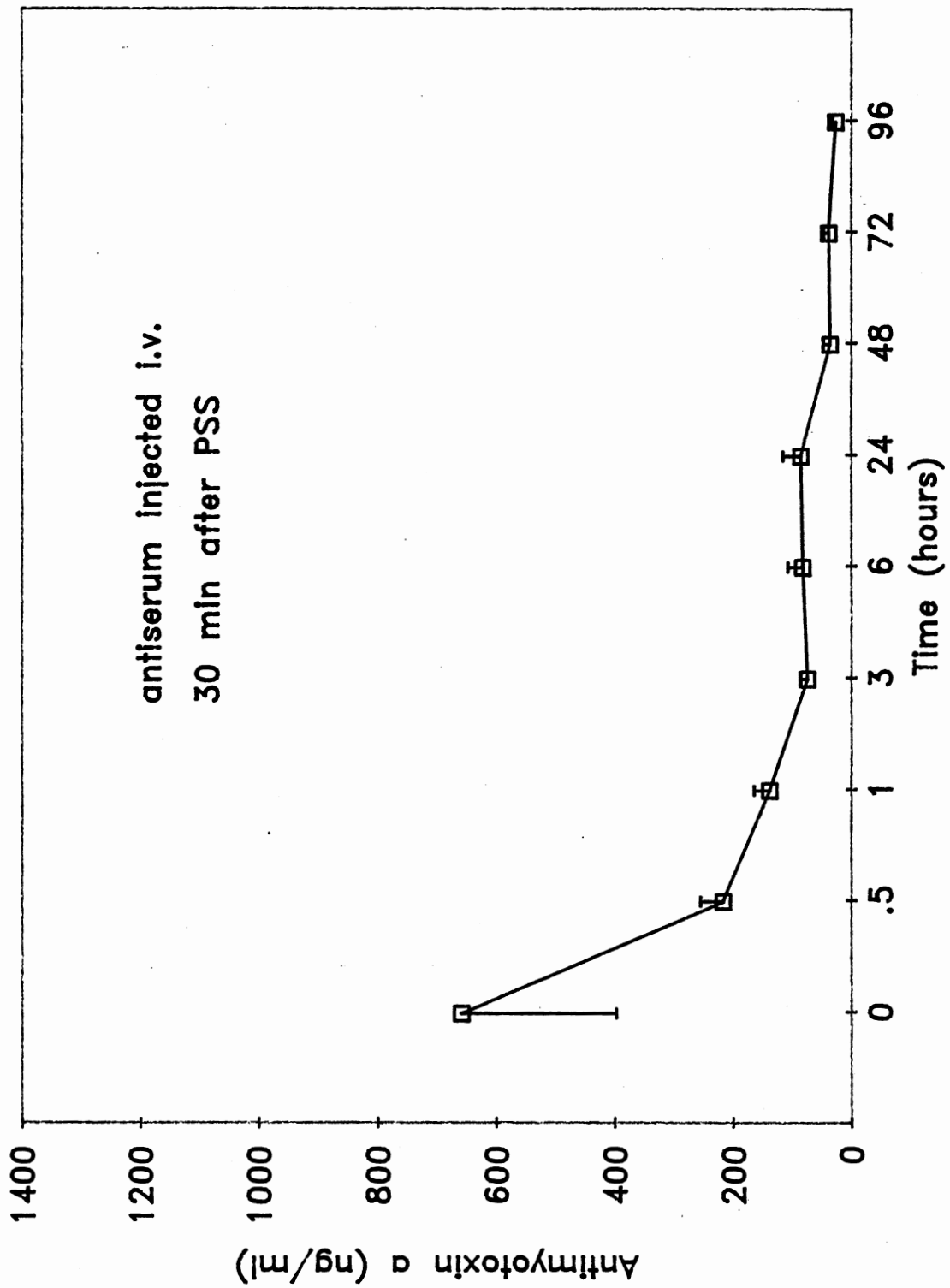
Antibody Detection ELISA

All blood samples were assayed in duplicate using the antibody detection ELISA as described in detail in Chapter IV. Pure myotoxin a (1µg/ml) was used to coat the microtiter plates and affinity purified antimyotoxin was used for the standard curve. A standard curve and control samples were included with each microtiter plate.

Results

Figure 10 shows the blood disappearance of antimyotoxin a at 0.0, 0.5, 1, 3, 6, 24, 48, 72 and 96h from mice injected (i.v.) with antiserum thirty minutes after an injection of PSS (i.m.). The blood level of antimyotoxin a at zero time was 675 ng/ml, however there was a large amount of variation between animals. The variation between animals was considerably less at the later time periods. Antimyotoxin was still detectable at twenty-four hours, however at 48, 72 and 96 hours antimyotoxin was non-detectable in the blood.

Figure 10. The disappearance of antimyotoxin a when injected (i.v.) 30min after PSS (i.m.).


antiserum injected i.v.
30 min after PSS
Antimyotoxin a (ng/ml)
1400
1200
1000
800
600
400
200
0
0
.5
1
3
6
24
48
72
96
Time (hours)

Figures 11 and 12 show the blood disappearance of antimyotoxin at 0.0, 0.5, 1, 3, 6, 24, 48, 72 and 96 hours from mice injected (i.v.) with antiserum thirty minutes after an injection (i.m.) of 0.75 and 1.5 μg/g myotoxin a, respectively. At zero time both groups had detectable levels of antimyotoxin a, although the group receiving 0.75 ug/g had a significantly ($p<0.05$) higher level of antibody at zero time than the 1.5 μg/g treated group. However, both groups showed a rapid decline in the level of blood-antimyotoxin. At 0.5h and for all later samplings the antibody was non-dectable.

Figure 13 shows the blood disappearance of antimyotoxin at 0.0, 0.5, 1, 3, 6, 24, 48, 72 and 96 hours from mice injected (i.v.) with crude antiserum immediately followed by an (i.m.) injection of PSS. Blood antimyotoxin remained constant at zero time and thirty minutes followed by a gradual decline at one and three hours. However, a fairly constant level of blood-antimyotoxin (268-236ng/ml) was maintained for all other sampling times. Antimyotoxin a was detectable at each time period.

Figures 14 and 15 show the blood disappearance of antimyotoxin at 0.0, 0.5, 1, 3, 6, 24, 48, 72 and 96 hours from mice when crude serum was injected (i.v) immediately after an (i.m.) injection of 0.75 and 1.5 μg/g myotoxin a, respectively. Mice injected with 0.75 μg/g myotoxin a had 427 ng/ml of antimyotoxin at zero time but rapidly declined to non-dectable levels for all other time periods. Mice

Figure 11. The disappearance of antimyotoxin a when injected (i.v.) 30min after 0.75µg/g myotoxin (i.m.).

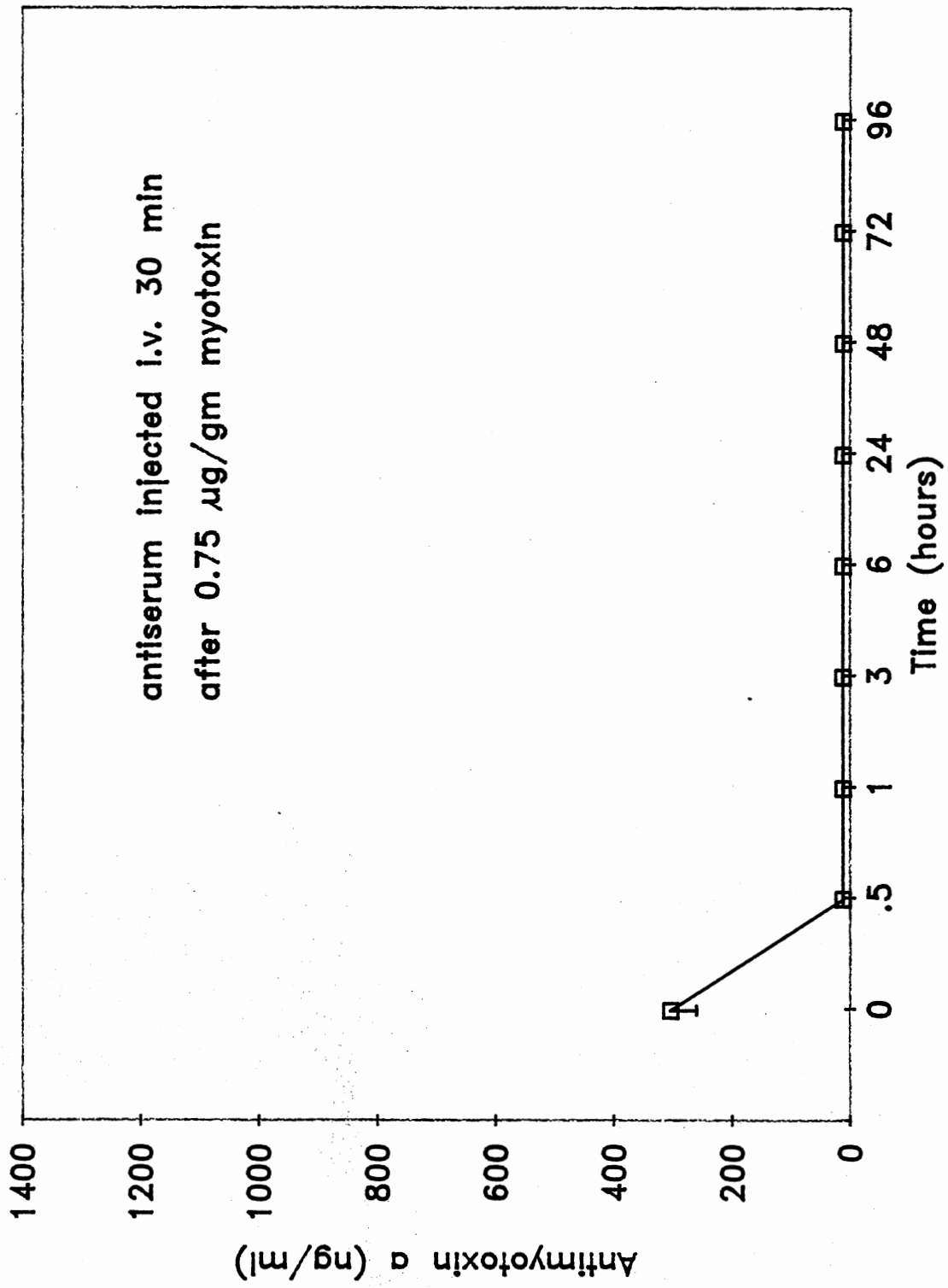

antiserum injected i.v. 30 min
after 0.75 µg/gm myotoxin
Antimyotoxin a (ng/ml)
1400
1200
1000
800
600
400
200
0
0
.5
1
3
6
24
48
72
96
Time (hours)

Figure 12. The disappearance of antimyotoxin a when injected (i.v.) 30min after 1.5μg/g myotoxin (i.m.).

