PATHOGENESIS OF HEMORRHAGE AND MYONECROSIS INDUCED BY VENOM AND PURIFIED VENOM TOXINS OF *AGKISTRODON CONTORTRIX LATICINCTUS* (BROAD-BANDED COPPERHEAD)

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PATHOGENESIS OF HEMORRHAGE AND MYONECROSIS

INDUCED BY VENOM AND PURIFIED VENOM TOXINS

OF *AGKISTRODON CONTORTRIX LATICINCTUS*

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CHAPTER I

INTRODUCTION

Venomous snakebite is a serious health problem worldwide with an estimated 30,000-40,000 human deaths each year (Russell, 1969). In the United States, the mortality from snakebite is considerably less than in other countries with usually fewer than 10-12 reported fatalities per year (Minton, 1971). The low mortality rate in the U.S. is due primarly to the widespread use of polyvalent (Crotalidae) antivenom in treating many of the serious bites. This antivenom, manufactured by Wyeth-Ayerst Laboratories, Inc., Marietta, PA., consists of hyperimmune serum collected from horses immunized with the venoms of four snake species, *Crotalus atrox, C. adamanteus, C. durissus terrificus, and Bothrops atrox*. Due to paraspecific neutralization of lethality by antibodies raised against these four venoms (Gingrich and Hohenadel, 1956), only one antivenom is produced in the U.S. to treat bites by all North American pit vipers.

Although the use of the polyvalent antivenom has been shown to have life-saving efficacy (Russell et al., 1973), its use does not always prevent the local tissue damage associated with bites by crotaline snakes (Ownby et al., 1983). This tissue damage, which includes both hemorrhage and myonecrosis, can result in significant tissue loss or in severe bites to a loss of function of the bitten region. Therefore, although the mortality from snakebite in the U.S. is relatively low, the tissue damage caused by the venoms continues to be a serious complication in the treatment of snakebite.

Snake venoms are complex mixtures of proteins that have a wide range of biological activities (Tu, 1977). The severity of a venomous snakebite is influenced by several factors which include the type and relative amounts of the different toxins in the venom. Because the specific toxins that produce tissue damage are important in the pathogenesis of snakebite, there has been considerable research on these toxins. It has been shown that the venoms of most crotaline snakes are especially rich sources of toxins that are capable of producing severe hemorrhage and myonecrosis due to their effects on capillary endothelium and skeletal muscle cells, respectively. Recently, many hemorrhagic toxins (Bjarnason and Fox, 1988) and myotoxins (Mebs and Ownby, 1990) have been purified from crotaline snake venoms and characterized as to their chemical and biological activities.

Although many of the myotoxins and hemorrhagic toxins in snake venom have been well studied, the mechanism of their action at the molecular level has not been unequivocally determined. In many cases the mechanism of action is unknown or there is considerable contradiction in the literature concerning their action. In addition, as more toxins are isolated from snake venoms, the number of unanswered questions is increasing at a much faster rate than the number of solutions. Because these toxins play a major role in crotaline snakebite, knowledge about their mechanism of action is important because it may allow for the development of alternatives to antivenom in treating tissue damage due to snakebite. This is especially important since the use of antivenom often has serious side effects, such as allergic reactions to the horse serum (Corrigan et al., 1978), and could be avoided in less severe cases where the lives of the patients are not threatened.

Differences in the composition of venoms from taxonomically distinct snake species have been shown by many researchers (Tu, 1977). On the other hand, homologous toxins present in a large number of different snake venoms have also been reported (Weinstein,

1985; Bober, 1988). Because many of these homologous toxins induce tissue damage and appear to be present in the venoms of a large number of snake species, it is possible that the local tissue damage produced by crotaline snake venoms may be due to only a few types of chemically related toxins. Therefore, the knowledge obtained from studying the mechanism of action of purified myotoxins and hemorrhagic toxins from one species may be useful in understanding the mechanism of action of a wide variety of snake venom toxins with similar biological activities.

In the United States most venomous snakebites are caused by snakes in the subfamily Crotalinae (pit vipers) (Parrish, 1980). These include the rattlesnakes, of which there are two genera, *Crotalus* and *Sistrurus* and the copperheads and water moccasins, genus *Agkistrodon.* Copperheads (*A. contortrix*) are responsible for approximately 30% of the human snakebites in the U.S. with about 850 bites per year (Parrish, 1980). Even though bites by copperheads represent a relatively high percentage of snakebite cases, the biological activities of copperhead venoms have not been as thoroughly investigated as those of venoms from other snake species. The venom of *A. c. laticinctus* (broad-banded copperhead), a subspecies found in southern Kansas, Oklahoma, and Texas, U.S.A., induces considerable tissue damage in both humans and experimental animals (unpublished data) yet virtually nothing is known about the hemorrhage and myonecrosis caused by this venom or its toxins. The purpose of the following investigation was to isolate both a myotoxin and hemorrhagic toxin from the venom of *A. c. laticinctus* and to study the myonecrosis and hemorrhage produced by these toxins and the crude venom. This is important because future research on the mechanism of action of these toxins may help in understanding the action of similar toxins in other snake venoms and may lead to the development of alternatives to antivenom therapy in treating the local tissue damage produced by crotaline snakebite.

CHAPTER II

ISOLATION AND INITIAL CHARACTERIZATION OF A MYOTOXIN FROM THE VENOM OF *AGKISTRODON CONTORTRIX LATICINCTUS* (BROAD-BANDED COPPERHEAD)

Introduction

Local tissue damage, including skeletal muscle necrosis, is a common sequela following bites by many species of venomous snakes. In many cases this damage can lead to a marked decrease in function, or in severe bites to amputation of the bitten limb. From a medical viewpoint, the myonecrosis induced by snake venoms is important since experimental research has shown that often the use of antivenom does not completely prevent this damage (Ownby et al., 1983).

Snake venom myotoxins are a diverse group of toxins (Mebs and Ownby, 1990) which include 1) small, basic polypeptides such as myotoxin a (Ownby et al., 1976; Cameron and Tu, 1977), peptide c (Maeda et al., 1978), and other homologous toxins isolated from several rattlesnake venoms; 2) highly toxic, pre-synaptic neurotoxins that possess phospholipase A_2 activity such as notexin (Harris et al., 1975), crotoxin (Gopalakrishnakone et al., 1984), and taipoxin (Harris and Maltin, 1982); 3) non-neurotoxic basic phospholipases A_2 (Gutierrez et al., 1984; Mebs, 1986); and 4) myotoxins devoid of detectable phospholipase A_2 activity (Gutierrez et al. 1989; Lomonte and Gutierrez, 1989).

The type of pathological change in skeletal muscle cells induced by snake venom myotoxins varies according to the type of myotoxin. One type of pathological change produced by many snake venom myotoxins is characterized by a hypercontraction of myofilaments which results in a pattern of densily clumped myofibrils in the muscle cells. This type of damage has been observed following injections of notexin (Harris et al., 1975), crotoxin (Gopalkrishnakone et al., 1984), myotoxin I from *Bothrops asper* venom (Gutierrez et al., 1984), myotoxin II also isolated from *B. asper* venom (Lomonte and Gutierrez, 1989), and a myotoxin from the venom of *B. nummifer* (Gutierrez et al., 1989).

The mechanism by which these myotoxins induce this pathological change has not been conclusively determined. Because many of the toxins that were initially isolated had phospholipase A_2 activity, it has been proposed that a hydrolysis of membrane phospholipids due to enzymatic activity may disrupt the plasma membrane so that it becomes leaky to calcium (Harris and MacDonell, 1981; Gutierrez et al., 1984). According to this hypothesis, the inability of the muscle cells to regulate fluxes of calcium would lead to the pathological changes produced by these myotoxins. However, recent studies suggest that enzymatic activity alone cannot totally explain this type of myonecrosis since two snake venom myotoxins which produce hypercontracted myofilaments, myotoxin II from *B. asper* venom (Lomonte and Gutierrez, 1989) and a myotoxin from *B. nummifer* venom (Gutierrez et al., 1989), lack detectable phospholipase A_2 activity. Therefore, additional research is needed on these newly discovered snake venom myotoxins which lack phospholipase A_2 activity yet produce a hypercontraction of myofilaments in skeletal muscle cells. The purpose of the following investigation was to isolate and characterize one of these non-phospholipase myotoxins from the venom of the broad-banded copperhead (*Agkistrodon contortrix laticinctus*).

Materials and Methods

Venom

A. c. laticinctus venom was obtained from 15 snakes collected in central Texas, U.S.A and maintained at the Venom Research Serpentarium, Department of Physiological Sciences, Oklahoma State University, U.S.A. The snakes were extracted approximately every two weeks during 1988-1989. The venom was extracted by allowing the snakes to bite through parafilm which covered a glass funnel that emptied into a polycarbonate centrifuge tube resting in an ice bath. Immediately after extraction the venom was centrifuged at 28,000 x *g* for 20 min using a Sorvall RCR5 refrigerated centrifuge (4 C) to remove insoluble material, frozen, and then lyophilized. Lyophilized venom was stored over dessicant at -20 C until needed.

Isolation of Myotoxin

Anion Exchange Chromatography. Samples of crude venom (125 mg) were reconstituted in 9.0 ml of 10 mM Tris buffer (pH 8.6), filtered using a Millipore 0.2 um FG filter, and applied to a Waters DEAE-5PW HPLC anion exchange column (21.5 mm x 15 cm) at a flow rate of 3.6 ml/min. The fractions were eluted by first washing the column with the same buffer for 20 min followed by a 0.0-0.4 M linear NaCl gradient in the same buffer for 50 minutes. Tris buffer (pH 8.6) containing 1.0 M NaCl was then applied for 10 minutes to elute any remaining fractions. The fractions were collected as peaks and checked for myotoxic activity in mice using histological techniques as described below. The myotoxic fraction eluted before the salt gradient, therefore a desalting step was not necessary. The myotoxic fraction in 10 mM Tris buffer (pH 8.6) was concentrated to about 10 ml using a

Millipore Minitan System with a 1000 MW exclusion membrane and further purified using a HPLC cation exchange column.

Cation Exchange Chromatography. The myotoxic fraction from the DEAE column was filtered using a Millipore 0.2 um FG filter and applied to a Waters Protein Pak SP-5PW HPLC cation exchange column (7.5 mm x 7.5 cm) equilibrated with 10 mM Tris buffer (pH 8.6). The fractions were eluted from the column at a flow rate of 1.0 ml/min using the same buffer and a 0.0-1.0 M linear NaCl gradient applied over 50 minutes. The fractions were checked for myotoxic activity as described below and for homogeneity by SDS-PAGE. If necessary, the fraction of interest was re-applied to the cation exchange for further purification. The purified fraction was concentrated to about 2.0 ml using both a Millipore Minitan system as described above and Centricon 10 microconcentrating units (molecular weight cutoff of 10,000) and frozen at -20 C until needed.

Electrophoresis

Homogeneity and molecular weight of the myotoxin were determined by SDS-PAGE using a Pharmacia Phastsystem mini-electrophoresis unit and 8-25% gradient gels. Twenty-five microliters of a 2.0 mg/ml solution of myotoxin was added to either an equal volume of sample buffer containing 10 mM Tris-HCl buffer (pH 8.6), 2.5% sodium dodecyl sulfate (SDS), and 5.0% β-mercaptoethanol and heated at 100 C for 3 minutes or to sample buffer without β-mercaptoethanol and heated at 100 C for 3 or 10 minutes. Four microliters of the sample were applied to the gels and the electrophoresis performed for 100 volt-hours following the procedure described in the Phastsystem Technical Bulletin No. 110. Upon completion of the electrophoresis, the gels were stained using a Pharmacia Phastsilver staining kit. Molecular weight markers were as follows: rabbit muscle phosphorylase B (97,400),

bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400).

Isoelectric focusing of the purified myotoxin was also performed using the Pharmacia Phastsytem. Pharmacia Phastgels with a pH gradient from pH 3-9 were used for the isoelectric focusing. Four microliters of a 1.0 mg/ml solution of ACL myotoxin were applied to the gels and the isoelectric focusing performed following the procedure described in the Pharmacia Technical Bulletin No. 100. Gels were stained using a Pharmacia Phastsilver staining kit. Bio Rad IEF standards (pI 4.65-9.60) were used for determination of the isoelectric point of the myotoxin.

Phospholipase A Activity

Phospholipase A activity of the purified myotoxin and crude venom was determined using L-1-phosphatidylcholine from egg yolk (Sigma, Inc.) as substrate using the method detailed in the Mannheim-Boehringer catalog No. 15057 as described by Johnson et al. (1987). The activity was determined by the amount of free fatty acid liberated from the substrate and was measured by a decoloration of the indicator dye, cresol red, at 578 nm. The specific activity was expressed in units/mg and was determined by dividing the volume activity (U/ml) by the amount of venom or toxin.

Amino Acid Analysis

Amino acid analysis of the myotoxin was determined using the pre-column phenylisothiocyanate (PITC) derivatization method of Heinrickson and Meredith (1984) and Koop et al. (1982). One micromole of myotoxin was added to 200 μl of 6 N HCl and heated at 110 C for 18-24 hours. Samples were then dessicated to dryness under vacuum and 100 μl

of coupling buffer (acetonitrile:pyridine:triethylamine:water, 16:5:2:3) and 5 μl PITC were added and incubated for 5 minutes. Samples were dried under vacuum and redissolved in 25 μl of a 1:1 dilution of acetonitrile and water. Amino acids were separated by HPLC using a RT 250-4 LiChromsorb RP 18 column (EM Sciences). This derivitazation procedure does not detect sulfur containing amino acids therefore cysteine and methionine were not determined. Tryptophan, which is destroyed by acid hydrolsis, also was not determined.