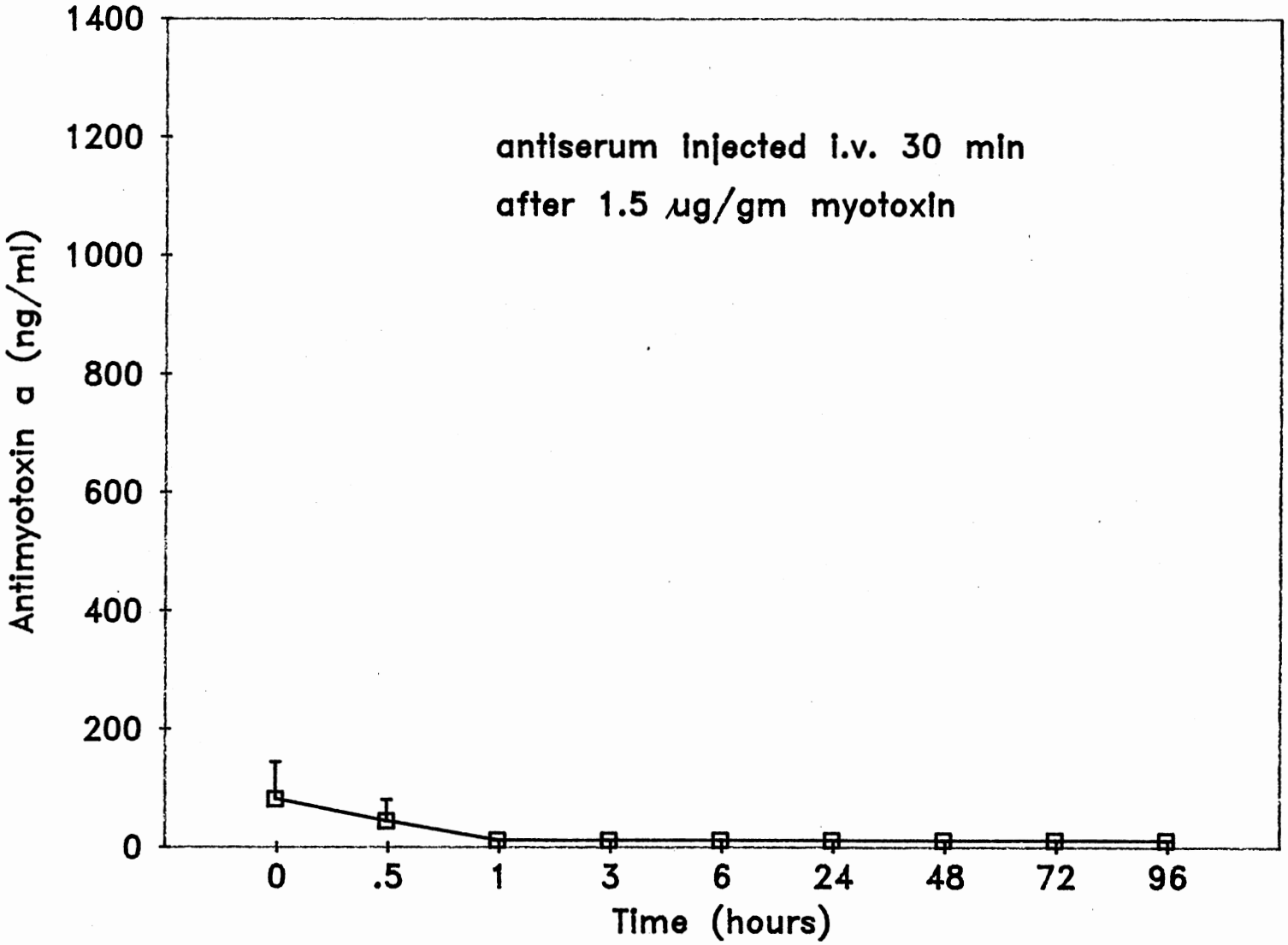

antiserum injected i.v. 30 min
after 1.5 µg/gm myotoxin
Antimyotoxin a (ng/ml)
1400
1200
1000
800
600
400
200
0
0
.5
1
3
6
24
48
72
96
Time (hours)

Figure 13. The disappearance of antimyotoxin a when injected (i.v.) immediately after PSS (i.m.).

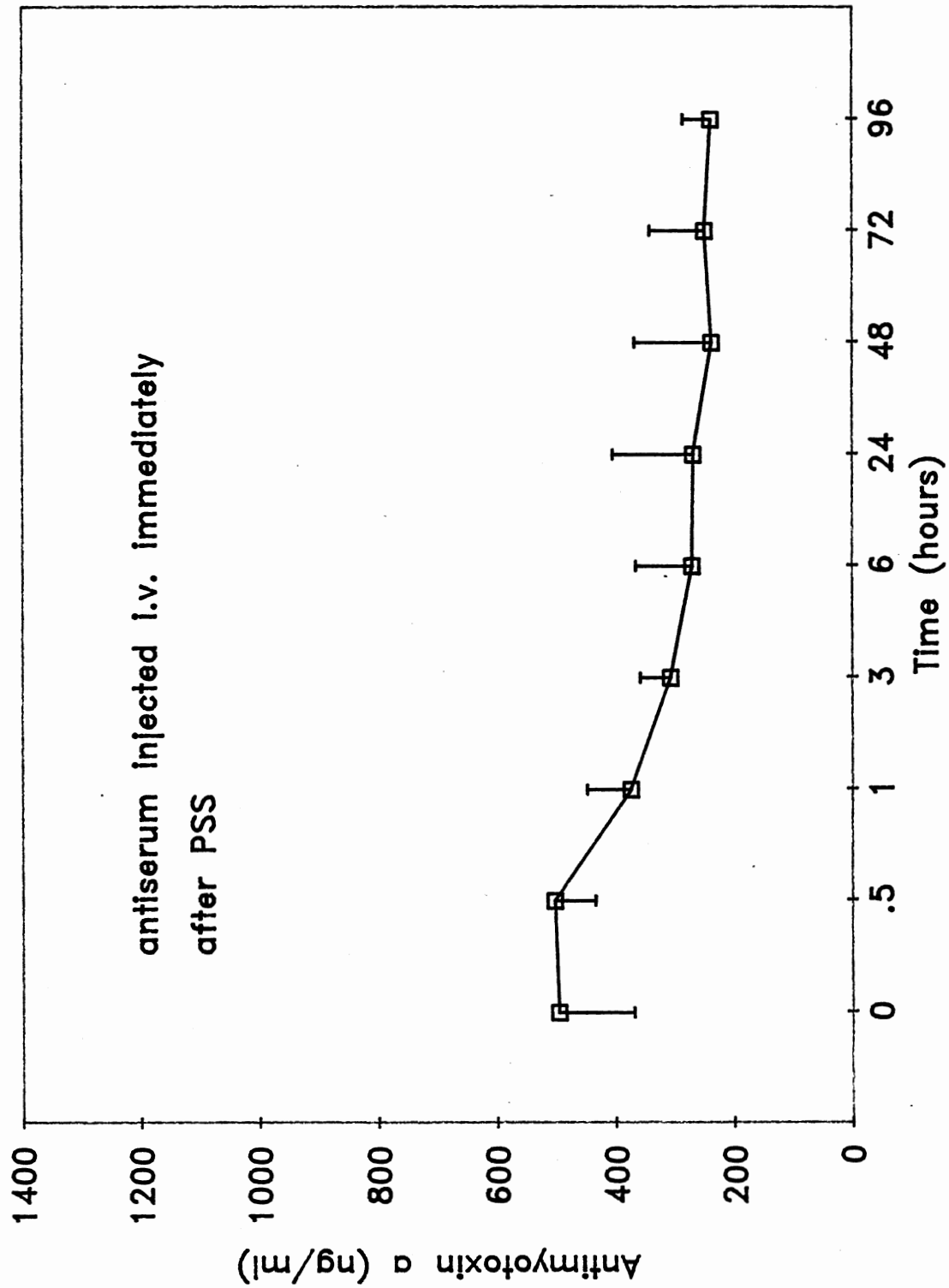

antiserum injected i.v. immediately
after PSS
Antimyotoxin α (ng/ml)
1400
1200
1000
800
600
400
200
0
0
.5
1
3
6
24
48
72
96
Time (hours)

Figure 14. The disappearance of antimyotoxin a when injected (i.v.) immediately after 0.75μg/g (i.m.).

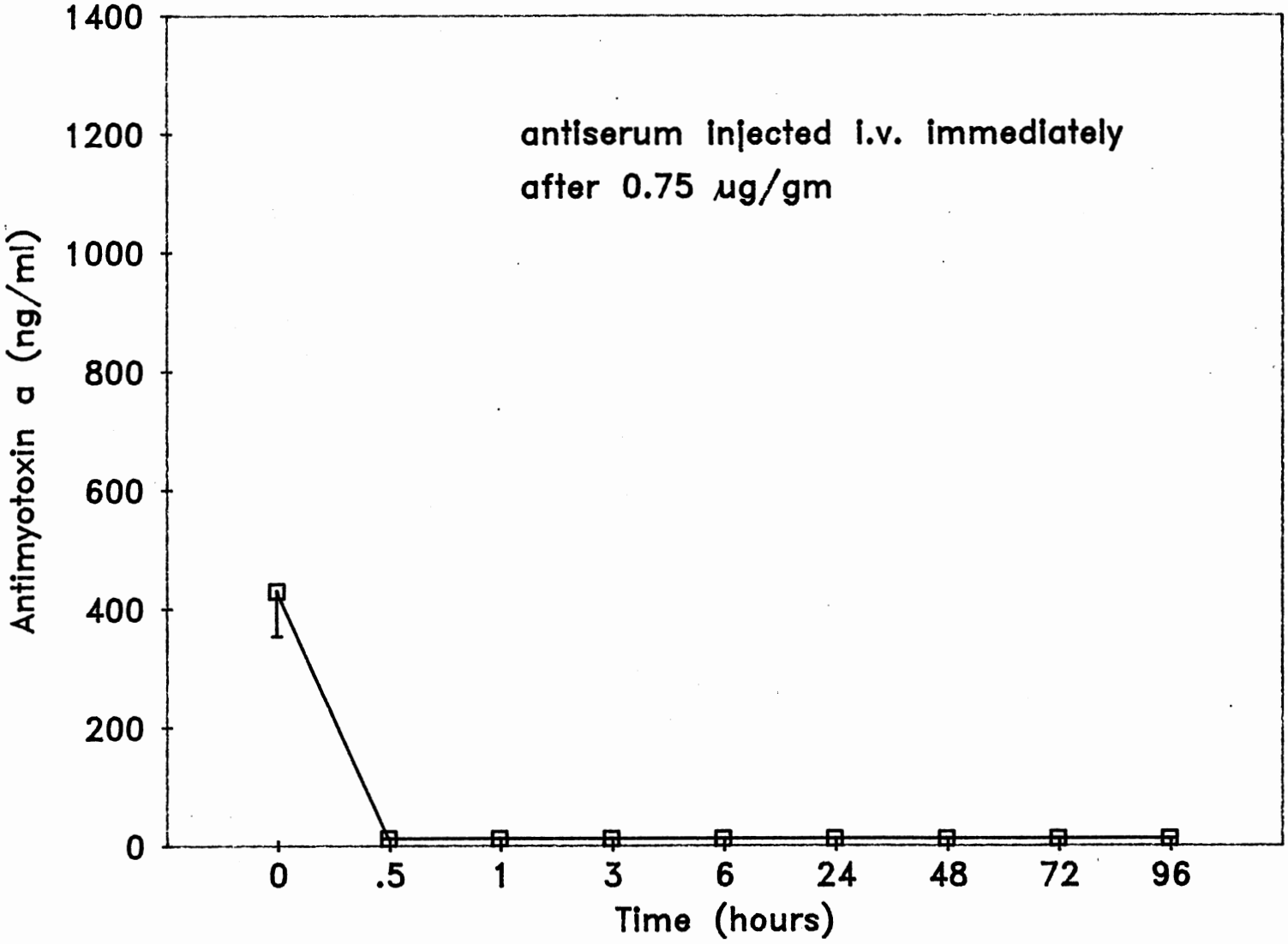
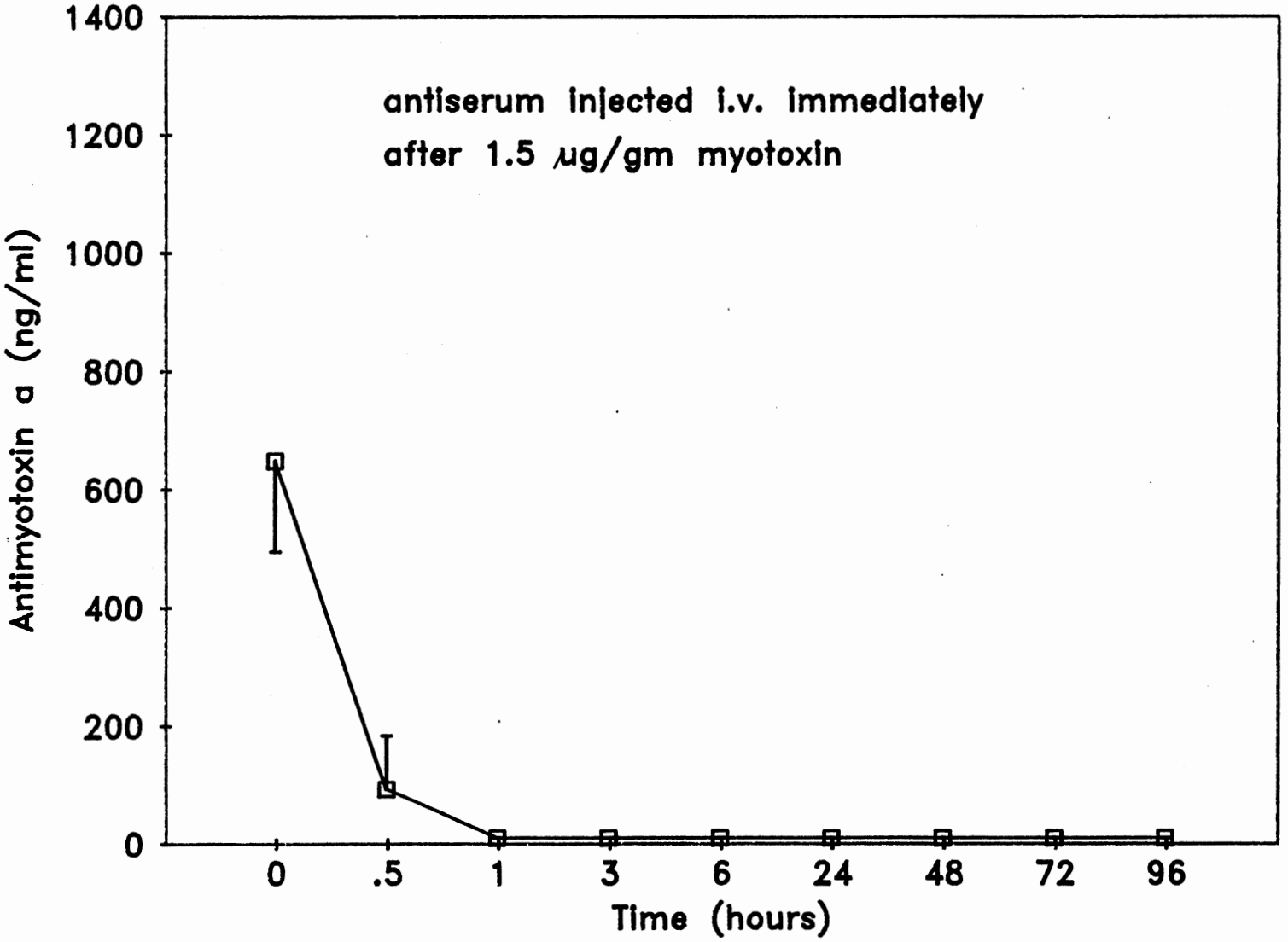

antiserum injected i.v. immediately
after 0.75 µg/gm
Antimyotoxin a (ng/ml)
1400
1200
1000
800
600
400
200
0
0
.5
1
3
6
24
48
72
96
Time (hours)

Figure 15. The disappearance of antimyotoxin a when injected (i.v.) immediately after 1.5μg/g myotoxin (i.m.).


antiserum injected i.v. immediately
after 1.5 µg/gm myotoxin
Antimyotoxin a (ng/ml)
1400
1200
1000
800
600
400
200
0
0
.5
1
3
6
24
48
72
96
Time (hours)

injected with 1.5 μg/g had 648 ng/ml blood-antimyotoxin at zero time. Antimyotoxin rapidly declined to a significantly less ($p<0.05$) value of 75 ng/ml. All other sampling times had non-detectable levels of antimyotoxin a.

Figure 16 shows the blood disappearance of antimyotoxin at 0.0, 0.5, 1, 3, 6, 24, 48, 72 and 96 hours from mice injected (i.v.) with crude antiserum five minutes before an (i.m.) injection of PSS. Blood-antimyotoxin was detectable at each sampling period. Antimyotoxin was 729 ng/ml at zero time and declined to 156 ng/ml at 96h.

Figures 17 and 18 show the blood disappearance of antimyotoxin at 0.0, 0.5, 1, 3, 6, 24, 48, 72 and 96 hours for mice injected (i.v.) with crude antiserum five minutes before an (i.m.) injection of 0.75 and 1.5 μg/g of myotoxin a, respectively. Mice injected with 0.75 μg/g had 799 ng/ml of antimyotoxin at zero time followed by a rapid decline to non-dectable levels at all other time periods. Mice injected with 1.5 μg/g had 1110 ng/ml antimyotoxin at zero time followed by a rapid decline to non-detectable levels of blood antimyotoxin at all other time periods.

Discussion

The presence of myotoxin a quickly and significantly ($p<0.05$) reduced the blood levels of antibody to myotoxin. Antibody levels were non-detectable at thirty and sixty minutes if the toxin was present, whereas if toxin is

Figure 16. The disappearance of antimyotoxin a when injected (i.v.) 5min before PSS (i.m.).

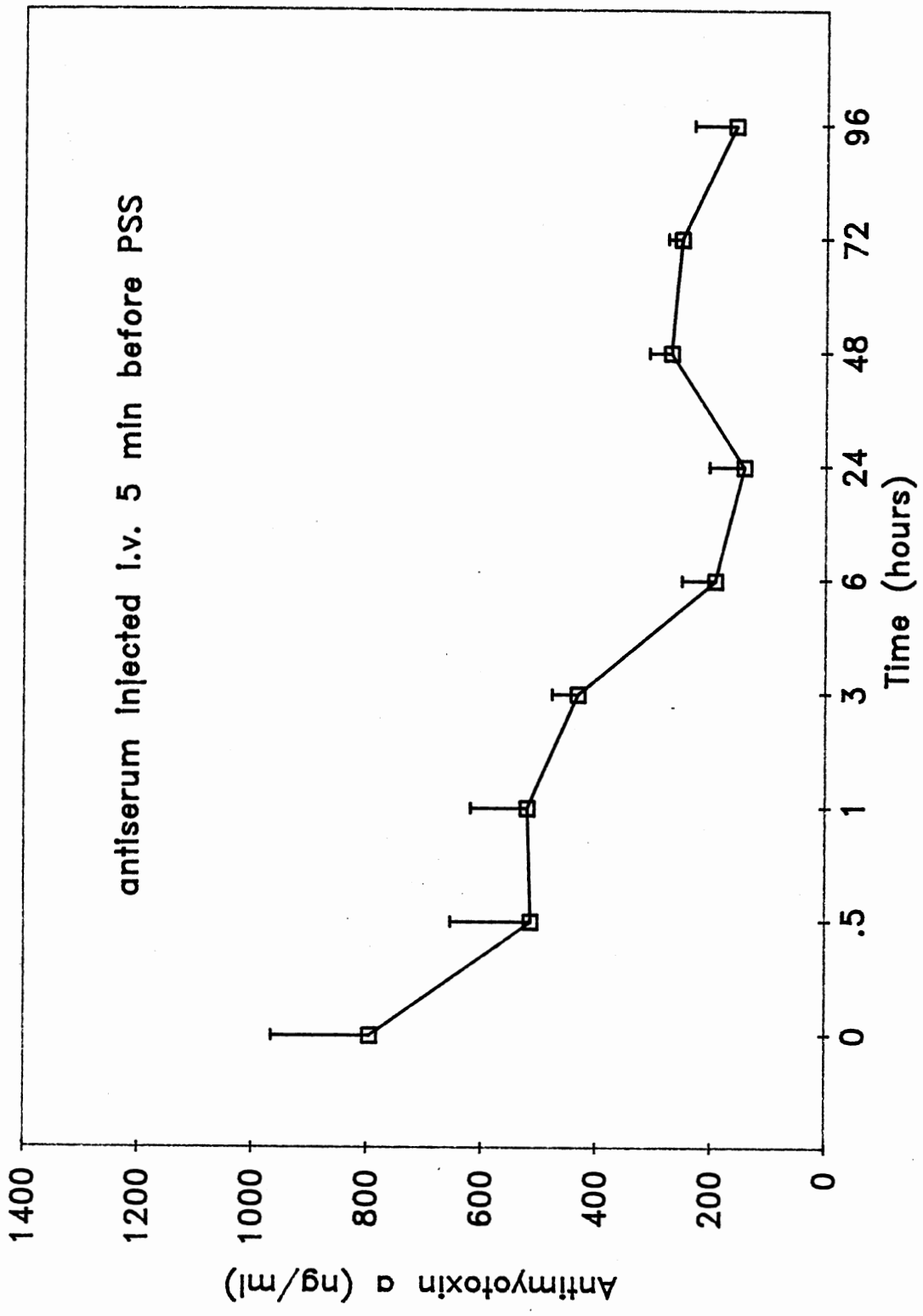

antiserum injected I.v. 5 min before PSS
Antimyotoxin a (ng/ml)
1400
1200
1000
800
600
400
200
0
0
.5
1
3
6
24
48
72
96
Time (hours)

Figure 17. The disappearance of antimyotoxin a when injected (i.v.) 5min before 0.75μg/g myotoxin (i.m.).