Histological Examination

Myonecrosis induced by fractions and the purified myotoxin was investigated using the method of Ownby et al. (1976). Fifty microliters of each fraction were injected into the right thigh of female mice (CD-1, Charles Rivers) weighing 25-30 g. Myotoxin in 10 mM tris buffer (pH 8.6) was adjusted to a solution concentration of 1.5 μg/μl and injected so that a 25 g mouse would receive 50 μl containing a 2.5 μg/g dose of myotoxin. Control mice were injected with 50 μl 10 mM tris buffer (pH 7.4). Mice were killed by cervical disslocation and tissue samples taken at one time period (1 hour) after injection of each fraction and at varying time periods (5, 15, and 30 min; 1, and 3 hours) after injection of the purified myotoxin. Experiments which consisted of injecting two mice per time period with myotoxin and one mouse with tris buffer alone as a control of fixation were performed in duplicate. Samples were fixed for 2 hours in 2.0% glutaraldehyde in 0.16 M cacodylate buffer (pH 7.4). The tissue was washed three times in 0.08 M cacodylate buffer containing 0.18 M sucrose and post-fixed for 1 hour in 1% aqueous osmium tetroxide. The tissue was dehydrated in ascending concentrations of acetone and embedded in Polybed 812 resin. Thick sections (1 μm) for light microscopic examination were obtained with a Sorvall MT 5000 ultramicrotome and stained with Mallory's azure II-toludine blue dye. A total of 50 sections

were examined.

Results

Purification of Myotoxin

Fractionation of *A. c. laticinctus* venom by DEAE-anion exchange chromatography yielded a total of 18 fractions, designated A1-A18 (Figure 1). Myotoxic activity was found in fraction A1 which had a considerable amount of protein based on absorbance at 280 nm and was chosen for further purification although it was not the only myotoxic fraction. Fraction A1 had weak affinity for the column and eluted before the NaCl gradient suggesting that at pH 8.6 the proteins in this fraction are cationic. Therefore, fraction 1 was further purified by cation exchange chromatography. Figure 2 shows the elution profile of the separation of fraction A1 from the DEAE column using a Waters SP-5PW cation exchange column. A total of 12 fractions, designated B1-B12, eluted from this column. Fraction B12 (shaded) eluted at a NaCl concentration of about 600 mM and contained myotoxic activity. This fraction was homogeneous as determined by SDS-PAGE using a silver stain and is herein referred to as ACL myotoxin.

Figure 3 shows the results of the SDS-PAGE analysis of ACL myotoxin. The molecular weight of ACL myotoxin was estimated to be 29,000 when the reducing agent β-mercaptoethanol was excluded from the sample buffer. However, in the presence of β-mercaptoethanol a single band was observed with a molecular weight of 14,500 which suggests that ACL myotoxin exists as two polypeptide chains. The two polypeptide chains were not disassociated by heating at 100 C for 3 minutes in the presence of 2.5% sodium dodecyl sulfate and only partially disassociated by heating at 100 C for 10 minutes.

Isoelectric focusing indicated that ACL myotoxin is a very basic protein with a pI >

Figure 1. Fractionation of *A. c. laticinctus* venom on a Waters DEAE-5PW anion exchange column (21.5 mm x 15 cm). Samples of 125 mg venom were dissolved in 10 mM Tris buffer (pH 8.6) and applied at a flow rate of 3.6 ml/min. Fractions were eluted with 10 mM Tris buffer for 20 minutes after which time a 0.0-0.4 M NaCl gradient in the same buffer was applied over 50 minutes. Myotoxic activity was found in fraction A1 (shaded) which was further purified by cation exchange chromatography.

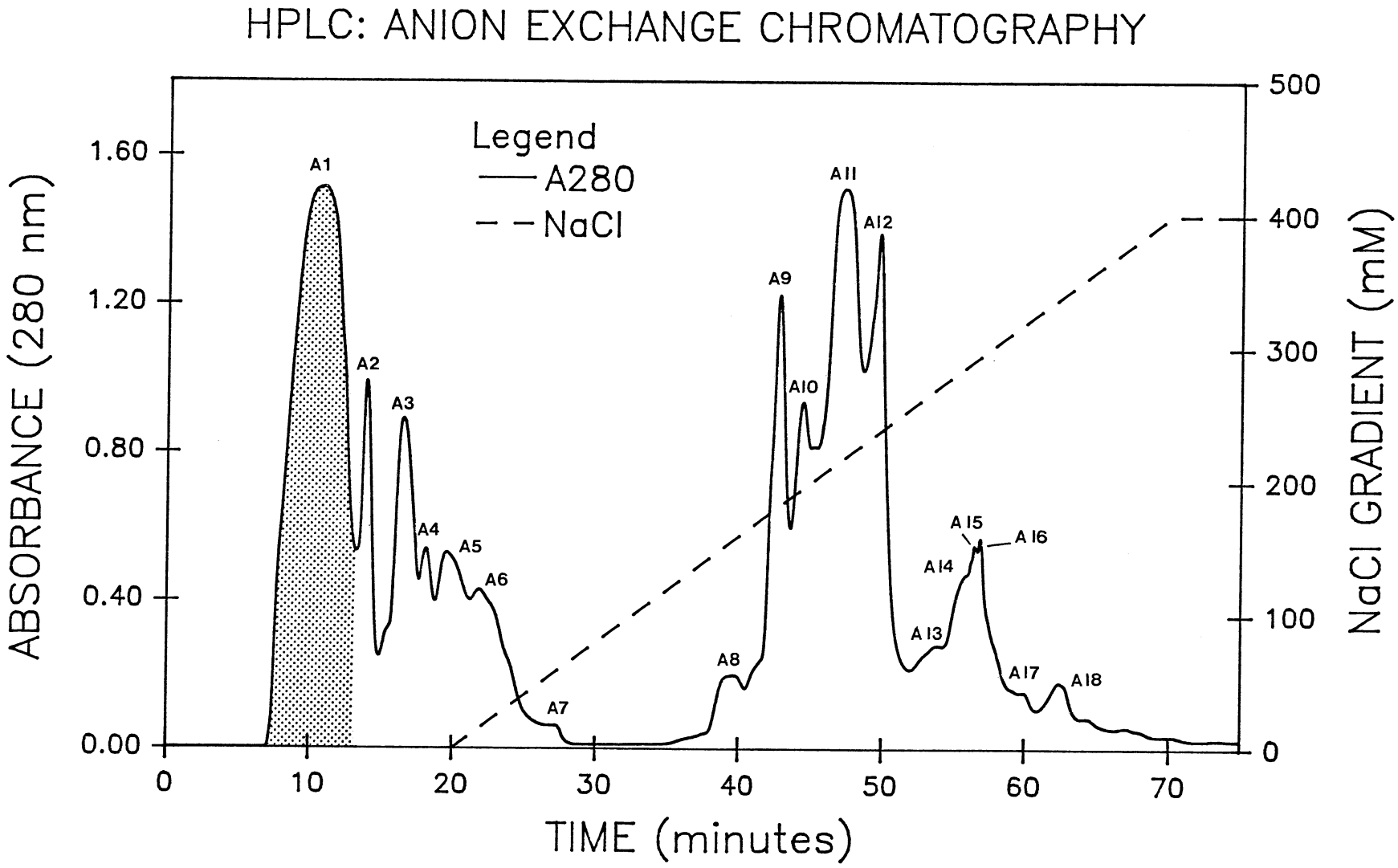

HPLC: ANION EXCHANGE CHROMATOGRAPHY
Legend
A280
NaCl
ABSORBANCE (280 nm)
1.60
1.20
0.80
0.40
0.00
NaCl GRADIENT (mM)
500
400
300
200
100
0
TIME (minutes)
0
10
20
30
40
50
60
70
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
A13
A14
A15
A16
A17
A18

Figure 2. Fractionation of fraction A1 from DEAE anion exchange column on a Waters Protein Pak SP-5PW cation exchange column (7.5 mm x 7.5 cm). Fraction A1 in 10 mM Tris buffer (pH 8.6) was applied to the column at a flow rate of 1.0 ml/min. Fractions were eluted using a linear 0.0-1.0 M NaCl gradient applied over 50 minutes. Myotoxic activity was in fraction B12.

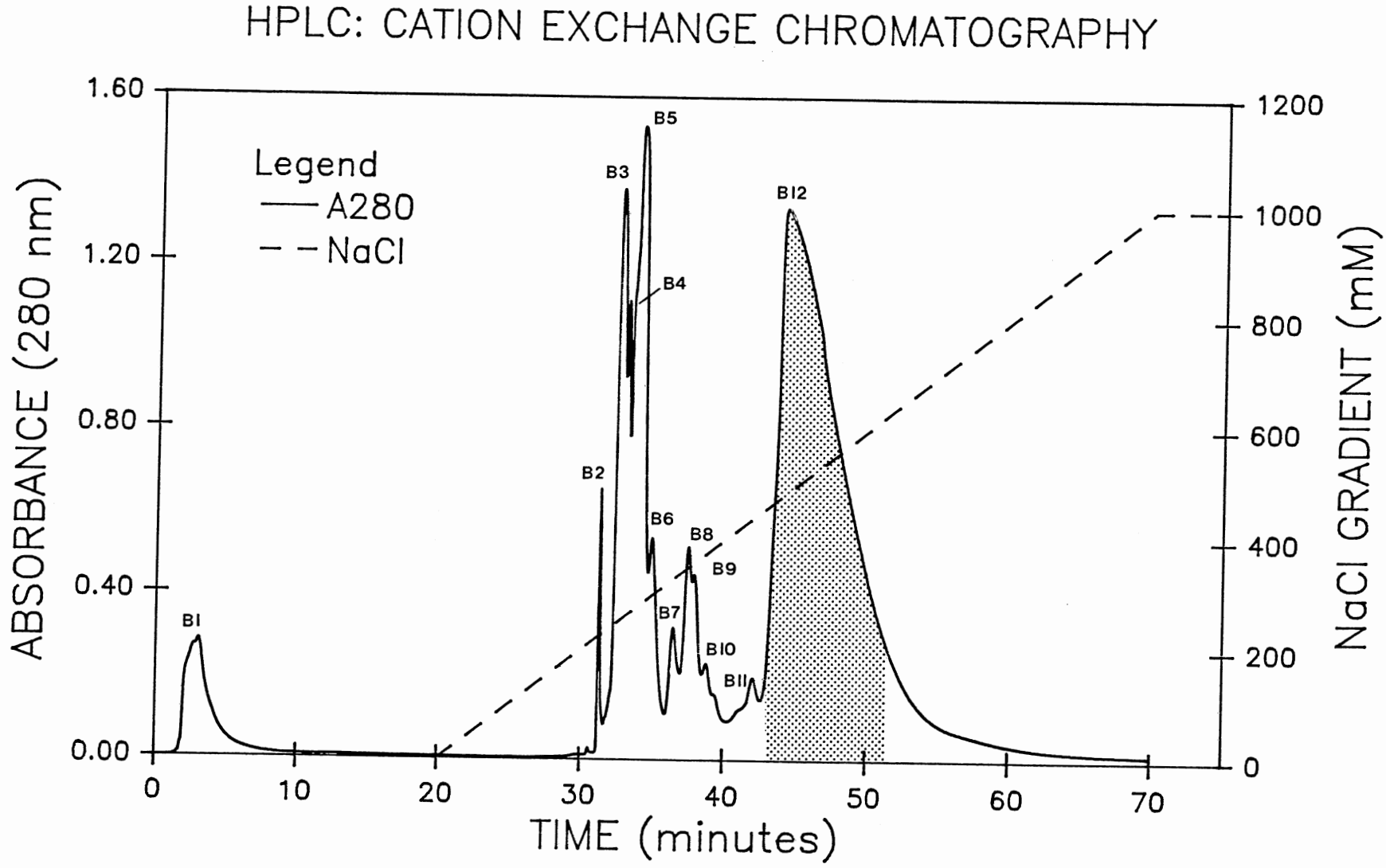
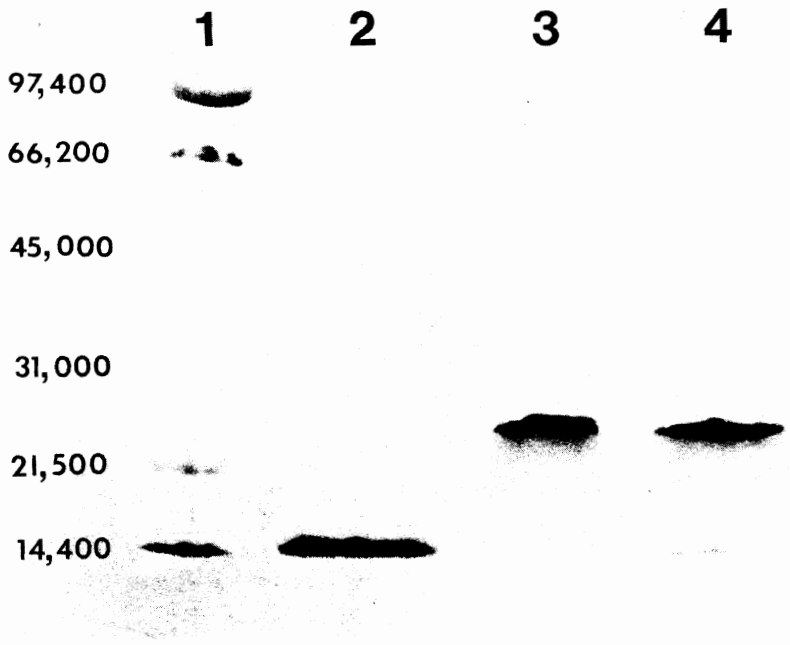

HPLC: CATION EXCHANGE CHROMATOGRAPHY
Legend
A280
NaCl
ABSORBANCE (280 nm)
NaCl GRADIENT (mM)
TIME (minutes)
1.60
1.20
0.80
0.40
0.00
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12

Figure 3. SDS-Polyacrylamide gel electrophoresis of ACL myotoxin.
Lane 1: molecular weight standards, Lane 2 ACL myotoxin (1 μg) in presence of 5% β-mercaptoethanol, Lane 3: ACL myotoxin (1 μg) in absence of β-mercaptoethanol; heated at 100 C for 3 minutes, Lane 4: ACL myotoxin (1 μg) in absence of β-mercaptoethanol; heated at 100 C for 10 minutes.