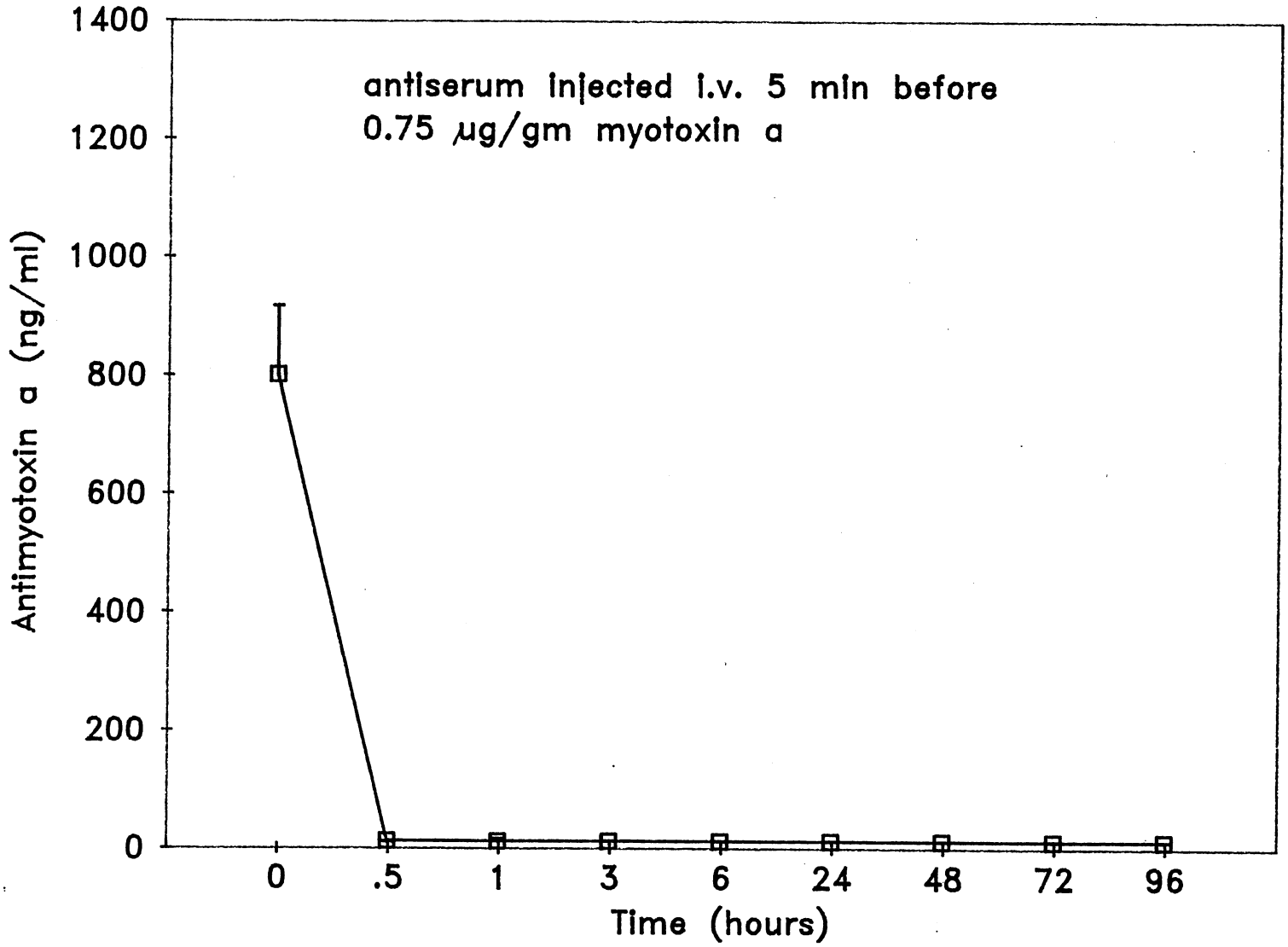
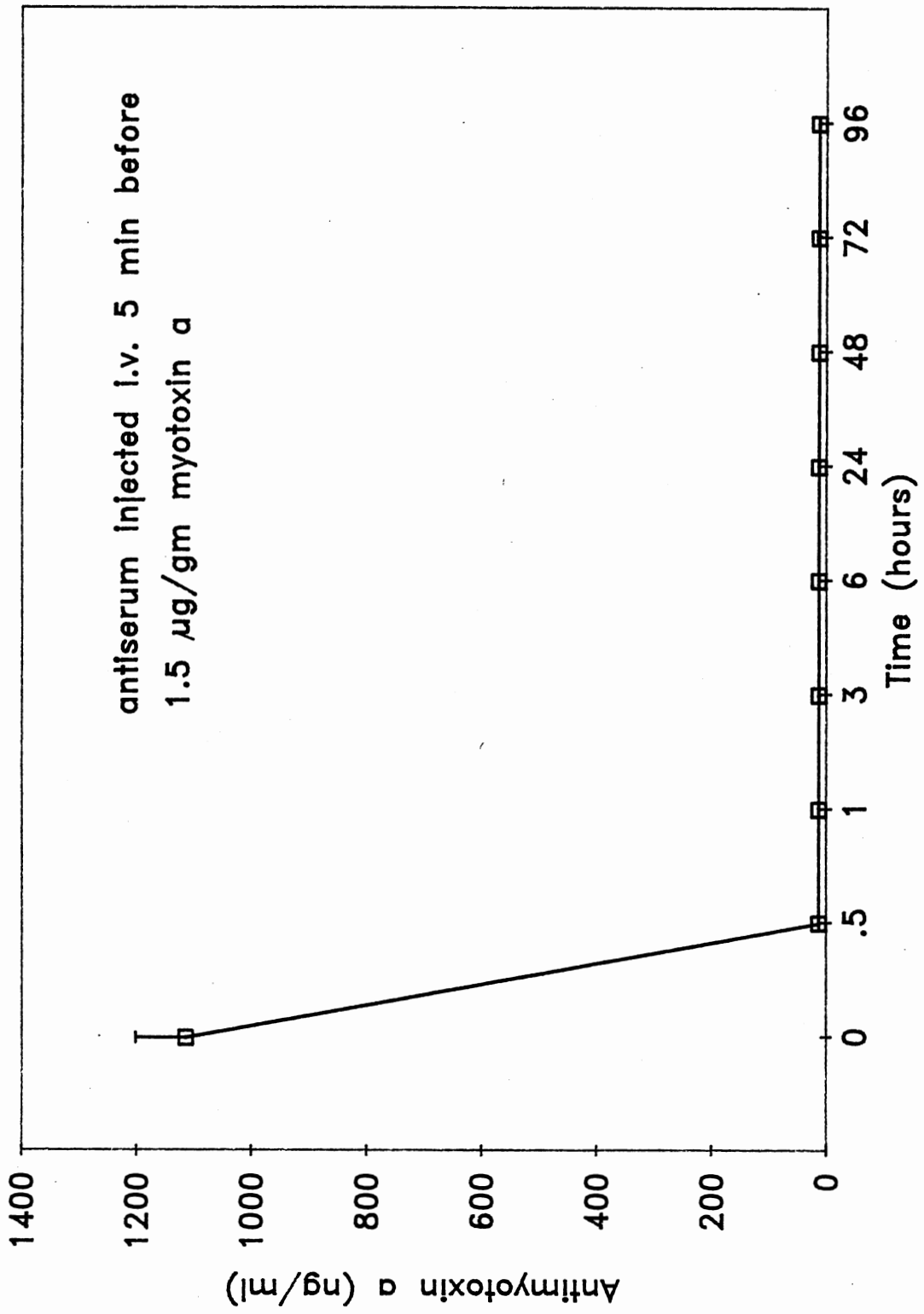

antiserum injected i.v. 5 min before
0.75 µg/gm myotoxin a
Antimyotoxin a (ng/ml)
1400
1200
1000
800
600
400
200
0
0
.5
1
3
6
24
48
72
96
Time (hours)

Figure 18. The disappearance of antimyotoxin a when injected (i.v.) 5min before 1.5μg/g myotoxin (i.m.).


antiserum injected i.v. 5 min before
1.5 µg/gm myotoxin a
Antimyotoxin a (ng/ml)
1400
1200
1000
800
600
400
200
0
0
.5
1
3
6
24
48
72
96
Time (hours)

absent antibody levels were detectable with each sampling. Injections of myotoxin at either (1.5 or 0.75μg/g) resulted in a sharp and rapid decrease of antibodies to myotoxin from the bloodstream. Animals receiving the antiserum thirty minutes after an injection of toxin 0.75 or 1.5μg/g had the lowest levels of antibody at zero time compared to the other toxin treated groups. This result is especially important since not only is the toxin still capable of binding to the antiserum, even though the antiserum is injected thirty minutes after the toxin, but in fact more toxin is binding to the antiserum than at any other time as indicated by the lower levels of antibody at zero time. Blood levels of antibody to myotoxin were non-detectable at thirty minutes for all groups receiving 0.75μg/g toxin, thus the addition of more myotoxin did little to change the disappearance rate of antibody from the blood.

This is the first report of the disappearance rate of antibodies to myotoxin in the presence of toxin. The values from the groups receiving toxin were obtained from thirty mice and without exception the same pattern of antibody disappearance was observed. This rapid decrease of antibodies to myotoxin was seen regardless of when the antiserum was injected, suggesting a sufficient amount of toxin was always available to bind the antiserum. Conversely, the animals injected with only antiserum demonstrated a gradual decline of antiserum from the bloodstream. A total of thirty mice were injected with

antiserum alone and although there was some variation between groups, without exception blood levels of antibodies to myotoxin were always detectable. These data show the striking effect myotoxin has on the disappearance rate of antibody and emphasises the need for physicians to monitor blood antivenom levels in snakebite patients. Snakebite victims may require more antivenom at some later time following the initial antivenom therapy since the presence of venom is quickly reducing blood antivenom concentrations.

This particular study measured the disappearance of crude serum containing antibodies to myotoxin a rather than neutralization of myonecrosis. However, Ownby et al. (1984) have studied the in vivo ability of this same lot of antiserum to neutralize local myonecrosis induced by myotoxin. Their results showed that antimyotoxin a serum neutralizes the myotoxicity of 0.75µg/g myotoxin a when injected five minutes before or immediately after the toxin, but not when injected fifteen minutes or more after the toxin. The rapid decrease of blood-antimyotoxin observed in our study when either 0.75 or 1.5µg/g of toxin was injected may reflect the in vivo binding of toxin and antibody even thirty minutes after toxin injection. It is important to realize that after thirty minutes of exposure to toxin that local muscle damage has probably already occured since Ownby et al. (1984) detected local myonecrosis fifteen minutes after an injection of myotoxin.

The decrease of blood levels of antibody to myotoxin observed even when antiserum is given thirty minutes after toxin may be due to 1) an excess of toxin that is available to bind to the antiserum or 2) a release of tissue bound myotoxin once local myonecrosis has already occured. The results indicate that blood level of antibodies to myotoxin quickly decrease regardless of when antiserum is injected. Antiserum administered as late as thirty minutes after the toxin can still bind the antigen, but myotoxin a-induced myonecrosis has probably already occured.

Although Ho et al. (1986b) did not measure blood-antivenom levels, they did observe an increase in blood-venom levels after antivenom therapy was discontinued. The results obtained in our experiments with known amounts of myotoxin a show the rapid effect the toxin has on blood-antimyotoxin levels and suggest serial injections of antiserum may be needed to maintain high blood levels of antiserum. The results indicate that the disappearance of antimyotoxin in the absence of toxin is considerably slower than when toxin is present. However, when the myotoxin has been present for thirty minutes prior to the antiserum injection the antiserum blood levels decline rapidly. Considering the length of time that can elapse in rural regions of the world prior to treatment for snake venom poisoning this finding may be of practical significance.

Thus, this study has shown that 1) the antibody detection ELISA can detect nanogram amounts of antibodies

to myotoxin *in vivo* 2) the decline of antimyotoxin *a* is very rapid when myotoxin *a* (0.75 or 1.5μg/g) is present and 3) the rapid decline of antimyotoxin is observed even when the antiserum is injected thirty minutes after the toxin although muscle damage has probably already occured.

CHAPTER VI

THE USE OF ELISA TO SCREEN VENOMS FOR MYOTOXIN a

Introduction

Myotoxin a is a small basic protein (4,400 dlatons, 9.8 pI) that has been isolated from Crotalus viridis viridis venom (Ownby et al., 1976; Cameron and Tu, 1977) and is known to induce local myonecrosis (Ownby et al., 1976). In addition, Ownby et al. (1984b) have shown that the pure myotoxin induces a myonecrosis similar to that of the crude venom. An antiserum produced against the pure myotoxin has been reported to neutralize myonecrosis (Ownby et al., 1983a). However, the commerically available Wyeth's polyvalent (Crotalidae) antivenin contains only trace amounts of antibodies to myotoxin a (Ownby et al., 1983b).

Basic toxins of small molecular weight have also been isolated from venoms other than C. viridis viridis, i.e. C. adamanteus, C. durissus terrificus and C. v. helleri, and have also been shown to induce myonecrosis. Crotamine a small basic protein isolated from C. d. terrificus var. Crotaminicus venom (Goncalves, 1956) can affect the muscle cell membranes in rats (Brazil et al., 1979), while peptide

C isolated from C. v. helleri also has similarities in amino acid composition to myotoxin a (Maeda et al., 1979). Mebs and Kornalik (1984) have isolated a basic toxin of approximately 5,800 daltons from the venom of C. adamanteus having amino acid similarities to crotamine of C. d. terrificus, myotoxin a of C. v. viridis and peptide C from C. v. helleri.

It is of practical significance to understand the similarities and differences between venoms and particularly important to be aware of the presence or absence of known deleterious snake venom toxins. If myotoxin a is a widely distributed protein among different venoms then an efficacious polyvalent antiserum must contain antibodies to myotoxin. Therefore, a study was performed using ELISA that screened eighty-two venoms from six genera having a wide geographic distribution for the presence of a protein known to induce local myonecrosis, myotoxin a (Ownby et al., 1976). Presence or absence of detectable levels of myotoxin was then compared with respect to the geographic orgin, genus and species of the snake.

Recently, antibodies to myotoxin a have been purified using affinity chromatography (Chapter III), thus providing and excellent reagent for an ELISA antigen detection system which was used in the current study.

Materials and Methods

Antigen Detection ELISA

Microtiter plates (Flow Laboratories, Mclean, VA) were coated with two-hundred microliters of affinity purified antimyotoxin (1 ug/ml). The antibody was diluted with coating buffer, pH 9.6. The plates were incubated overnight at 4 C. The following day the plates were rinsed three times with washing buffer, allowing a five minute soaking period between each washing. Following the final rinsing two-hundred microliters of known amounts of antigen or sample were added to each well and incubated one hour at 37 C. The myotoxin a solutions for the standard curve were diluted with incubation buffer. The antigen solutions for the standard curve consisted of the following concentrations: 100, 80, 60, 40, 20, 10, 5 and 1 ng/ml. Four wells coated with antimyotoxin received two-hundred microliters of incubation buffer alone and were assayed for non-specific binding. After the one hour incubation plates were rinsed three times with washing buffer and following the final rinsing two-hundred microliters of 1 µg/ml affinity purified antibody in incubation buffer were added to each sample well. Microtiter plates were allowed to incubate one hour at 37 C and then rinsed three times with washing buffer. Following the final rinsing two-hundred microliters of goat anti-rabbit IgG (Sigma, St. Louis, MO) were added to each sample well. The goat anti-rabbit IgG

was diluted 1:2000 with incubation buffer containing 1% BSA. Plates were again incubated at 37 C for one hour and then rinsed three times with washing buffer. Following the last rinsing two-hundred microliters of phosphatase substrate (p-nitrophenyl phosphate disodium, Sigma, St. Louis, MO) were added to each well. The substrate was diluted to 1 mg/ml with diethanolamine buffer, pH 9.8. Substrate was allowed to incubate for thirty minutes at room temperature. The reaction was stopped with 0.050ml 3N NaOH and the absorbance of each well was read with an EIA manual reader at a wavelength of 405nm. The amount of color formed is proportional to the amount of antigen present.

Screening Venoms
for Myotoxin a

Eighty-two snake venoms from the genera Crotalus, Agkistrodon, Bothrops, Bitis, Naja and Vipera were provided by James L. Glenn, Veterans's Administration Medical Center, Salt Lake City, Utah. Venoms samples were from various geographic orgins including Arizona, California, Florida, Georgia, Kansas, Louisana, North Carolina, New Mexico, Texas, Utah, Virginia, Wyoming, Brazil, India, Mexico Thailand and Venezuela. All venom samples were received lypholized and later diluted with PBS, pH 7.4 to 1 mg/ml. All venom samples were assayed using the ELISA antigen detection plates for the presence of myotoxin a. If the absorbance reading obtained for any venom sample was

over 2.0 the sample was diluted 1:1000 and assayed again.

Results

Antigen Detection ELISA

Figure 19 shows a flow chart of the steps involved in the myotoxin a detection assay. Non-specific binding was very low with absorbance readings between 0.110 and 0.122. Table VIII shows the absorbance readings for three standard curves using known amounts of purified myotoxin. The correlation coefficients are 0.97, 0.96 and 0.99 for the three antigen standard curves. The small differences between duplicate samples are reflected in the very low standard errors. Differences in absorbance readings for the same concentration of antigen is due at least in part to differences between microtiter plates.

Screening Venoms

Table IX lists the eighty-two venoms, their geographical orgins and the results of the myotoxin a detection assay. Venoms from the genera Agkistrodon, Bothrops, Bitis, Naja and Vipera did not have detectable levels of myotoxin a at a concentration of 1mg/ml regardless of their geographic orgin. Myotoxin a is present most frequently in the crotaline venoms of Montana, Colorado and Wyoming. Crotalus viridis viridis, C. durissus and C. molossus commonly have detectable levels of myotoxin a, however C.

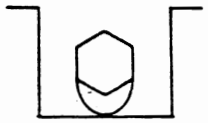
Figure 19. A flow chart illustrating the steps involved in the myotoxin a detection plate.

ELISA FOR MYOTOXIN a DETECTION

1. ANTI-MYOTOXIN a ADSORBED TO PLATE

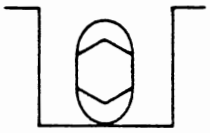
WASH

2. SOLUTION CONTAINING MYOTOXIN a ADDED



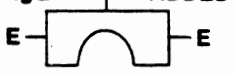
WASH

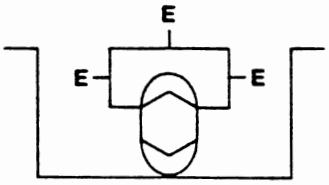
3. SOLUTION CONTAINING ANTI-MYOTOXIN a ADDED



WASH

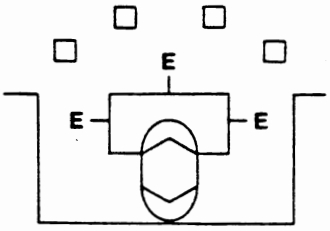
4. ALKALINE PHOSPHATASE LABELLED ANTIBODY TO RABBIT IgG ADDED





WASH

5. ENZYME SUBSTRATE □ ADDED



INCUBATE

6. READ ABSORBANCE OF REACTION PRODUCT □ AT 405nM

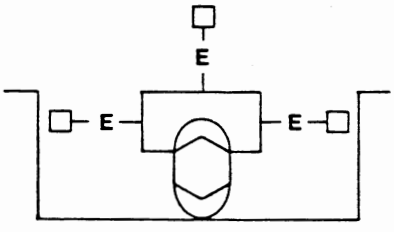


TABLE VIII

DETECTION OF MYOTOXIN A USING ELISA

Ag (ng/ml)*	Plate 1	Plate 2	Plate 3
	Absorbance 405 (nm)		
100	1.39 ± .01	1.00 ± .22	1.64 ± .23
80	1.29 ± .23	1.28 ± .11	1.42 ± .01
60	0.75 ± .01	0.84 ± .14	1.00 ± .02
40	0.87 ± .13	0.58 ± .01	0.71 ± .05
20	0.25 ± .02	0.19 ± .02	0.24 ± .03
10	0.09 ± .01	0.08 ± .01	0.12 ± .003
.5	0.04 ± .002	0.04 ± .003	0.04 ± .004
1	0.01 ± .000	0.01 ± .01	0.00
IB	0.11 ± .001	0.12 ± .003	0.12 ± .01

* Ag = myotoxin a. The antigen standard curve was assayed on three different microtiter plates. All samples were assayed in duplicate. Expressed as mean ± standard error.

** IB = incubation buffer 1% BSA. Sample wells were coated with affinity purified antibody and IB was assayed for non-specific binding. The background values have ben subtracted from each absorbance.

TABLE IX

SCREENING VENOMS FOR MYOTOXIN A WITH ELISA

Venom	1 mg/ml	1 µg/ml	1 ng/ml
A. b. bilineatus ** unknown orgin	-		
A. c. mokesona ** Miami Serpentarium	-		
A. c. rhodostoma ** Thailand	-		
A. p. leucastoma ** Louisiana	-		
Bitis arietans ** unknown orgin	-		
Bothrops asper unknown orgin	-		
Bothrops atrox comerical venom Brazil	-		
Crotalus adamanteus Florida	-		
C. adamanteus * Florida	-		
C. atrox * Texas	-		
C. b. basiliscus ** unknown orgin	+	+	
C. catalinensis * Catalina Island	+	-	
C. cerastes * Miami Serpentarium	-		
C. c. cerastes ** Utah	+		

TABLE IX (Continued)

Venom	1 mg/ml	1 µg/ml	1 ng/ml
C. d. culminatus ** Houston Zoo	+		
C. d. durissus ** unknown	+	+	
C. d. terrificus ** Venezuela	+		
C. d. totonacus ** unknown	+	+	
C. d. tzabcan ** Yucatan	+		
C. exsul * unknown orgin	+		
C. exsul * unknown orgin	+	+	+
C. e. enyo ** Baja Sur	-		
C. h. atricaudatus ** Georgia	-		
C. h. atricaudatus ** North Florida	-		
C. h. atricaudatus ** unknown orgin	+		
C. h. horridus ** Kansas	-		
C. h. horridus ** North Carolina	+	+	

Table IX (Continued)

Venom	1 mg/ml	1 µg/ml	1 ng/ml
C. l. klauberi ** Durango	-		
C. l. klauberi ** Mexico	-		
C. l. klauberi ** Mexico	-		
C. l. klauberi ** New Mexico	+		
C. m. mitchelli ** Baja Sur	-		
C. m. mitchelli ** Mulege, Baja	-		
C. m. pyrrhus ** Arizona	-		
C. m. pyrrhus ** Bajia de los Angeles	+		
C. m. pyrrhus ** Bajia de los Angeles	-		
C. m. pyrrhus ** Mexico	-		
C m. pyrrhus ** Smith Isle	-		

TABLE IX (Continued)

Venom	1 mg/ml	1 µg/ml	1 ng/ml
C. m. pyrrhus ** Utah	-		
C. m. pyrrhus ** Utah	-		
C. m. pyrrhus ** Utah	-		
C. m. pyrrhus ** Utah	-		
C. m. pyrrhus ** Utah	-		
C. m. pyrrhus ** Utah	-		
C. m. pyrrhus ** Utah	-		
C. m. pyrrhus ** Utah	-		
C. m. pyrrhus ** Utah	-		
C. m. stephensi ** orgin unknown	-		
C. m. molossus *** Texas	+	+	
C. m. nigrescens *** Durango, Mexico	+	+	
C. ruber ruber San Ignacio	+	+	
C. ruber ruber San Marcos Isl.	-		
C. vegrandis * Venezuela	-		

TABLE IX (Continued)

Venom	1 mg/ml	1 µg/ml	1 ng/ml
C. v. abysuss ** Arizona	+		
C. v. abyssus ** Sonoran Desert Museum	-		
C. v. cerberus ** unknown orgin	+		
C. v. concolor ** Wyoming	+		
C. v. helleri ** orgin unknown	+		
C. v. lutosus ** Arizona	-		
C. v. lutosus ** Neveda	+		
C. v. lutosus ** Utah	-		
C. v. nuntius ** Utah	-		
C. v. organus ** Washington	+	+	+
C. v. viridis ** Colorado	+	-	
C. v. viridis ** Colorado	+		
C. v. viridis ** Colorado	+	+	-

TABLE IX (Continued)

Venom	1 mg/ml	1 µg/ml	1 ng/ml
C. v. viridis ** Colorado	-		
C. v. viridis ** Montana	+	+	
C. v. viridis ** Montana	+		
C. v. viridis ** New Mexico	-		
C. v. viridis ** New Mexico	-		
C. v. viridis ** New Mexico	+		
C. v. viridis ** New Mexico	-		
C. v. viridis ** South Dakota	+		
C. v. viridis ** Texas	-		
C. v. viridis ** Texas	-		
C. v. viridis ** Wyoming	+		
C. v. viridis ** Wyoming	+		
C. v. viridis ** Wyoming	+		

TABLE IX (Continued)

Venom	1 mg/ml	1 μg/ml	1 ng/ml
C. v. viridis ** Wyoming	+		
C. v. viridis ** Wyoming	+		
N. n. kaouthia ** unknown orgin	-		
Vipera r. russelli ** India	-		

* Venom samples were diluted with PBS, pH 7.4 to 1 mg/ml then assayed with ELISA. If a positive result was obtained the venom sample was diluted 1:1000 and assayed again. Beneath each venom is given the geographical orgin. A. = Agkistrodon, C. = Crotalus, N. = Naja.