1
2
3
4
97,400
66,200
45,000
31,000
21,500
14,400

9.0. However, it was not possible to determine the exact pI since the myotoxin migrated off the anode side of the gel using a pH gradient from pH 3-9.

The partial amino acid composition of ACL myotoxin is shown in Table 1. The toxin contains a large number of glysine, lysine and leucine, as well as hydrophobic and polar amino acids. Using partial amino acid composition, the formula weight of ACL myotoxin is 14,487.

Phospholipase A Activity

The myotoxin was devoid of detectable phospolipase A activity using L-1-phosphotidylcholine as substrate under the experimental conditions used in this experiment. The crude venom had considerable phospholipase A activity with a mean specific activity of 5,230 U/mg.

Histological Examination

ACL myotoxin induced a rapid myonecrosis of mouse skeletal muscle cells with necrotic cells present as early as 5 min after i.m. injection of the myotoxin. The predominant pattern of myonecrosis induced by ACL myotoxin was characterized by clumped myofilaments in damaged muscle cells. However, three types of pathological changes could be identified in muscle cells damaged by the myotoxin (Figure 4). These included 1) muscle cells that contained clear vacuoles, 2) muscle cells with disorganized myofibrils, referred to as cells with a "mottled" appearance, and 3) muscle cells that contained densely clumped myofibrils.

Discussion

A toxin capable of inducing the necrosis of mouse skeletal muscle cells was isolated

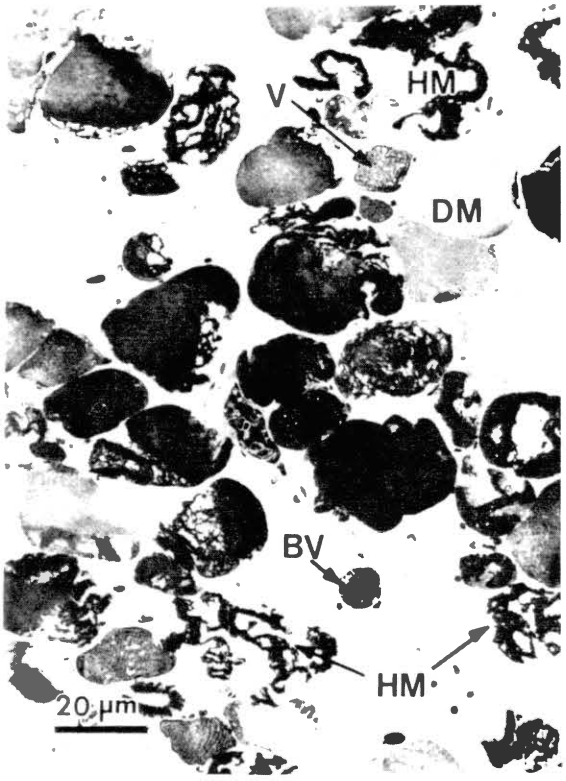
Table I

AMINO ACID COMPOSITION OF ACL MYOTOXIN

Amino acid	Moles amino acid/ Moles protein	Nearest integer
Asx	3.60	4
Glx	2.80	3
Ser	8.60	9
Gly	13.40	13
His	6.80	7
Arg	2.00	2
Thr	11.40	11
Ala	5.40	5
Pro	9.00	9
Tyr	3.80	4
Val	3.60	4
Ile	7.80	8
Leu	12.20	12
Phe	10.60	11
Lys	11.80	12
Total residues*		114
Formula weight		14,487

*Tryptophan, cystine, and methionine not determined

Figure 4. Light micrograph of mouse skeletal muscle taken 5 minutes after injection of ACL myotoxin. Note muscle cells with clear vacuoles (V), muscle cells with disorganized myofibrils (DM), muscle cells containing hypercontracted myofilaments (HM), and congested blood vessel (BV).


V
HM
DM
BV
HM
20 µm

from *A. c. laticinctus* venom by a two column procedure using HPLC. The toxin, designated ACL myotoxin, was homogeneous as determined by SDS-PAGE analysis using a silver stain. The molecular weight of ACL myotoxin was estimated to be 14,500 by SDS-PAGE. However, in the absence of β-mercaptoethanol, the molecular weight of ACL myotoxin was 29,000 which suggests that the myotoxin exists as two polypeptide chains, each with a molecular weight of 14,500.

ACL myotoxin is a basic protein that contains a high content of lysine residues. The large number of lysine and histidine combined with relatively low amounts of glutamate and aspartate most likely contribute to the high isoelectric point (> 9.0) of the myotoxin. The high content of lysine and basic isoelectric point of ACL myotoxin are similar to that described for a large number of snake venom myotoxins. Maeda et al. (1978) reported that peptide c, a polypeptide myotoxin isolated from *Crotalus viridis helleri* venom, has an isoelectric point greater than 10.0 and contains approximately 20% lysine residues. Similarly, myotoxin I (Gutierrez et al., 1984) and myotoxin II (Lomonte and Gutierrez, 1989) from *B. asper* venom are both basic proteins that contain 15% and 14% lysine residues, respectively. Bothropstoxin, a non-phospholipase myotoxin isolated from the venom of *B. jararacussu*, also is a basic protein (pI = 8.2) with lysine comprising about 15% of the total amino acids (Homsi-Brandeburgo et al., 1988).

ACL myotoxin was devoid of detectable phospholipase A activity using L-1-phosphatidylcholine as substrate. In this regard, ACL myotoxin is similar to bothropstoxin (Homsi-Brandeburgo et al., 1988), myotoxin II isolated from *B. asper* venom (Lomonte and Gutierrez, 1989) and a myotoxin isolated from the venom of *B. nummifer* from Costa Rica (Gutierrez et al., 1989). Myotoxin II from *B. asper* venom is a dimeric protein with a monomeric molecular weight of 13,000 whereas the myotoxin from *B. nummifer* venoms

consists of a single polypeptide chain with a molecular weight of 13,000. Bothropstoxin from the venom of *B. jararacussu* also is a single chain polypeptide with a molecular weight of 13,000. It has been shown that these myotoxins cause a type of myonecrosis similar to the type induced by ACL myotoxin (Homsi-Brandeburgo et al., 1988; Lomonte and Gutierrez, 1989; Gutierrez et al., 1989).

ACL myotoxin induces a rapid myonecrosis of murine skeletal muscle cells characterized by a hypercontraction of myofilaments resulting in areas of densely clumped myofibrils in the cytoplasm. The hypercontracted myofilaments suggest elevated levels of free intracellular calcium which has been proposed to cause this type of pathologic change (Harris and MacDonell, 1981). If this hypothesis in correct, increased levels of free intracellular calcium could be produced by at least two different mechanisms. These include 1) an increased influx of calcium from the extracellular fluid due to a disruption of permeability of the plasma membrane and/or 2) a release of calcium from the sarcoplasmic reticulum. Although the exact mechanism by which ACL myotoxin may induce an increase in free intracellular calcium is not known, several possibilities exist and are discussed below.

ACL myotoxin contains a large number of both hydrophobic and polar amino acids. Although the sequence of the toxin was not determined, it is possible ACL myotoxin contains both a hydrophobic domain and a more polar, hydrophilic domain. This configuration has been reported for several cytolytic toxins including α-toxin from *S. aureus* (Tobkes et al., 1985). It is possible that ACL myotoxin may behave as an amphiphilic molecule and insert a hydrophobic domain into the plasma membrane of skeletal muscle cells thus disrupting the phospholipids by a detergent-like action. A detergent-like action has been proposed for other cytolytic toxins including mellitin, a polypeptide isolated from honeybee (*Apis mellifera*) venom (Shipolini, 1984). The conformation of mellitin is an amphiphilic alpha-helix that has

a high content of hydrophobic amino acids oriented to one side of the helix (Shipolini, 1984). It has been suggested that mellitin may insert a hydrophobic portion of the toxin into the plasma membrane and change the vectoral orientation of phospholipids from tilted to vertical and thereby disrupt the permeablilty barrier of the membrane (Shipolini, 1984). Disruption of phospholipid organization would cause the plasma membrane to become leaky to ions and abolish the regulation of ion fluxes. Such a disruption may allow high concentrations of ions such as sodium and calcium to enter the cell along their concentration gradients and produce pathological changes in the cell. It is possible that ACL myotoxin acts in a similar manner to mellitin since ACL myotoxin also contains a high content of hydrophobic amino acids. The large number of polar amino acids in ACL myotoxin may comprise a domain that is located at the lipid-water interface.

If ACL myotoxin contains both hydrophobic and polar domains, another possible mechanism is that ACL myotoxin may form pores in the plasma membrane which allows the influx of ions into muscle cells. Such a mechanism has been implicated for a large number of toxins from a wide variety of organisms including bacteria such as *Staphlococcus* and *Streptococcus*, various coelenterate venoms, and bee, wasp, and hornet venoms (Harvey, 1990). For example, $\propto$-toxin, a 33,000 dalton cytotoxin from the bacterium *S. aureus*, has been shown to form 2-3 nm pores in the plasma membrane of rabbit erythrocytes (Bhakdi and Tranum-Jensen, 1986). The pores are believed to be formed from hexamers of the toxin which are inserted into the membrane (Harvey, 1990). In order for an amphiphilic toxin to insert into the lipid-rich environment of the plasma membrane, polar amino acids must be shielded from the phospholipids of the membrane. One way this could be accomplished by the formation of complexes of the toxin arranged so that hydrophobic amino acids face outward and polar amino acids face inward away from the phospholipids. This has been

reported for various other cytotoxins, including ∝ and delta-toxins from *S. aureus*, cytolysin II from venom of the sea anemone *Stoichactus helianthus*, mellitin from honeybee (*A. mellifera*) venom, and the gramicidins from *Basillus brevis* (Harvey, 1990). It is possible that ACL myotoxin forms complexes with a similar configuration. The polar interior of such a complex allows interactions with water molecules and could function as an aqueous pore which is permeable to various ions. Ions present in much higher concentrations in the extracellular fluid compared to intracellular fluid, such as sodium and calcium, would follow their concentration gradients and enter the muscle cells through these pores. High levels of intracellular calcium have been implicated in several pathological processes (Trump et al., 1981; Wrogemann and Pena, 1976) and may produce the pathological changes induced by ACL myotoxin, such as hypercontracted myofilaments (Harris and MacDonell, 1981).

Although hypercontracted myofilaments in skeletal muscle cells suggest increased free intracellular calcium, it has not been conclusively determined if this results from an influx of calcium from the extracellular fluid. Elevated levels of free intracellular calcium induced by ACL myotoxin could also be due to a release of calcium from the sarcoplasmic reticulum, either by a direct action on the terminal cisternae or indirectly through a disruption of other cellular processes. The concentration of calcium in the terminal cisternae of the sarcoplasmic reticulum is normally about 50 mM (Ruegg, 1988). Most of the calcium in the terminal cisternae is bound to calsequestrin, although levels of free calcium can be as high as 1 mM (Ruegg, 1988). An uncontrolled release of free calcium from the terminal cisternae could result in the accumulation of this ion in the cytoplasm. The contraction of myofilaments has been shown to be dependent on free calcium concentrations with maximum contraction occuring at about 10 μM (Thomas, 1982). If calcium in the terminal cisternae was released into the cytoplasm, the levels could possibly rise above this concentration, especially if all the

bound calcium was released, and may reach levels sufficient to produce hypercontracted myofilaments. However, the mechanism by which ACL myotoxin may induce a release of calcium from the sarcoplasmic reticulum is not clear and additional research is needed to conclusively determine the mechanism of action of ACL myotoxin.