** Species and subspecies are as follows: A. bilineatus b.; A. contortrix m.; A. callesolasma r.; A. piscivorus l.; C. basiliscus b.; C. cerastes c.; d. = durissus; e. = enyo; h. = horridus; l. = lepidus; m. = mitchellii; v. = viridis; n = naja; r = russelli.

*** m. = molossus

mitchelli has almost consistently non-detectable levels of the toxin.

Discussion

The use of the antigen detection ELISA to screen venoms for the presence of myotoxin a has helped to identify genera and species with particularly high levels of the toxin. A total of eighty-two venoms from six genera (Agkistrodon, Bitis, Bothrops, Crotalus, Naja and Vipera) were assayed for the presence of detectable levels of myotoxin a. Only venom from the genus Crotalus had detectable levels of myotoxin a. Seventy-four crotaline venoms were assayed and of these thirty-four (45%) had detectable levels of myotoxin a. Myotoxin appeared predominately in the species C. viridis, C. molossus and C. durissus with a greater distribution of toxin appearing in the north and southwest regions of the United States, as well as Mexico and South America. Ownby et al. (1986b) have shown that the commerically available Wyeth's polyvalent (Crotalidae) antivenin has an extremely low titer of antibodies to myotoxin a when tested with ELISA. Considering the number of of crotaline venoms which have detectable amounts of myotoxin a an efficious antivenom should contain a neutralizing antibody to myotoxin a.

Our ELISA results indicate that the presence of myotoxin a in venom samples varied with genera and

geographic orgin. Glenn and Straight (1978) have also observed variation in toxicity with geographic orgin for the Mojave rattlesnake (Crotalus scutulatus scutulatus) Venom from specimens collected in southern California and southwest Arizona was different in lethality and reactivity with the commerical antivenin from venom of specimens collected in central and northwest Arizona. Venom samples from southern California and southwest Arizona were poorly neutralized by the antivenin in comparison to venom samples from northwest and north central Arizona. The geographic variation seen within the subspecies could possibly help explain some of the varying clinical aspects of Mojave rattlesnake bites (Glenn and Straight, 1978).

Interestingly, Glenn et al. (1983) have observed a higher proteolytic activity in venoms of C. s. scutulatus that had little to none of the lethal toxin, Mojave toxin. These data are similar to results obtained with C. atrox venom (Minton and Weinstein, 1986). Crotalus atrox venom differs with geographic orgin with an inverse relationship between proteolytic activity and lethality being observed.

A geographic distribution has also been noted for crotamine, a basic protein known to induce myonecrosis, from Crotalus durissus terrificus venom. Venom from C. d. terrificus inhabitating northeastern Brazil are missing the basic polypeptide, whereas specimens from southern Brazil, Argentina and Venzezuela possess this particular venom component (Glenn and Straight, 1982). Variation between

venom samples however, does not require large geographic distances, on the contrary variation between venom samples from individual snakes of same litter can occur. Mebs and Kornalik (1984) found that venom samples from four specimens of C. adamanteus from the same litter differed in the presence or absence of a small basic toxin. Venom from two of the snakes possessed the toxin and the venom from the two other snakes were essentially free of the toxin.

Individual or geographical variation within a subspecies however, are often times not observed since many of the lethal toxicities reported are derived from pooled venoms, especially if the venoms are commerically supplied (Glenn and Straight, 1982). The study of variations in venom composition and venom toxicity with geographic orgin is important in order to provide better treatment for snakebite poisoning and to better understand taxonomic differences. The ELISA is possibly the most economical, rapid and sensitive test for screening venoms for specific toxins and detecting the individual and geographic variations of snake venoms.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Antibody produced against myotoxin a from Crotalus viridis viridis venom was purified using affinity chromatography. Myotoxin a was conjugated to Affi-Gel 10 and samples of crude antiserum were applied to the column, followed by washings with PBS, DW and acetic acid. Antimyotoxin a was eluted with each of the effluents. The antibody was measured with the antibody detection enzyme-linked immunosorbent assay (ELISA) and later tested for its ability to neutralize myotoxin-induced myonecrosis. The affinity purified antimyotoxin a was used to develop a sensitive antigen and antibody ELISA. Antibody eluted with DW had the largest amount of protein as determined by A_{280} and was used routinely to coat our ELISA antigen plates or for a standard curve with the ELISA antibody detection system. Antimyotoxin a eluted with DW, acetic acid and PBS each reduced the myotoxin-induced myonecrosis when mixed and incubated together prior to intramuscular injections in mice, however there was a great deal of animal variation and the decrease was not significant ($p<0.05$).

The antibody detection ELISA was used to measure the disappearance of three concentrations of crude antiserum

from the blood of mice during a four week period. Regardless of the amount of antibody injected a slow decrease in the amount of antibody occurred during the first ninety-six hours resulting in non-detectable levels at two and four weeks. The mice injected with the greatest concentration of antibody had initially higher blood-antibody levels however, antibody was non-detectable at four weeks. The antibody detection ELISA is capable of detecting and monitoring changes in blood-antibody levels at nanogram concentrations.

Animal variation is a significant problem when using the ELISA systems to measure disappearance of antibodies from the blood and demonstrates the need to use either very large numbers of small laboratory animals or larger animals whereby indwelling cathers could be used. However, either because of cost limitations or quantity of purified antiserum and toxin such experiments are not always feasible. These limitations do not preclude the use of ELISA to study the disappearance of antibody from the blood in laboratory animals. The ELISA is capable of monitoring and detecting decreases in antibodies to myotoxin from the blood and distinct patterns in the rate of antibody disappearance in the presence or absence of toxin can be observed using the ELISA system with mice. It is equally important to realize the ELISA systems are sensitive and reliable assays as indicated by the small variation assoicated with the standard curves. Also, non-specific

binding never posed a problem and remained always low for all experiments.

The disappearance of antibodies to myotoxin from the blood in the presence of known amounts of myotoxin a (0.75 and 1.5μg/g) or physiological saline (PSS) has been compared. A marked difference was observed in the disappearance rate of the antiserum in the presence or absence of toxin. Whenever myotoxin a was injected, regardless of amount of toxin or time of administration, a rapid decline in blood levels of antibodies to myotoxin also occurred. Conversely, a gradual decline of blood-antiserum was observed over a 96h period when PSS was injected instead of toxin. The presence of the toxin causes a rapid clearance of the antiserum even when the toxin is injected thirty minutes prior to the antiserum. Both concentrations of toxin selected (0.75 and 1.5μg/g) caused a rapid decline of specific antibodies to myotoxin from the blood. Antibody declined in a similar manner when the antiserum was injected 1) five minutes before the toxin, 2) immediately after the toxin or 3) thirty minutes after the toxin. The latter finding has considerable clinical significance since it indicates either that there is still enough toxin available to bind the antibody even after thirty minutes or the toxin might induce local myonecrosis and then bind the antibody. Multiple injections of antiserum may be necessary to maintain high blood levels of antimyotoxin.

The ELISA antigen detection system has been used to screen eighty-two venoms of six genera for the presence of myotoxin a. Venoms from the genera Agkistrodon, Bothrops, Bitis, Naja and Vipera did not have detectable levels of myotoxin a. Numerous crotaline venoms (45%) did have myotoxin a present including Crotalus viridis, C. durissus and C. molossus. Crotaline venoms of the north, northwest and southwest often times were positive for myotoxin a compared to the crotaline venoms of the southeast which frequently had non-detectable levels of myotoxin a. The antigen ELISA detection system is a rapid, reliable and relatively inexpensive means to screen venoms of various geographic orgins for the presence of specific toxins.

SELECTED BIBLIOGRAPHY

Alden, N.C. (1969) Treatment of rattlesnake bite. JAMA 207: 159.

Arnold, R.E. (1975) Results of treatment of Crotalus envenomation. Am. Surg. 41: 643-647.

Arnold, R.E. (1976) Treatment of snakebite. JAMA 236: 1843-1844.

Arnold, R.E. (1979) Controversies and hazards in the treatment of pit viper bites. Southern Med. J. 72: 902-910.

Bennett, J.E., Brelsford, H.G., Lewis, S.R. and Blocker, T.G. (1961) Distal extremity necrosis after snakebite. Plastic Reconstr. Surg. 28: 385-393.

Bieber, A.L., Tu, T. and Tu, A.T. (1975) Studies of an acidic cardiotoxin isolated from the venom of Mojave rattlesnake (Crotalus scutulatus). Biochim. biophys. Acta. 400: 178-188.

Bolanos, R., Cerdas, L. and Taylor, R. (1975) The production and characteristics of a coral snake (Micrurus mipartitus hertwigi) antivenin. Toxicon 13: 139-142.

Boulain, J.C., Menez, A., Couderc, J., Faure, G., Liacopoulos, P. and Fromageot, P. (1982) Neutralizing monoclonal antibody specific for Naja nigricollis toxin-α: preparation, characterization and localization of the antigenic binding site. Biochem. 21: 2910-2915.

Brazil, O.V., Prado-Franceschi, J. and Laure, C.J. (1979) Repetitive muscle response induced by crotamine. Toxicon 17: 61-67.

Bureau, D. and Daussant, J. (1983) Desorption following immunoaffinity chromatography; generalization of a gentle procedure for desorbing antigen. J. of Immunol. Methods 57: 205-213.

Cameron, D.L. and Tu, A.T. (1977) Characterization of myotoxin a from the venom of prairie rattlesnake (Crotalus viridis viridis). Biochem. 16: 2546-2553.

Cameron, D.L. and Tu, A.T. (1978) Chemical and functional homology of myotoxin a from prairie rattlesnake venom and crotamine from South American rattlesnake venom. Biochim. Biophys. Acta. 532: 147-154.

Chandler, H.M. and Hurrell, J.G. (1982) A new enzyme immunoassay system suitable for field use and its application in a snake venom detection kit. Clinica Chimica Acta. 121: 225-230.

Chang, C.C. and Hayaski, K. (1969) Chemical modification of the tryptophan residue in cobrotoxin. Biochim. Biophys. Res. Commun. 37: 841-846.

Christian, C.L. (1970) Character of non-precipitating antibodies. Immunol. 18: 457-466.

Clark, R.W. (1971) Cryotherapy and cortiosteroids in the treatment of rattlesnake bite. Milit. Med. 136: 42-44.

Clausen, J. (1969) Immunological techniques for the identification and estimation of macromolecules. In: Laboratory Techniques in Biochemistry and Molecular Biology, Vol 2, part III (Work, T.S. and Work, E., Eds). Amsterdam: North Holland.