An interesting aspect of ACL myotoxin in regard to its ability to damage skeletal muscle cells is its high isoelectric point and high content of the amino acid lysine. Suarez-Kurtz (1985) showed that polycations such as polymers of lysine and ornithine caused an increase in the loss of creatine kinase and lactate dehydrogenase from frog skeletal muscle which suggests damage to the plasma membrane. Enzyme release from cells was related to the degree of polymerization of the cations in such a way that the more cationic polymers produced a greater loss of enzymes. It is possible that the high content of cationic amino acids in ACL myotoxin may contribute to its necrotic action on skeletal muscle cells, however, the mechanism through which this would occur is not clear. One possibility is that the charged groups on ACL myotoxin may aid in attachment of the toxin to the plasma membrane. The cytotoxic polypeptide mellitin is composed of 26 amino acids of which 4 of the last 6 C-terminus amino acids are positively charged (Harvey, 1990). It has been suggested that the charged terminus of mellitin may form electrostatic interactions with negatively charged groups of the phospholipids and thereby aid in aligning the toxin in the membrane (Haberman, 1980). The positive charge of ACL myotoxin may serve a similar function. The presence of a positive charge appears to be an important feature of snake venom myotoxins since all myotoxins isolated to date have basic isoelectric points (Ownby, 1990).

The similarity of molecular weights, the lack of phospholipase A activity, and the type of myonecrosis induced by ACL myotoxin, myotoxin II from *B. asper* venom, and a

myotoxin from the venom of *B. nummifer*, suggests that these toxins may have a similar mechanism of action. This is interesting since ACL myotoxin was isolated from the venom of *A. c. laticinctus* which belongs to a different genus from these other snakes. It is possible that myotoxins with similar mechanisms of action may be present in the venoms of a large number of venomous snake species. This has important implications because in treating the tissue damage induced by snakebite it is desirable to have an antivenom capable of neutralizing the tissue damage produced by a large number of snake species. Because ACL myotoxin induces considerable myonecrosis in mice and homologs may be present in the venom of a large number of snake species, it may be an important immunogen which can be used to make an improved antivenom. In addition, it may be possible to antagonize the myonecrosis produced by these toxins using a non-antibody treatment if their mechanism of action is determined. Therefore, future research on the mechanism of action of ACL myotoxin and related myotoxins may prove useful in developing an improved treatment for viperid snakebite.

CHAPTER III

MYONECROSIS INDUCED BY ACL MYOTOXIN AND CRUDE VENOM OF *AGKISTRODON CONTORTRIX LATICINCTUS* (BROAD-BANDED COPPERHEAD)

Introduction

Snake venoms are complex mixtures of proteins that induce severe pathological changes in numerous tissues of the body. These changes can be divided into 1) systemic effects, such as changes in blood pressure, respiration, and hemostasis, and 2) local effects which include tissue damage such as hemorrhage, myonecrosis, and edema (Lee, 1975). In the United States, the mortality caused by venomous snakebite is relatively low due primarily to the use of antivenom in treating the serious bites. Although the use of antivenom is effective in treating many of the systemic effects produced by snakebite, its use does not always prevent the local tissue damage caused by the venoms (Ownby et al., 1983). Since these local reactions often have debilitating effects on surviving snakebite victims, the abatement of hemorrhage and myonecrosis is an important part of the treatment of crotaline snakebite.

Snake venom myotoxins are a diverse class of molecules that induce several types of skeletal muscle cell necrosis (Mebs and Ownby, 1990). One type of necrosis produced by a large number of snake venom myotoxins is characterized by a hypercontraction of myofilaments in damaged muscle cells. This kind of necrosis is produced by both the

phospholipase A_2 myotoxins isolated from venoms of elapid, viperine, and crotaline snakes (Harris et al., 1975; Gopalakrishnakone et al., 1984; Mebs, 1986) and non-phospholipase myotoxins isolated from several *Bothrops* venoms (Gutierrez et al., 1989; Lomonte and Gutierrez, 1989). The mechanism of action of both of these types of myotoxins has not been unequivocally determined.

Harris and MacDonell (1981) proposed that hypercontracted myofilaments in skeletal muscle cells damaged by notexin, a phospholipase A_2 myotoxin isolated from the venom of *Notechis scutatus scutatus*, result from an influx of extracellular Ca^{2+} through a disruption of the sarcolemma due to a hydrolysis of phospholipids by the enzymatic activity of the toxin. Gutierrez et al. (1984) proposed that myotoxin I from *Bothrops asper* venom produced hypercontracted myofilaments by a similar mechanism. Although hypercontracted myofilaments suggest increases in intracellular Ca^{2+}, it has not been conclusively determined that this pathological change results from Ca^{2+} entering the cell from the extracellular fluid. Another possibility is that Ca^{2+} could be released from the sarcoplasmic reticulum. The terminal cisternae of the sarcoplasmic reticulum of skeletal muscle cells normally store large amounts of Ca^{2+} (Ruegg, 1988). The concentration of Ca^{2+} in the sarcoplasmic reticulum is much higher than in the extracellular fluid and represents a large source of Ca^{2+} which could potentially be released into the cytoplasm. Therefore, it is possible that some snake venom myotoxins induce hypercontracted myofilaments in skeletal muscle cells through a release of Ca^{2+} from the sarcoplasmic reticulum and not by an influx of extracellular Ca^{2+}.

In the United States, copperheads (*Agkistrodon contortrix*) are responsible for a high percentage of venomous snakebites (Parrish, 1980). In general, bites by copperheads are not as serious as are bites by other species, although occasionally these bites result in local tissue

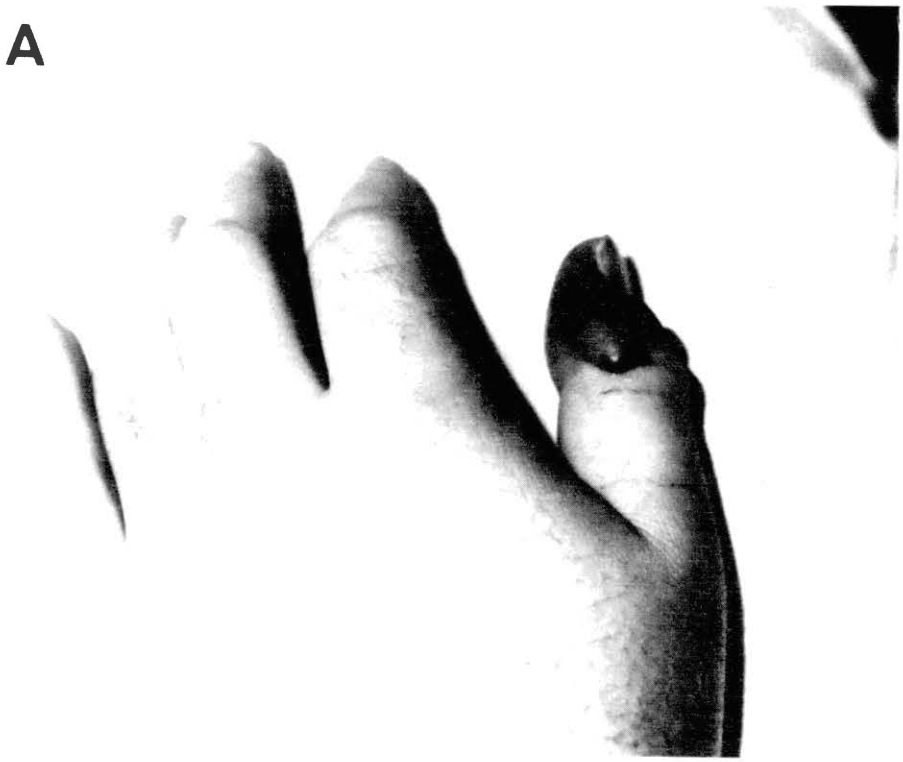
damage. This is especially true for certain subspecies, most notibly *A. c. laticinctus*, whose venom can produce tissue necrosis following a bite (figure 5). A non-phospholipase A_2 myotoxin has been isolated from the venom of *A. c. laticinctus* and characterized as to its chemical and biological properties (see chapter 2). This myotoxin, designated ACL myotoxin, produces necrotic skeletal muscle cells which contain hypercontracted myofilaments similar in morphology to cells damaged by other snake venom myotoxins. The purpose of the following investigation was to study the myonecrosis produced by ACL myotoxin and crude venom of *A. c. laticinctus* and to investigate the role of extracellular Ca^{2+} and other ions in producing pathological changes in skeletal muscle cells *in vitro* and to compare these pathological changes to those induced by ACL myotoxin *in vivo*.

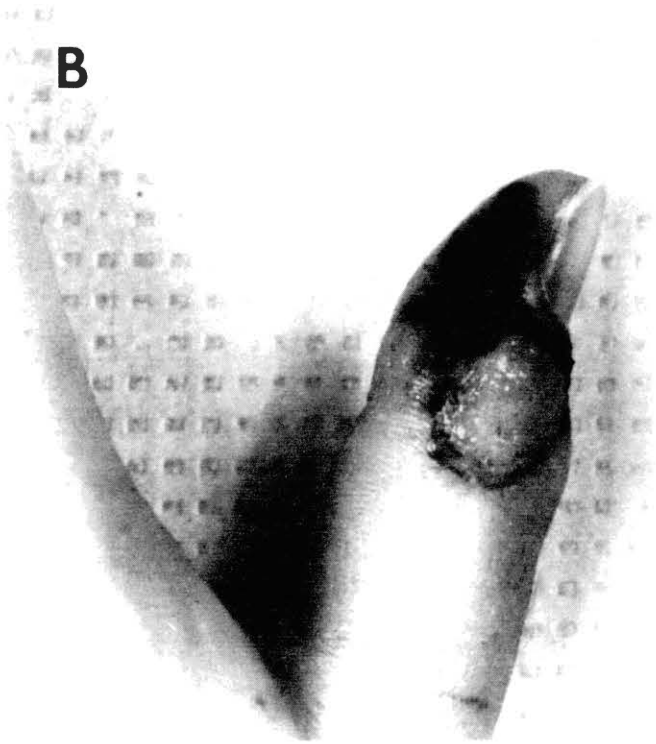
Material and Methods

Venom

A. c. laticinctus venom was obtained from 15 snakes collected in central Texas, U.S.A., and maintained at the Venom Research Serpentarium, Department of Physiological Sciences, Oklahoma State University, U.S.A. The snakes were extracted approximately every two weeks during 1988-1989. The venom was extracted by allowing the snakes to bite through parafilm which covered a glass funnel that emptied into a polycarbonate centrifuge tube resting in an ice bath. Immediately after extraction, the venom was centrifuged at 28,000 x *g* for 20 minutes using a Sorvall RCR5 refrigerated centrifuge (4 C) to remove insoluble material, frozen, and lyophilized. Lyophilized venom was stored over dessicant at -20 C until needed.

Figure 5. *A. c. laticinctus* bite on medial aspect of first digit of left thumb.
A. 24 hours. Note edema of hand and blood filled bullae on thumb.
B. 1 week. Note necrotic tissue surrounding bite site.


A


B

Isolation of Myotoxin

ACL myotoxin was isolated from *A. c. laticinctus* venom as described in chapter 2. The toxin was purified from the venom using HPLC and a two column procedure. Briefly, samples of 125 mg of crude venom were reconstituted in 10 mM Tris buffer (pH 8.6) and applied to a Waters DEAE-5PW HPLC anion exchange column at a flow rate of 3.6 ml/minute. The fractions were tested for myotoxic activity in mice using histological analysis, and the fraction of interest concentrated and lyophilized. The myotoxic fraction from the DEAE column was then applied to a Waters Protein Pak SP-5PW HPLC cation exchange column at flow rate of 1.0 ml/minute. The myotoxic fraction from this column was shown to be homogeneous by SDS-PAGE and was designated ACL myotoxin.

Histological Examination

Myonecrosis induced by ACL myotoxin and crude venom of *A. c. laticinctus* was investigated using the method of Ownby et al. (1976). Lyophilized venom was reconstituted in phosphate-buffered saline to a concentration of 1.5 μg/ul and injected into the right thigh of female mice (CD-1, Charles River) weighing 25-30 gram. Injection volumes were adjusted so that a 25 gram mouse would receive 50 μl containing a final dose of 2.5 μg/g body weight. Myotoxin in 10 mM tris buffer (pH 8.6) was adjusted to a solution concentration of 1.5 μg/μl and similarly injected. Mice were killed by cervical dislocation and tissue samples were taken at 5, 15, and 30 minutes and 1 and 3 hour after injection of the toxin and 5, 15, and 30 min and 1, 3, 6, 12, and 24 hours after injection of crude venom. Experiments which consisted of injecting two mice per time period with venom and one mouse with 50 μl phosphate-buffer saline and experiments consisting of injecting two mice per time period with myotoxin and one mouse with 50 μl tris buffer alone as a control of fixation were performed in duplicate.

Samples were fixed for 2 hours in 2.0% glutaraldehyde in 0.16 M cacodylate buffer (pH 7.4). The tissue was washed three times in 0.08 M cacodylate buffer containing 0.18 M sucrose and post-fixed for 1 hour in 1.0% aqueous osmium tetroxide. The tissue was dehydrated in ascending concentrations of acetone and embedded in Polybed 812 resin. Tissue was oriented in the resin so that both cross-sections and longitudinal sections of damaged muscle cells could be examined. Thick sections (1 μm) for light microscopic examination were obtained using a Sorvall MT 5000 ultramicrotome and stained with Mallory's toludine blue-azure II dye. A total of 260 thick sections were examined. Thin sections (silver to light gray) for electron microscopy were cut with glass or diamond knives and stained with 4% methanolic uranyl acetate and 4% aqueous lead citrate. The thin sections were observed using a JEOL 100CX STEM electron microscope. A total of about 50 thin sections were examined.

In Vitro Skeletal Muscle Organ Preparation

Pathological changes in skeletal muscle cells induced by various ions were investigated using an *in vitro* skeletal muscle organ preparation. Mice were killed by cervical dislocation and the biceps femoris muscle removed from both thighs and cut into small pieces (approximately 1 x 3 mm) so that at least some muscle cells were cut to destroy the permeability barrier of the plasma membrane. Approximately 10 pieces of muscle were placed in 1.0 ml of 10 mM tris buffer (pH 7.4) containing various concentrations of ions and incubated for 1 hour at room temperature. Concentrations (in mM) were as follows: NaCl-100, 150 and 300; LiCl- 150; $CaCl_2$-1, 10, 50, 150 and 600; KCl-150 and 300. Each treatment consisted of two vials of ion solutions, each containing the cut pieces of biceps femoris muscle from one thigh of a mouse. After incubation, the muscle tissue was processed

for histological examination as described above. Pathological changes induced by the diffusion of ions into the cut ends of skeletal muscle cells were determined using light microscopic analysis. In order to verify that the plasma membrane of muscle cells in the organ preparation were cut, trypan blue was added to the medium and incubated for 20-30 minutes. Movement of the dye into cut skeletal muscle cells was determined using a Nikon TMS phase-contrast microscope.

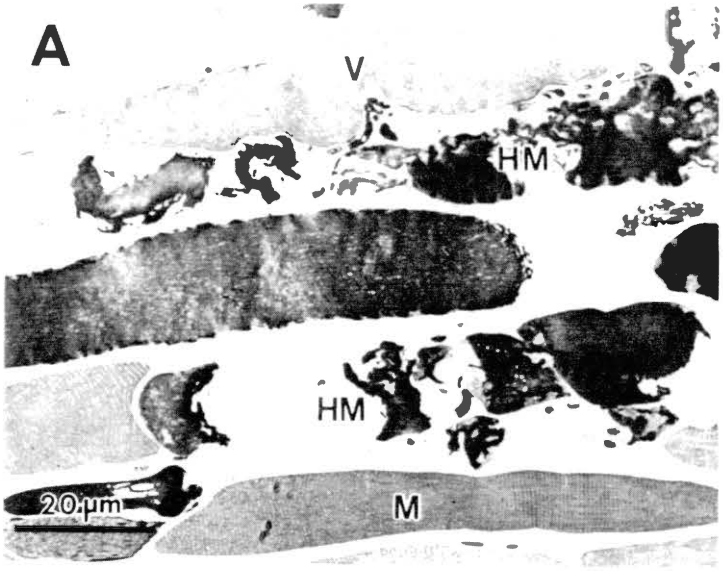
Results

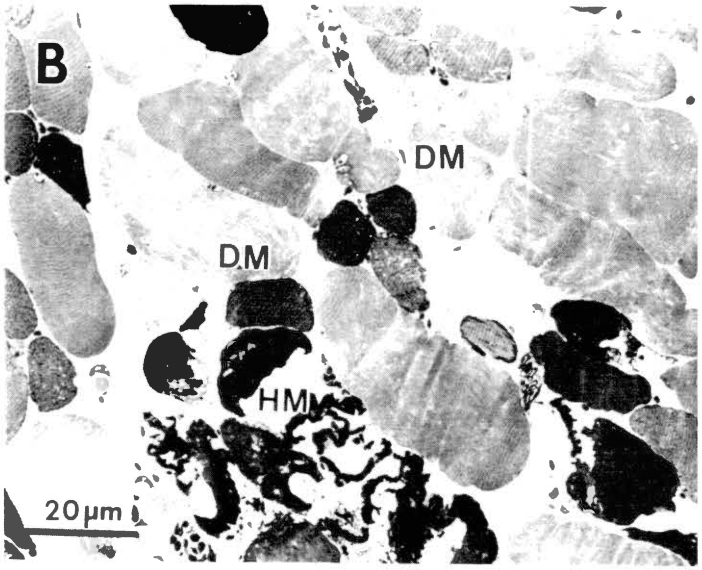
Myonecrosis Induced by ACL Myotoxin

Light Microscopic Examination. ACL myotoxin produced considerable myonecrosis of mouse skeletal muscle cells. The myonecrosis had a rapid onset with damaged muscle cells present as early as 5 minutes after injection of the toxin. Muscle spindles and nerves appeared normal although many small blood vessels were congested with erythrocytes. Skeletal muscle taken from mice injected with phosphate-buffered saline or tris buffer showed no pathological changes.

At the light microscopic level, three types of pathological changes, designated types I-III, could be observed in skeletal muscle cells damaged by ACL myotoxin (Figure 6). The types of pathological change were as follows; type I which consists of clear vacuoles (Figure 6A), type II which consisted of disorganized myofibrils, referred to as a "mottled" appearance (Figure 6B), and type III which consisted of clumped myofilaments (Figure 6A). Although in cross-sections muscle cells appeared to show only one of the three types of pathological changes, when viewed in a longitudinal plane one muscle cell often had regions of cytoplasm showing one, two, or all three of these types of changes. This suggests that these types of

Figure 6. Light micrographs of mouse skeletal muscle taken 5 minutes after injection of ACL myotoxin.
A. Note normal muscle cells (M), muscle cells with clear vacuoles (V) and muscle cells with hypercontracted myofilaments (HM).
B. Note muscle cells with disorganized myofibrils (DM) and muscle cells with hypercontracted myofilaments (HM).


A
V
HM
HM
20 µm
M


B
DM
DM
HM
20 µm

pathological changes may correspond to different stages of degeneration, which can occur in different regions of one cell simultaneously.

Electron Microscopic Examination. These three types of pathological changes could also be observed at the electron microscopic level. Type I pathological change, in which damaged muscle cells appeared vacuolated at the light microscopic level, was characterized by swollen sarcoplasmic reticulum at the electron microscopic level (figure 7a). The swollen sarcoplasmic reticulum ranged from a slight dilation of the cisternae to large dilitations that formed vacuoles which extended for several sarcomere lengths (figure 7b). The swollen sarcoplasmic reticulum cisternae often appeared to be fragmenting into small vesicles. Mitochondria in these regions of damage also appeared swollen and contained dilated intercristal spaces. Transverse tubules in these regions of damage appeared normal.

Type II pathologic change ("mottled") consists of regions of damaged muscle cells that contained disorganized myofibrils. In regions of muscle cells showing this pattern of necrosis the cytoplasm between myofibrils was greatly expanded so that adjacent myofibrils were no longer in contact (figure 7c). In some areas, the myofibrils appeared to be splitting (figure 7d) whereas other myofibrils were broken which gave the appearance that sections of 4-5 sarcomeres in length were missing from the myofibrils (figure 8a). Z disks of the disorganized myofibrils had normal fine structure and did not appear to be severely affected by the damage.

Type III pathological change consists of a hypercontraction of myofilaments in the cells which correspond to areas of clumped myofilaments with the light microscope. Early changes associated with this type of myonecrosis included a contraction of myofilaments which produced a shortening of sarcomeres (figure 8b). The myofibrils in these areas were usually intact and remained properly oriented with each other. In some areas, the

Figure 7. Electron micrographs of mouse skeletal muscle taken 5 minutes after injection of ACL myotoxin.
A. Note sarcomeres (S), swollen sarcoplasmic reticulum (SR), and mitochondria (Mi) with dilitated intercristal spaces.
B. Note large dilitations of sarcoplasmic reticulum, T tubules (T), and vesicles of fragmented cisternae membranes (arrowheads).
C. Area of disorganized myofibrils (Mf). The cytoplasm between myofibrils was greatly expanded so that adjacent myofibrils were no longer in contact.
D. Higher magnification of disorganized myofibrils. Note areas where myofibrils appear to be breaking apart (arrows); Mi = mitochondria.

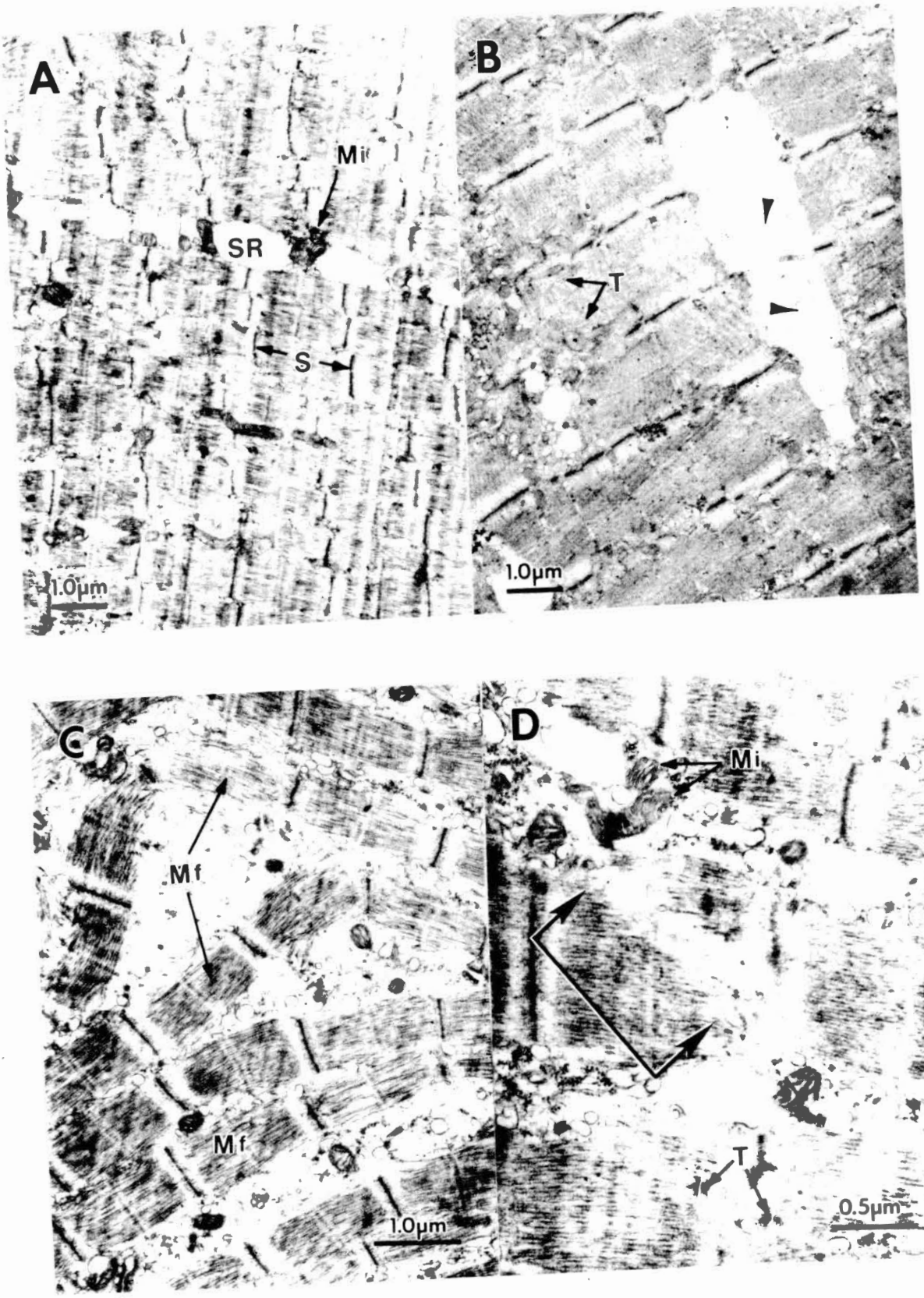

A
Mi
SR
S
1.0μm
B
T
1.0μm
C
Mf
Mf
1.0μm
D
Mi
T
0.5μm

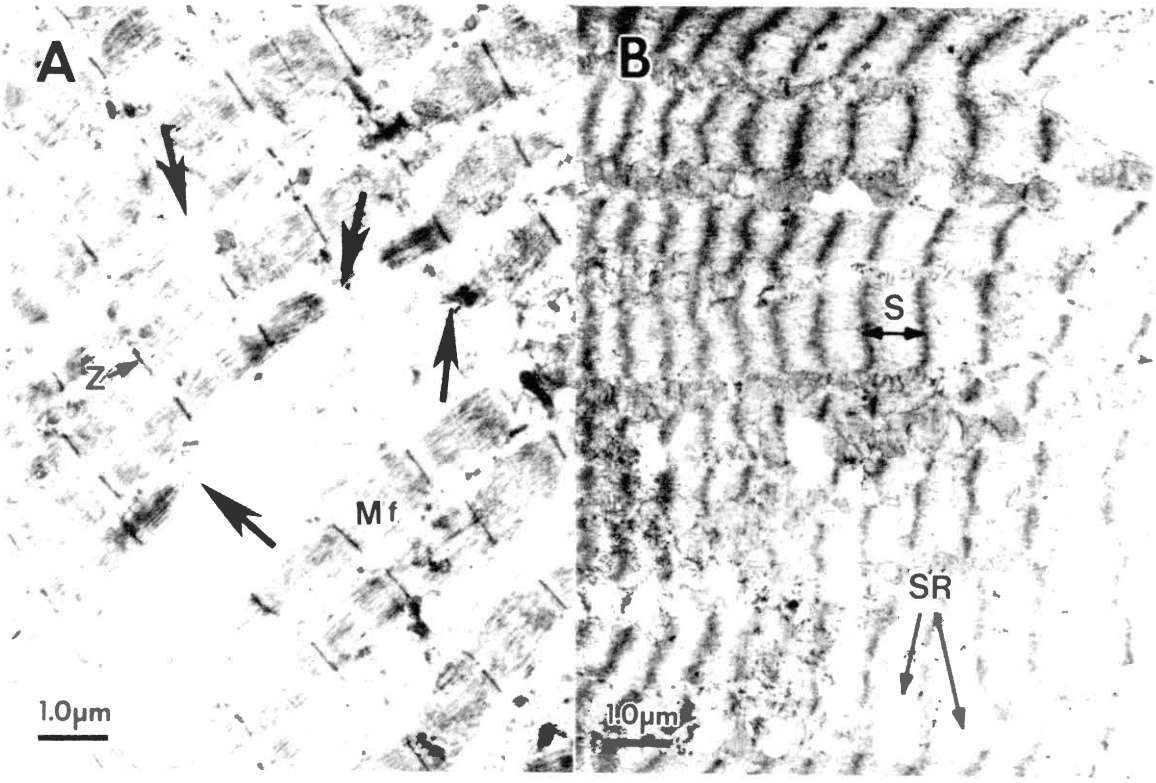
Figure 8. Electron micrographs of mouse skeletal muscle taken at various time periods after injection of ACL myotoxin.