Clement, J.F. and Pietrusko, R.G. (1979) Pit viper snakebite envenomation in the United States. Clinical Toxicol. 14: 515-538.

Coulter A.R., Cox, J.C., Sutherland, S.K. and Waddell, C.J. (1978) A new solid-phase sandwich radioimmunoassay and its application to the detection of snake venom. J. of Immunological Methods 23: 241-252.

Coulter, A.R., Harris, R.D. and Sutherland, S.K. (1980) Enzyme-immunoassay for the rapid clinical identification of snake venom. Med. J. Aust. 1: 433-435.

Cuatrecasas, P. and Anfinsen, C.B. (1971) Affinity chromatography. In: Methods in Enzymology: Enzyme Purification and Related Techniques, Vol XXII, p 345 (Jakoby, W.B., Ed.) Academic Press: New York.

Danse, J.M., Toussaint, J.L. and Kempf, J. (1986) Neutralization of Bungarotoxin by monoclonal antibodies. Toxicon 24: 141-151.

Deubel, A., Mouly, J.J., Salaun, C., Adam, Mo, Diop, M. and Digoutte, J.P. (1983) Comparison of the enzyme-linked immunosorbent assay (ELISA) with standard tests used to detect yellow fever virus antibodies. The Am. Proc. of Trop. Med. and Hyg. 3: 565-568.

Dhaliwal, I.S., Lim, T.W. and Sukumaran, K.D. (1983) A double sandwich micro-ELISA for the rapid diagnosis of snakebite. Southeast Asian J. of Trop. Med. and Public Health 14: 367-373.

Egorov, T.A., Svenson, A. and Rydien, L. (1975) A rapid and specific method for isolation of thiol-containing peptides from large proteins by thiol-disulfide exchange on a solid support. Proc. Natl. Acad. Sci. USA 72: 3029-3033.

Gaebert, A.K. (1977) Isolation of thrombin-like factors from Malayan pit viper venom by affinity chromatography. Toxicon 15: 217-224.

Galloway, D.R., Hedstrom, R.C. and Pavlovskis, O.R. (1984) Production and characterization of monoclonal antibodies to exotoxin A from Pseudomonas aeruginosa. Infect. Immunol. 44: 262-267.

Ganthavorn, S. (1969) Toxicities of Thailand snake venoms and neutralization capacity of antivenin. Toxicon 7: 239-241.

Garcia, E.V. and Perez, J.C. (1983) The purification and characterization of an antihemorrhagic factor in wood-rat (Neotoma microopus). Toxicon 22: 129-138.

Gelb, W.G. (1973) Affinity chromatography for separation of biological materials. American Lab. Oct: 61-67.

Gennaro, J.F. (1963) Observations on the treatment of snakebite in North America. In: Venomous and Poisonous Animals and Noxious Plants of the Pacific Region, p 427 (Keegan, H.L. and MacFarlane, W., Eds.) Pergamon Press: Oxford.

Gennaro, J.F. and McCullough, N.C. (1961) Comments on the contemporary treatment of poisonous snakebite in North American. Med. Rec. Ann. 46: 224-225.

Gennaro, J.F. and Watt, C.H. (1977) Snakebite emergency. Emergency Press 9: 36-38.

Gill, K.A., Jr. (1970) The evaluation of cryotherapy in the treatment of snake envenomation. Southern Med. J. 63: 552-556.

Glass, T.G., Jr. (1976) Early debridement in pit viper bites. JAMA 235: 2513-2516.

Glenn, J.L. and Straight, R.C. (1977) The midget faded rattlesnake (Crotalus viridis concolor) venom: lethal toxicity and individual variability. Toxicon 15: 129-133.

Glenn, J.L. and Straight, R.C. (1978) Mojave rattlesnake Crotalus scutulatus scutulatus venom: Variation in toxicity with geographical orgin. Toxicon 16: 81-84.

Glenn, J.L. and Straight, R.C. (1982) The rattlesnakes and their venom yield and lethal toxicity. In: Rattlesnake Venoms: Their Actions and Treatment, p 3 (Tu A.T., Ed.) Marcel Dekker: New York.

Glenn, J.L., Straight, R.C., Wolfe, M.C. and Hardy, D.L. (1983) Geographical variation in Crotalus scutulatus scutulatus (mojave rattlesnake) venom proteins. Toxicon 21: 119-130.

Goncalves, J.M. (1956) Purification and properties of crotamine. In: Venoms, pp 261-274 (Buckley, E.E. and Porges, N., Eds.) Am. Assoc. Adv. Sci.: Washington D.C.

Gopalakrishnakone, P., Hawgood, B.J. and Theakston, R.D.G. (1981) Specificity of antibodies to the reconstituted crotoxin complex, from the venom of South American rattlesnake (Crotalus durissus terrificus), using enzyme-linked immunosorbent assay (ELISA) and double immunodiffusion. Toxicon 19: 131-139.

Greenwood, B.M. and Whittle, H.C. (1981) Immunological changes in healthy individuals living in the tropics. In: Immunology of Medicine in the Tropics, p 1 (Arnold, E., Ed.) London.

Gutierrez, J.M. and Chaves, F. (1980) Efectos proteolitico, hemorragico y mionecrotico de los venenos de serpientes costaricenses de los generos Bothrops, Crotalus y lachesis. Toxicon 18: 315-321.

Gutierrez, J.M., Ownby, C.L. and Odell, G.V. (1984) Pathogenesis of myonecrosis induced by crude venom and a myotoxin of Bothrops asper. Exp. Molec. Path. 40: 367-369.

Habermann, E. and Breithaupt, H. (1978) Mini-review. The crotoxin complex: an example of biochemical and pharmacological protein complementation. Toxicon 16: 19-30.

Hawgood, B.J. and Smith, J.W. (1977) The mode of action at the mouse neuromuscular junction of the phospholipase A-crotapotin complex isolated from the venom of the South American rattlesnake. Br. J. Pharmac. 61: 597-606.

Hayakawa, S., Uchida, T., Mekada, E., Moynihan, M.R. and Okada, Y. (1983) Monoclonal antibody against diptheria toxin. Effect on toxin binding and entry into cells. J. Biol. Chem. 258: 4311-4317.

Henderson, B.M and Dujun, E. (1973) Snakebites in children. Pediatr. Surg. 8: 729-733.

Ho, M., Warrell, M.J., Warrell, D.A., Bidewell, D. and Voller, A. (1986a) A critical reappraisal in the use of enzyme-linked immunosorbent assays in the study of snakebite. Toxicon 24: 211-221.

Ho, M., Warrell, D.A., Looareesuwan, S., Phillips, R.E., Chanthavanich, P., Karbwang, J., Supanaranond, W., Viravan, C., Hutton, R.A. and Vejcho, S. (1986b) Clinical significance of venom antigen levels in patients envenomed by the Malayan pit viper (Calloselasma rhodostoma). Am. J. Trop. Med. Hyg. 35: 579-587.

Hopkins, L.T. (1976) Snakes and snakebites; characteristics, management and prognosis. J. Kansas Med. Soc. 77: 193-196.

Huang, T.T., Lynch, J.B., Larson, D.L. and Lewis, S.R. (1974) The use of excisional therapy in the management of snakebite. Ann. Surg. 179: 598-603.

Huang, T.T., Steven, J.B. and Stephen, R.L. (1978) Hand deformities in patients with snakebite. Plastic and Reconstructive Surg. 62: 32-36.

Jacob, J.L. (1977) An evaluation of the possibility of hybridization between the rattlesnake Crotalus atrox and Crotalus scutulatus in the southwestern United States. Swest. Nat. 22: 469.

Jeter, W.S., Russell, F.E. and Sullivan, J.B. (1983) Anaphylaxis in guinea pigs challenged with antivenin preparations. Toxicon 21: 729-730.

Kabat, E.A. and Mayer, M.M. (1961) In: Experimental Immunochemistry, p 22 (Thomas, C.C. Ed.) Springfield.

Kfir, R., Johannsen, E. and Boles, D.P. (1986) Monoclonal antibody specific for cyanoginosin LA: preparation and characterization. Toxicon 24: 543-552.

Kirchner, K. and Faulstich, H. (1986) Purification of Amatoxin-specific antibodies from rabbit sera by affinity chromatography, their characterization and use in toxicological studies. Toxicon 24: 273-283.

Klauber, L.M. (1956) In: Rattlesnakes: Their Habits, Life Histories, and Influence on Mankind, p 817 (U. Calif. Press) Berkeley: California.

Kocholaty, W.F., Ledford-Bowles, E., Daly, J.G. and Billings, T.A. (1971a) Preparation of a coral snake antivenin from goat serum. Toxicon 9: 297-298.

Kocholaty, W.F., Ledford-Bowles, E., Daly, J.G. and Billings, T.A. (1971b) Toxicity and some enzymatic properties and activities in the venoms of Crotalidae, Elapidae and Viperidae. Toxicon 9: 131-138.

Kochwa, S., Gitter, S., Strauss, A., DeVries, A. and Leffkowitz, M. (1959) Immunologic study of Vipera xanthina palestinae venom and preparation of potent antivenin in rabbits. J. Immunology 82: 107-115.

Kornalik, F. and Taborska, E. (1983) Neutralization of local and systemic effects of snake venoms by antivenoms. Toxicon Suppl. 3: 359-360.

Kuekarni, M.E. and Rao, S.S. (1956) Antigenic composition of the venoms of poisonous snakes of India. In: Venoms, p 175 (Buckley, E. and Proges, N., Eds.) Washington: Am. Assoc. for the Adv. of Sci.

Lew, A.M. (1984) The effect of epitope density and antibody affintiy on the ELISA as analysed by monoclonal antibodies. J. of Immunological Methods 72: 171-176.

Lockhart, W.E. (1965) Treatment of snakebite. JAMA 193: 36-38.

Lockhart, W.E. (1977) Treatment of snakebite. Rhode Island Medical J. 60: 13-15.

Lomonte, B., Gutierrez, J.M. and Mata, E. (1985) Isolation from a polyvalent antivenom of antibodies to a myotoxin in Bothrops asper snake venom. Toxicon 23: 807-813.

Loprinzi, C.L., Hennesse, J., Tamsicy, L. and Johnson, T.E. (1983) Snake antivenin administration in a patient allergic to horse serum. Southern Medical J. 76: 501-502.

Lwin, K.O., Myint, A.A., Pe, T., Nwe, T. and Naing, M. (1984) Russell's viper venom in serum of snakebite victims in Burma. Trans. R. Soc. Trop. Med. Hyg. 78: 165-168.

Maeda, N. Tamiya, N. Pattabhiraman, T.R. and Russell, F.E. (1978) Some chemical properties of the venom of the rattlesnake Crotalus viridis helleri. Toxicon 16: 431-442.

McCullough, N.C. and Gennaro, J.F. (1968) Diagnosis, symptoms, treatment and sequelae of envenomation by Crotalus adamanteus and genus Ankistrodon. J. Fla. Med. Assoc. 55: 327-329.

McCullough, N.C. and Gennaro, J.F. (1970) Treatment of venomous snakebite in the United States. Clinical Toxicology 3: 483-500.

Mebs, D. and Kornalik, F. (1984) Intraspecific variation in content of a basic toxin in eastern diamondback rattlesnake (Crotalus adamanteus) venom. Toxicon 22: 831-833.

Minton, S.A. (1954) Polyvalent antivenin in the treatment of experimental snake venom poisoning. J. Trop. Med. Hyg. 3: 1077-1082.

Minton, S.A. (1967) Paraspecific protection by elapid and sea snake antivenins. Toxicon 5: 47-55.