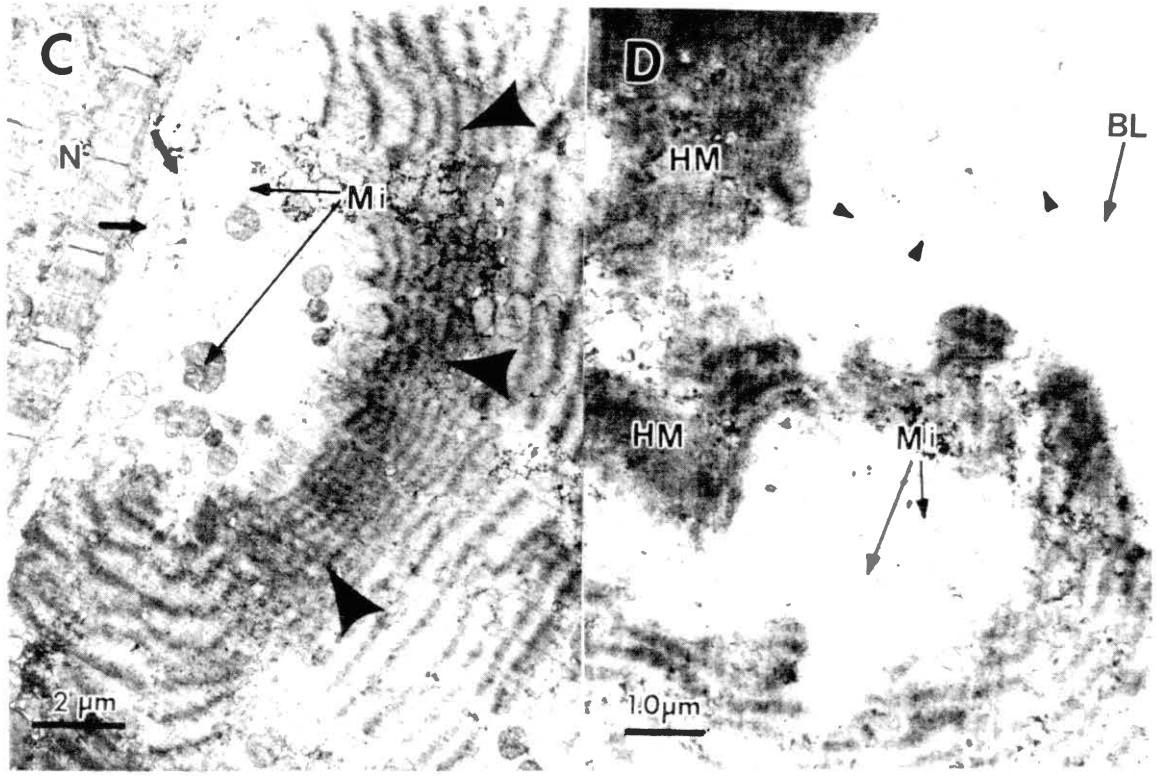
A. 5 minutes. Areas of disorganized myofibrils. Note breakage of myofibril (arrows); Mf = myofibrils; Z = Z disk.

B. 5 minutes. Areas of damaged muscle cell with overcontracted myofilaments. Note shortening of sarcomeres (S) and dilitated sarcoplasmic reticulum (SR).

C. 5 minutes. Note focal disruption of plasma membrane (arrows). Myofilaments beneath damaged membrane are overcontracted (arrowheads); Mi = mitochondria, N = portion of normal muscle cell.

D. 15 minutes. Note area of damaged muscle cell with hypercontracted myofilaments (HM), swollen mitochondria (Mi), and sarcoplasmic reticulum vesicles (arrowheads). Plasma membrane is absent although the basal lamina is present (BL).


A
Z
Mf
1.0μm
B
S
SR
1.0μm


C
N
Mi
2 μm
D
HM
BL
HM
Mi
1.0μm

myofilaments were beginning to hypercontract (figure 8c) which resulted in dense contraction bands. Other areas of muscle cells with this pattern of necrosis contained highly contorted masses of hypercontracted myofilaments that were contracted to the extent that individual sarcomeres could not be identified (figure 8d). This resulted in alternating areas in the cytoplasm of very dense clumps of myofilaments adjacent to areas that contained flocculent material and were devoid of contractile proteins. Numerous cytoplasmic organelles such as swollen mitochondria, sarcoplasmic reticulum vesicles, and pyknotic nuclei were located in the areas devoid of myofilaments.

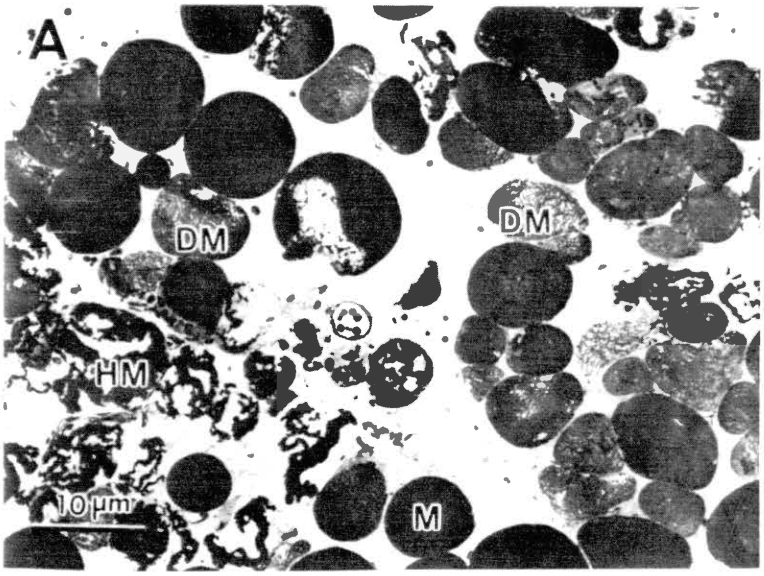
Myonecrosis Induced by Crude

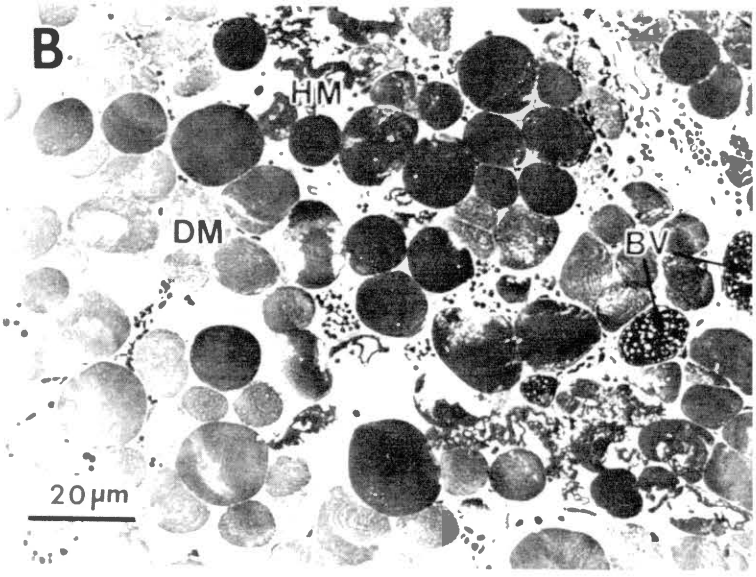
A. c. laticinctus Venom

The pathogenesis of myonecrosis induced by crude *A. c. laticinctus* venom at early time intervals was virtually identical to that induced by ACL myotoxin. Light microscopic examination revealed regions of damaged muscle cells showing the same three types of pathological change as described for ACL myotoxin. Figure 9a is typical of the myonecrosis induced by *A. c. laticinctus* venom. Similar to the purified myotoxin, a single damaged muscle cell often contained regions of cytoplasm showing more than one of the three types of pathological change. At early time periods (5 and 15 minutes), morphological changes at the electron microscopic level induced by the crude venom were indistinguishable from those produced by ACL myotoxin.

At later time periods (6-12 hours), crude *A. c. laticinctus* venom induced an additional type of necrosis which consisted of disorganized myofibrils that were similar in appearance to the muscle cells seen at earlier time periods (figure 9b). However, this type of pathological change appears to be different from that induced by ACL myotoxin since electron microscopy

Figure 9. Light micrographs of mouse skeletal muscle taken at various time periods after injection of *A. c. laticinctus* venom.
A. 30 minutes. Note muscle cells with disorganized myofibrils (DM) and muscle cells with hypercontracted myofilaments (HM); N = normal muscle cells.
B. 12 hours. Note muscle cells with hypercontracted myofilaments (HM) and muscle cells with disorganized myofibrils (DM) similar to cells seen at earlier time periods; BV = blood vessels congested with erythrocytes.


A
DM
DM
HM
10 µm
M


B
HM
DM
BV
20 µm

revealed that mitochondria in these cells often contained flocculent densities. Mitochondria with flocculent densities were not observed in muscle cells with disorganized myofibrils from earlier times periods. This type of pathological change was usually present in areas of hemorrhage and could be due to hypoxia caused by the destruction of the microvasculature by hemorrhagic toxins in the venom (Gleason et al., 1983).

In Vitro Skeletal Muscle Organ Preparation

In order to investigate the role of different ions in producing the pathological changes induced by ACL myotoxin, an *in vitro* skeletal muscle organ preparation was developed. Small pieces of skeletal muscle were cut and then incubated in tris buffer (to control pH) containing various ions to allow diffusion of these solutes from the medium into the cells. Muscle incubated in trypan blue alone for 20-30 minutes showed that the plasma membrane of some muscle cells had been cut (Figure 10a). The dye entered through the ends of the cells which indicates that the permeability of the plasma membrane was eliminated in these regions. However, the plasma membrane along the rest of the length of the muscle cells appeared intact.

As a control for the experiments in which ions were added, muscle was first incubated in 10 mM tris buffer alone. Morphological changes were minimal although two types of pathological changes could be identified. These included 1) swollen muscle cells and 2) muscle cells that contained small, clear vacuoles (Figure 10b). The clear vacuoles were usually present in small muscle cells. In addition, the distance between muscle cells was increased suggesting an influx of water into the endomysium.

Muscle was incubated in buffer containing different concentrations of NaCl. Four

types of pathological changes were produced by incubation in 300 mM NaCl. These included 1) swollen muscle cells, 2) muscle cells that contained clear vacuoles, 3) muscle cells that contained disorganized myofibrils (Figure 10c), and 4) muscle cells that contained densely clumped myofibrils (Figure 10c). Muscle cells incubated in 150 mM NaCl produced the same four types of pathological changes although there were differences in the relative numbers of cells showing each change compared to 300 mM NaCl treatments. At 150 mM NaCl cells that were swollen and contained disorganized myofibrils (Figure 10d) were predominant whereas cells with densely clumped myofibrils were predominant in the 300 mM NaCl treatments.

When muscle cells were incubated in 10 mM $CaCl_2$, the predominant type of pathological change consisted of an area of disorganized myofibrils which was often restricted to the center of cells when viewed in cross-section (Figure 11a). In addition, there were fewer muscle cells showing any of the pathological changes when compared to other ions. At a concentration of 50 mM $CaCl_2$, the pathological changes were similar to those produced by 10 mM $CaCl_2$ (data not shown). However, at 600 mM $CaCl_2$ numerous muscle cells contained uniformly disorganized myofibrils that were not restricted to the center of the cells (Figure 11b). Other muscle cells contained dense bands (Figure 11b) of myofibrils that were not as densely clumped as in some muscle cells incubated in 300 mM NaCl.

Two types of pathological changes were produced by incubation in 150 mM KCl (Figure 11c). These included 1) swollen muscle cells that contained disorganized myofibrils and 2) muscle cells that contained large clear vacuoles. The clear vacuoles produced by 150 mM KCl appeared to be larger and fewer in number that the clear vacuoles produced by various concentrations of NaCl. There were no muscle cells with hypercontracted myofilaments in the 150 mM KCl treatments.

Figure 10. Light micrographs of mouse skeletal muscle taken from an *in vitro* preparation after incubation in various solutions.
A. Trypan blue. Note dye entering through cut ends of muscle cells indicating that the permeability barrier of the plasma membrane has been eliminated.
B. 10 mM Tris buffer. Many of the muscle cells are swollen although some small muscle cells also have clear vacuoles (V).
C. 300 mM NaCl. Note muscle cells with disrorganized myofibrils (DM) and muscle cells with hypercontracted myofilaments (HM). Many of the cells with hypercontracted myofilaments also had a clear ring of cytoplasm around the edge that was devoid of contractile proteins.
D. 150 mM NaCl. Many of the muscle cells are swollen and have disorganized myofibrils (DM).

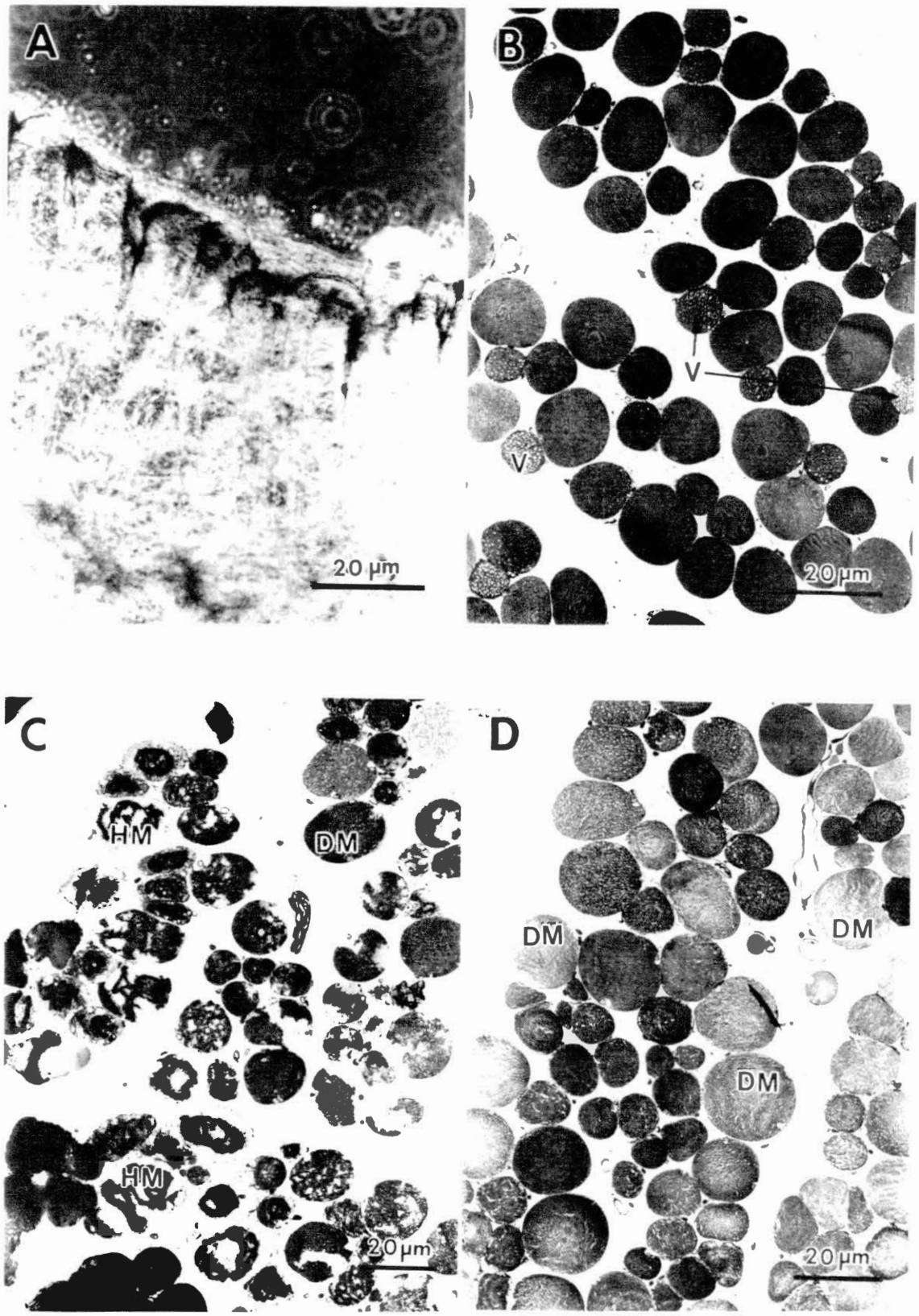
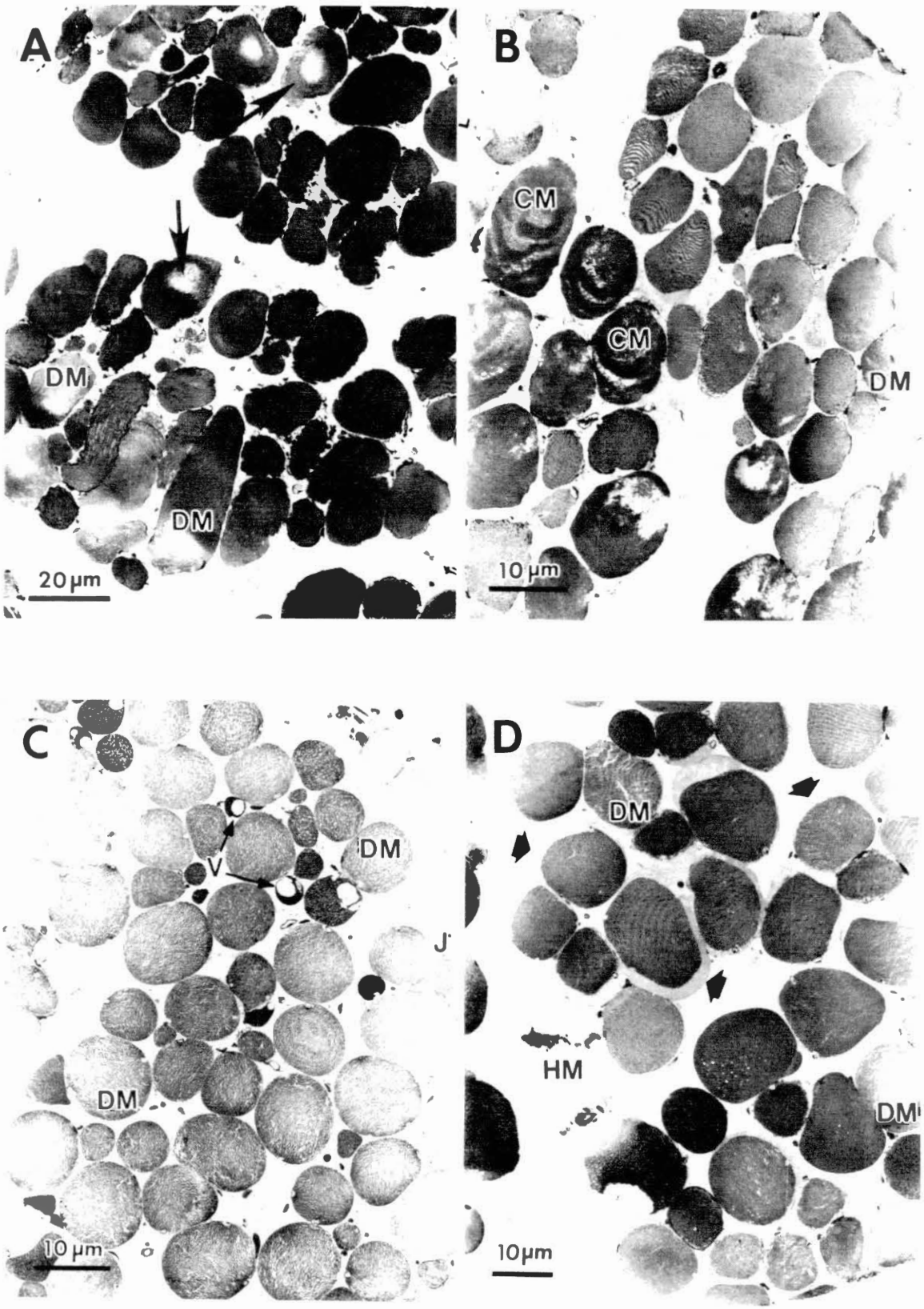

A
20 µm
B
V
V
20µm
C
HM
DM
HM
20µm
D
DM
DM
DM
20µm

Figure 11. Light micrographs of mouse skeletal muscle taken from an *in vitro* preparation incubated in solutions of various ions.
A. 10 mM $CaCl_2$. Note muscle cells with disorganized myofibrils (DM). This pathological change was often restricted to the center of muscle cells (arrows).
B. 600 mM $CaCl_2$. Note muscle cells with disrupted myofibrils (DM) and muscle cells with dense bands of contracted myofilaments (CM).
C. 150 mM KCl. Note muscle cells with disorganized myofibrils (DM) and muscle cells with large, clear vacuoles (V).
D. 300 mM LiCl. Many muscle cells had a ring of cytoplasm directly beneath the plasma membrane that was devoid of contractile proteins directly beneath the plasma membrane (arrows). Note muscle cells with disorganized myofibrils (DM) and muscle cells with hypercontracted myofilaments (HM).


A
DM
DM
20 µm
B
CM
CM
DM
10 µm
C
V
DM
DM
10 µm
D
DM
HM
DM
10µm

Five types of pathological changes were observed in muscle cells incubated in 300 mM LiCl. These included 1) swollen muscle cells, 2) muscle cells which contained clear vacuoles, 3) muscle cells that contained disorganized myofibrils (Figure 11d), 4) muscle cells that contained a ring of cytoplasm located beneath the plasma membrane which was devoid of contractile proteins (Figure 11d), and 5) muscle cells that contained hypercontracted myofibrils (Figure 11d), although these were not as prevalent as in 300 mM NaCl treatments.

Discussion

ACL myotoxin induced considerable myonecrosis of mouse skeletal muscle cells following intramuscular injection of the toxin. The myonecrosis was characterized by a hypercontraction of contractile proteins in damaged muscle cells which produced areas of densely clumped myofilaments in the cytoplasm. This type of damage was present as early as 5 minutes after injection of the toxin.

The hypercontraction of myofilaments produced by ACL myotoxin suggest that the toxin induces a change in skeletal muscle cells that results in an increase in free intracellular calcium. Calcium is involved in many cellular processes and plays an important role in muscle contraction by initiating the interaction of contractile protein in muscle cells. Skeletal muscle cells normally contain low amounts of free Ca^{2+} with concentrations that range from 0.1-0.3 μM (Thomas, 1982). The initiation of contraction requires a rise in free Ca^{2+} to approximately 1 μM whereas maximum contraction of the myofilaments has been shown to occur at a concentration of approximately 10 μM (Thomas, 1982). It is possible that sustained levels of free Ca^{2+} above this concentration could result in a contraction of myofilaments beyond normal limits thereby producing a hypercontraction. This has been proposed to explain the hypercontracted myofilaments produced by notexin, a myotoxin from

N. s. scutatus venom (Harris and MacDonell, 1981) and myotoxin I from *B. asper* venom (Gutierrez et al., 1984).

Electron microscopic studies show that focal segments of the sarcolemma of muscle cells damaged by ACL myotoxin appear disrupted at early time periods (5 and 15 minutes). These disruptions of the sarcolemma may eliminate the permeability barrier of muscle cells thereby allowing the influx of various ions. Although it has been proposed that the hypercontraction of myofilaments in muscle cells damaged by certain snake venom myotoxins may be due to extracellular Ca^{2+} entering through disrupted regions of plasma membrane, it is possible that other ions entering the cell may induce this pathological change through a release of Ca^{2+} from the sarcoplasmic reticulum which then accumulates in the cytoplasm and produces the hypercontracted myofilaments. Experiments using skeletal muscle cells *in vitro* were designed to investigate this possibility.

Skeletal muscle cells cut so that the plasma membrane permeability barrier was eliminated and then incubated in tris buffer containing 300 mM NaCl contained densely clumped myofibrils similar to muscle cells damaged by ACL myotoxin *in vivo*. Three hundred millimolar NaCl gives a solution concentration of 150 mM Na^+ which is approximately the same concentration of Na^+ (145 mM) in the extracellular fluid of animal tissues (Best and Taylor, 1985). Fewer muscle cells containing densely clumped myofibrils were observed when cut muscle cells were incubated in 150 mM NaCl compared with 300 mM NaCl. Muscle cells incubated in 150 mM KCl did not produce this change suggesting that densely clumped myofibrils are not due to Cl^- entering the cells. These results suggest that an influx of Na^+ into skeletal muscle cells initiates a change that results in hypercontracted myofilaments and that the damage is dependent on the concentration of Na^+. In addition, these results suggest that a disruption of the plasma membrane by ACL myotoxin

may be all that is required to produce hypercontracted myofilaments in muscle cells and that the myotoxin does not have directly disrupt other cellular processes to produce these pathological changes since these changes were induced by NaCl *in vitro* in the absence of myotoxin.