Minton, S.A. (1971) Snakebite: an unpredictable emergency. J. Trauma 11: 1053-1054.

Minton, S.A. and Weinstein, S.A. (1986) Geographic and ontogenic variation in venom of the western diamond-back rattlesnake (Crotalus atrox). Toxicon 24: 71-80.

Minton, S.A., Weinstein, S. and Wilde, C.E. (1984) An enzyme-linked immunoassay for detection of North American pit viper venoms. Clinical Toxicology 22: 303-316.

Mochly-Rosen, D. and Fuchs, S. (1981) Monoclonal anti-acetylcholine receptor antibodies directed against the cholinergic binding site. Biochemistry 20: 5920-5924.

Mohamed, A.H., Fawzia, K.K. and Baset, A.A. (1977) Immuno-logical studies on polyvalent and monovalent snake antivenins. Toxicon 15: 271-275.

Mohamed, A.H. and Kamel, A.H. (1963) Preparation of Egyptian black snake antivenin. Toxicon 1: 77-79.

Morgan, M.R., Brown, P.J., Leyland, M.J. and Dean, P.D. (1978) Electrophoresis: a new preparative desorption technique in affinity chromatography and immunoadsorption. FEBS Letters 87: 239-243.

Ovadia, M. (1978) Purification and characterization of an antihemorrhagic factor from the serum of the snake Vipera palaestinae. Toxicon 16: 661-672.

Ownby, C.L., Cameron, D. and Tu, A.T. (1976) Isolation of myotoxic component from rattlesnake (Crotalus viridis viridis) venom. Electron microscopic analysis of muscle damage. Am. J. Pathol. 85: 149-166.

Ownby, C.L., Woods, W.M. and Odell, G.V. (1979) Antiserum to myotoxin a from prairie rattlesnake (Crotalus viridis viridis) venom. Toxicon 17: 373-380.

Ownby, C.L. Gutierrez, J.M., Colberg, T.R. and Odell, G.V. (1982) Quantitation of myonecrosis induced by myotoxin a from prairie rattlesnake (Crotalus viridis viridis) venom. Toxicon 20: 877-885.

Ownby, C.L., Odell, G.V., Woods, W.M. and Colberg, T.R. (1983a) Ability of antiserum to myotoxin a from prairie rattlesnake (Crotalus viridis viridis) venom to neutralize local myotoxicity and lethal effects of myotoxin a and homologous crude venom. Toxicon 21: 35-45.

Ownby, C.L., Odell, G.V. and Theakston, R.D.G. (1983b) Detection of antibodies to myotoxin a and prairie rattlesnake (Crotalus viridis viridis) venom in three antisera using enzyme-linked immunosorbent assay and immunodiffusion. Toxicon 21: 849-855.

Ownby, C.L., Colberg, T.R. and Odell, G.V. (1984a) A new method of quantitating hemorrhage induced by rattlesnake venoms: ability of polyvalent antivenoms to neutralize hemorrhagic activity. Toxicon 22: 227-233.

Ownby, C.L. Colberg, T.R., Claypool, P.L. and Odell, G.V. (1984b) In vivo test of the ability of antiserum to myotoxin a from prairie rattlesnake (Crotalus viridis viridis) venom to neutralize local myonecrosis induced by myotoxin a and homologous crude venom. Toxicon 22: 99-105.

Ownby, C.L., Colberg, T.R. and Odell, G.V. (1986) In vivo ability of antimyotoxin a serum plus polyvalent (Crotalidae) antivenom to neutralize prairie rattlesnake venom. Toxicon 24: 197-200.

Parrish, H.M. (1980) Incidence of treated snakebites in the United States. In: Poisonous Snakebites in the United States, pp 1-64 Vantage Press: New York.

Parrish, H.M. and Hayes, R.H. (1970) Hospital management of pit vipers venenations. Clinical Toxicology 3: 501-511.

Pool, W.R. and Bieber, A.L. (1981) Fractionation of midget faded rattlesnake (Crotalus viridis concolor) venom. Toxicon 17: 373-380.

Pukrittayakamee, S., Esnouf, M.P. and McMichael, A.J. (1983) Purificiation of the factor X activator of Russell's viper venom with monoclonal antibodies. Molecular Biol. Med. 1: 123-135.

Rael, D.E. Salo, R.J. and Hector, Z. (1986) Monoclonal antibodies to mojave toxin and use for isolation of cross-reacting proteins in Crotalus venoms. Toxicon 7: 661-668.

Reid, H.A. (1964) Cobra-bites. British Med. J. 2: 540-545.

Reid, H.A. and Theakston, R.D.G. (1983) The management of snakebite. Bulletin of the WHO 61: 885-895.

Reid, H.A., Thean, P.C. and Martin, W.T. (1963) Specific antivenene and prednisone in viper bite poisoning: controlled trial. British Med. J. 2: 1378-1380.

Rosenfeld, G. and Kelen, E.M. (1966) Cross neutralization of the coagulant activity of some snake venoms by antivenins. Toxicon 4: 7-15.

Russell, F.E. (1965) Effects of cortisone during immunization with Crotalus venom. A preliminary report. Toxicon 3: 65-67.

Russell, F.E. (1969) Snakebite: In Current Therapy, pp 873-876 (Conn, H.F. Ed.) Saunders: Philadelphia.

Russell, F.E. and Emery, J.A. (1961) Incision and suction following the injection of rattlesnake venom. Am. J. Med. Sci. 241: 160-166.

Russell, F.E. and Lauritzen, L. (1966) Antivenins. Trans. R. Soc. Trop. Med. Hyg. 60: 797-810.

Russell, F.E., Timmerman, W.F. and Meadows, P.E. (1970) Clinical use of antivenin prepared from goat serum. Toxicon 8: 63-65.

Russell, F.E., Ruzic, N. and Gonzlaz, H. (1973) Effectiveness of antivenin (Crotalidae) polyvalent following injection of Crotalus venom. Toxicon 11: 461-464.

Russell, F.E., Carlson, R.W., Wainschel, J. and Osborne, A.H. (1975) Snake venom poisoning in the United States: experiences with 550 cases. JAMA 4: 341-344.

Russell, F.E., Sullivan, J.B., Egen, N.B., Jeter, W.S., Markland, F.S., Wingert, W.A. and Gar-Or, D. (1985) Preparation of a new antivenin by affinity chromatography. Toxcion 34: 141-150.

Schöttler, W.H. (1954a) Miscellanous observations on snake venoms and antivenins. Mem. Inst. Butantan 27: 73-105.

Schöttler, W.H. (1954b) Antihistamine, ACTH, cortisone, hydrocortisone and anesthetics in snakebite. Am. J. Trop. Med. 3: 1083-1091.

Skelley, D.S., Brown, L.P. and Besch, P.K. (1973) Radioimmunoassay. Clin. Chem. 19: 146-186.

Soloman, K.A., Tarkkanen, L. and Narvanen, S. (1968) Metabolic changes in the upper limb during tourniquet anethesia. Acta. Orthop. Scand. 39: 20-32.

Stahnke, H.L. and Mcbride, A. (1966) Snakebite and cryotherapy. J. of Occuptional Med. 8: 72-76.

Stanic, M. (1969) Critical comments on the quality of some commerical antivenins. Toxicon 6: 287-292.

Stringer, J.M., Kainer, R.A. and Tu, A.T. (1972) Myonecrosis induced by rattlesnake venoms. An electron microscopic study. Am. J. Pathol. 67: 127-136.

Strong, P.N., Wood, J.N. and Ivanji, J. (1984) Characterization of monoclonal antibodies against β-bungarotoxin and their use as structural probes for related phospholipase A2 enzymes and presynaptic phospholipase neurotoxins. Eur. J. Biochem. 142: 145-151 .

Sullivan, J.B., Jr. and Russell, F.E. (1982) Isolation and purification of antibodies to rattlesnake venom by affinity chromatography. Proc. West Pharmacol. 25: 185-192.

Sullivan, J.B. and Russell, F.E. (1983) Isolation, quantitation,and subclassing of IgG antibody to crotalidae venom by affinity chromatography and protein electrophoresis. Toxicon Suppl. 3: 492-432.

Sutherland, S.K. (1976) Treatment of snakebite in Australia and Papua New Guiena. Aust. Fam. Phy. 5: 272-288.

Synder, C.C, Straight, R. and Glenn, J. (1972) The snake-bitten hand. Plastic Reconstr. Surg. 49: 275-282.

Tan, N.H. (183) Improvement of Malayan cobra (Naja naja sputatrix) antivenin. Toxicon 21: 75-79.

Tarng, S.F., Huang, S.V. and Perez, J.C. (1986) Isolation of antihemorrhagic factors in opossum (Didelphis virginiana) serum using a monclonal antibody immuno-sorbent. Toxicon 24: 567-573.

Taub, A.M. (1964) Antivenins available for the treatment of snakebite. Toxicon 2: 71-77.

Teouanne, B., Nicolas, J.C., Descomps, B. and Crastes de Paulet, A. (1980) Affinity chromatography: a tool for enzyme immunoassay. J. of Immunol. Methods 35: 277-284.

Theakston, R.D.G. (1983) The application of immunoassay techniques , including enzyme-linked immunosorbent assay (ELISA), to snake venom research. Toxicon 21: 341-352.

Theakston, R.D.G. and Reid, H.A. (1979) Enzyme-linked immunosorbent assay (ELISA) in assessing antivenom potency. Toxicon 17: 511-515.

Theakston, R.D.G., Lloyd-Jones, M.J. and Reid, H.A. (1977) Micro-ELISA for detecting and assaying snake venoms and venom antibody. Lancet 2: 639-641.

Theakston, R.D.G., Pugh, R.N. and Reid, H.A. (1981a) Enzyme-linked immunosorbent assay of venom-antibodies in human victims of snakebite. J. Trop. Med. Hyg. 84: 109-112.

Theakston, R.D.G., Reid, H.A., Larrick, J.W., Kaplan, J. and Yost, J.A. (1981b) Snake venom antibodies in Ecuadorian Indians. J. of Trop. Med. and Hyg. 84: 199-202.

Timmerman, W.F. and Russell, F.E. (1969) Comparative studies on the effectiveness of an antivenin and its fractions. Toxicon 6: 311-314.

Trinca, G.F. (1963) Medical surveys: the treatment of snakebite. The Med. J. of Australia 50: 275-280.

Tu, A.T. (1982) Chemistry of rattlesnake venoms. In: Rattlesnake Venoms: Their Actions and Treatment, p 247 (Tu, A.T., Ed.) Marcel Dekker: New York.

Voller, A., Bartlett, A. and Bidwell, D.E. (1976) Enzyme immunoassays for parasitic diseases. Trans. of the Royal Soc. of Trop. Med. and Hyg. 70: 98-106.

Voller, A., Bidwell, D.E. and Bartlett, A. (1979) In: The Enzyme-linked Immunosorbent Assay (ELISA): A Guide with Abstracts of Microplate Applications, p 7 (Dynatech Laboratories, Inc.) Alexandria: Virginia.

Wisdom, G.B. (1976) Enzyme-immunoassay. Clinical Chem. 22: 1243-1255.

Yang, C.C. (1965) Crystallization and properties of cobrotoxin from Formosan cobra venom. J. Biol. Chem. 240: 1616-1618 .

Yang, C.C., Lin, M.F. and Chang, C.C. (1977) Purification of anticobrotoxin antibody by affinity chromatography. Toxicon 15: 51-62.

Yap, P.M. and Perry, J.F. (1960) Experimental evaluation of methods for the early treatment of snakebite. Surgery 47: 975-981.

VITA

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