The exact mechanism by which a large influx of Na^+ into skeletal muscle cells may induce a release of Ca^{2+} from the sarcoplasmic reticulum leading to hypercontracted myofilaments is not precisely known. One possibility is that the influx of Na^+ produces a sustained depolarization of the muscle cell. Disruption of the sarcolemma by ACL myotoxin or by a physical cutting of the muscle cells would eliminate the permeability barrier of the cells and allow a continuous influx of ions. The depolarization of excitable membranes normally involves a transient increase in permeability of Na^+ which results in increased concentrations of this ion in the cytoplasm (Best and Taylor, 1985). Under physiological conditions, the Na^+ is rapidly pumped out of the cell by the Na^+/K^+ ATPase. Possibly, when the sarcolemma is disrupted the influx of Na^+ is greater than the ability of the cell to actively pump Na^+ out of the cell. This would result in an accumulation of Na^+ in the cytoplasm which may both depolarized the membrane and prevent its repolarization. The release of Ca^{2+} from the terminal cisternae of the sarcoplasmic reticulum of skeletal muscle cells has been proposed to be linked to a depolarization of the transverse tubule system although the exact mechanism has not been conslusively determined (Ruegg, 1988). It is possible that a sustained depolarization of muscle cells due to a continuous influx of Na^+ through disruptions of the sarcolemma may lead to a continuous release of Ca^{2+} from the sarcoplasmic reticulum. This is supported by the 300 mM LiCl treatments which also produced hypercontracted myofilaments although not to the same extent as 300 mM NaCl. The terminal cisternae of the sarcoplasmic reticulum have been shown to contain concentrations of Ca^{2+} as high as 50 mM

(Ruegg, 1988). This represents a large potential source of Ca^{2+}, which if released into the cytoplasm, may lead to hypercontracted myofilaments.

In vitro, even high concentrations of Ca^{2+} did not produce muscle cells with densely clumped myofibrils. Skeletal muscle cells incubated in tris buffer containing Ca^{2+} concentrations as high as 200 mM contained bands of contracted myofilaments that were less densely clumped compared to muscle cells damaged by ACL myotoxin *in vivo* or incubated in 300 mM NaCl. This concentration of Ca^{2+} is much higher than the physiological concentration of Ca^{2+} in the extracellular fluid which is normally about 1.2 mM (Best and Taylor, 1985). The inability of high concentrations of Ca^{2+} to produce densely clumped myofibrils in these preparations is unexpected and suggests that skeletal muscle cells are capable of binding large amounts of Ca^{2+} that enter the cytoplasm. Therefore, extracellular Ca^{2+} alone may not produce the hypercontracted myofilaments in skeletal muscle cells damaged by ACL myotoxin or other snake venom myotoxins *in vivo*.

In addition to hypercontracted myofilaments, ACL myotoxin induced swollen sarcoplasmic reticulum. The swollen sarcoplasmic reticulum suggests a large influx of water into the cytoplasm which could be due to osmotic pressure produced by an influx of Na^{+}. The sarcoplasmic reticulum plays an important role in cell volume regulation by taking up excess water entering the cell resulting in a swelling of the organelle (Trump et al., 1975). Osmotic swelling has been suggested to produce similar pathological changes in skeletal muscle cells damaged by other snake venom myotoxin. For example, swollen sarcoplasmic reticulum in muscle cells damaged by myotoxin *a*, a polypeptide myotoxin isolated from *Crotalus viridis viridis* venom, has been suggested to result from excess water entering the cells in response to an increase in intracellular Na^{+} (Ownby et al., 1976). This is further evidence that swollen sarcoplasmic reticulum induced by ACL myotoxin may result from an

influx of Na^+ and other ions through disruptions of the sarcolemma.

ACL myotoxin also caused areas of disorganized myofibrils in affected muscle cells. This change was also observed in the *in vitro* experiments with ions which are normally present in much higher concentrations in the extracellular fluid. Disrupted myofibrils could be explained by osmotic swelling of the muscle cells. The areas of the damaged cells containing disrupted myofibrils appear swollen to a greater extent than areas containing only dilated sarcoplasmic reticulum. The myofibrils in these areas were pulled apart along their long axis and many of the myofibrils appeared to be broken. Both of these changes could be explained by a large increase in volume of the cytoplasm. Such a swelling may expand the cytoplasm surrounding the myofibrils to the point where myofibrils are physically forced apart. The swelling may also produce shear forces that result in breakage of some of the myofibrils.

The hypercontracted myofilaments, swollen sarcoplasmic reticulum, and disrorganized myofibrils may all be explained by an influx of Na^+ into the muscle cell which produces osmotic swelling. These pathological changes often were present in different regions of a single damaged muscle cells which suggests a graded reponse to different concentrations of Na^+ within the cells. This further supports the hypothesis that ACL myotoxin produces focal disruptions of the sarcolemma. Sodium would enter through these focal disruptions and diffuse throughout the cytoplasm although a gradient would be established due to the size and geometric shape of skeletal muscle cells. Initially, the concentration of Na^+ would be highest near the site of disrupted sarcolemma and lowest at points away from the damage. Therefore, it is possible that areas of hypercontracted myofilaments occur first in regions with the highest levels of Na^+ that are close to the disrupted sarcolemma. The disorganized myofibrils, which were not contracted, may occur in regions of cytoplasm where the Na^+ concentrations are not

high enough to cause changes resulting in a hypercontraction, but there is enough osmotic swelling to force the myofibrils apart. According to this hypothesis, swollen sarcoplasmic reticulum would occur in regions distant to the disrupted sarcolemma where Na^+ concentrations are raised only slightly above normal values. However, enough Na^+ may eventually diffuse into the cell to cause hypercontracted myofilaments throughout the cytoplasm.

In summary, ACL myotoxin induced a rapid myonecrosis in mice characterized by a hypercontraction of myofilaments which produced densely clumped myofibrils in the muscle cells. In addition, two other types of pathological changes that included swollen sarcoplasmic reticulum and disorganized myofibrils were induced by the toxin. These three types of pathological change also were observed in cut muscle cells in an *in vitro* skeletal muscle preparation which suggests that these changes may be produced by influxes of various ions. Concentrations of 150 mM and 300 mM NaCl in tris buffer produced muscle cells with densely clumped myofibrils, whereas treatments of 300 mM KCl in tris buffer did not produce this change suggesting that densely clumped myofibrils can be caused by an influx of Na^+. In addition, 150 and 300 mM NaCl treatments as well as 300 mM KCl and various concentrations of $CaCl_2$ produced swollen cells and muscle cells with disorganized myofibrils. At the electron microscopic level, ACL myotoxin appeared to cause a disruption of the sarcolemma. Therefore, the pathological changes, including hypercontracted myofilaments, induced by ACL myotoxin *in vivo* could be due to influxes of Na^+ through these disruptions of the sarcolemma. The hypercontracted myofilaments may result from Ca^{2+} released from the sarcoplasmic reticulum in response to a sustained depolarization of the cell due to high levels of intracellular Na^+. The high levels of intracellular Na^+ could also explain the swollen sarcoplasmic reticulum and disorganized myofibrils due to osmotic swelling.

However, these results suggest that extracellular calcium, which has been proposed to produce densely clumped myofibrils in muscle cells damaged by other snake venom myotoxins (Harris and MacDonell, 1981; Gutierrez et al., 1984) may not be responsible for this pathological change since extracellular Ca^{2+} concentrations as high as 200 mM did not produce this change in cut skeletal muscle cells *in vitro*.

CHAPTER IV

ISOLATION AND INITIAL CHARACTERIZATION OF A HEMORRHAGIC TOXIN FROM THE VENOM OF *AGKISTRODON CONTORTRIX LATICINCTUS* (BROAD-BANDED COPPERHEAD)

Introduction

The venoms of many snake species are capable of inducing pathological changes of the vascular system. These changes include 1) a disruption of hemostasis (Kamiguti and Cardoso, 1989), 2) alterations of vascular permeability (Miller and Tu, 1989), and 3) the production of both local and systemic hemorrhage (Ownby, 1982). Hemorrhage induced by snake venoms is caused by specific toxins in the venoms that are capable of producing a loss of blood from the vascular system. These toxins, referred to as hemorrhagic toxins, are found in many snake venoms, however, the venoms of snakes in the subfamily Crotalinae (pit vipers) are especially rich sources of hemorrhagic toxins. Recently, many of these toxins have been purified and characterized as to their chemical and biological activities (Bjarnason and Fox, 1988).

Virtually all hemorrhagic toxins isolated from snake venoms have been shown to be metalloproteases with activities toward substrates such as the B chain of insulin, casein, and collagen (Bjarnason and Fox, 1988). These metal-dependent enzymes usually contain high amounts of zinc, often in equimolar concentrations with the toxin, which is required for

activity (Friederich and Tu, 1971). Removal of the zinc by metal chelators such as EDTA and 1,10 phenathroline has been shown to result in a complete inhibition of both hemorrhagic and proteolytic activities (Bjarnason and Tu, 1978). However, the degree of enzymatic activity of these toxins does not always directly correlate with the degree of hemorrhagic activity since in many cases the toxins that produce the most hemorrhage, as determined by minimum hemorrhagic dose, are not the most proteolytic (Bjarnason and Tu, 1978).

In the United States, copperheads (*Agkistrodon contortrix*) are responsible for a large number of the venomous snakebites. Parrish (1980) reported that in 1959 a total of 850 human bites by *A. contortrix* were reported; this represents approximately 30% of the total number of bites that year. In general, *A. contortrix* bites are not as serious as are bites by other species, although occasionally these bites result in considerable hemorrhage around the bite site. However, very little is known about the hemorrhagic toxins present in *A. contortrix* venoms. The purpose of the present investigation was to isolate and characterize a hemorrhagic toxin from the venom of *A. c. laticinctus* (broad-banded copperhead).

Materials and Methods

Venom

A. c. laticinctus venom was obtained from 15 snakes collected in central Texas, U.S.A., and maintained at the Venom Research Serpentarium, Department of Physiological Sciences, Oklahoma State University, U.S.A. Venom was extracted from the snakes approximately every two weeks during 1988-1989. The venom was extracted by allowing the snakes to bite through a parafilm covered glass funnel that emptied into a polycarbonate centrifuge tube resting in an ice bath. Immediately after extraction, the venom was centrifuged at 28,000 x *g* for 20 minutes at 4 C to remove insoluble material, frozen, and

lyophilized. Lyophilized venom was stored over dessicant at -20 C until needed.

Isolation of Hemorrhagic Toxin

Gel Filtration Chromatography. Five hundred milligrams of *A. c. laticinctus* venom was reconstituted in 5.0 ml of 10 mM Tris buffer (pH 8.6) containing 100 mM NaCl, filtered using a Millipore 0.45 um filter, and applied to a Sephacryl S-200 HR column (2.6 cm x 100 cm) at a flow rate of 20 ml/hr. Fractions were eluted from the column at 4 C for 38 hours. A total of 150 tubes were collected with each tube containing 5.3 ml of buffer. Absorbance was monitored at 280 nm. Every fifth tube was tested for hemorrhagic activity by giving s.c. injections of 50 μl of each solution between the scapulas of mice. The mice were killed by cervical dislocation after 2 hours and the skins removed and examined for the presence of hemorrhage. The tubes showing hemorrhagic activity were combined as peaks, concentrated to about 10 ml using an Amicon concentrating cell with a YM 2 membrane (molecular weight cutoff = 2,000), and lyophilized. The hemorrhagic fraction of interest was then further purified using high performance liquid chromatography.

Anion Exchange Chromatography. The hemorrhagic fraction from the Sephacryl S-200 HR column was reconstituted in 9.0 ml of 10 mM Tris buffer (pH 8.6), filtered using a Millipore 0.2 um FG filter, and applied to a Waters DEAE-5PW HPLC anion exchange column (21.5 mm x 15 cm) at a flow rate of 3.6 ml/minute. The fractions were eluted by first washing the column with the same buffer for 20 minutes after which time a 0.0-0.4 M linear NaCl gradient in the same buffer was applied over 50 minutes. Absorbance was monitored at 280 nm. The fractions were collected as peaks in tubes resting in a refrigerated test tube rack (4 C) and concentrated to about 10 ml using a Millipore Minitan System with a 1000 MW exclusion membrane. The fractions were tested for hemorrhagic activity as

previously described, and the fraction of interest was lyophilized. The hemorrhagic fraction was further purified by HPLC gel filtration chromatography.

HPLC Gel Filtration Chromatography. The hemorrhagic fraction from the DEAE column was reconstituted in 10 mM MOPS buffer (pH 6.5) containing 100 mM NaCl and applied to a Waters Protein Pak 125 gel filtration column (7.5 mm x 30 cm). Fractions were eluted from the column at a flow rate of 0.5 ml/min using the same buffer. Total elution time was 60 minutes. Absorbance was monitored at 280 nm and the fractions were collected as peaks, tested for hemorrhagic activity, and frozen at -20 C.

Electrophoresis

The homogeneity and molecular weight of the hemorrhagic toxin were determined by SDS-PAGE using a Pharmacia Phastsystem mini-electrophoresis unit and 8-25% gradient gels. Twenty-five micrograms of the hemorrhagic toxin were reconstituted in 25 μl of sample buffer containing 10 mM Tris-HCl (pH 8.0), 2.5% sodium dodecyl sulfate, and 5.0% β-mercaptoethanol, and heated at 100 C for 3 minutes. Four micrograms of the hemorrhagic toxin were applied to the gel, and electrophoresis was carried out for 100-volt hours following the procedure described in the Phastsystem Technical Bulletin No. 110. Upon completion of the electrophoresis, the gels were stained using a Pharmacia Phastsilver staining kit. Molecular weight markers were as follows: rabbit muscle phosphorlyase B (97,400), bovine serum albumin (66,200), hen egg white ovalalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400).

Isoelectric focusing electrophoresis of the purified hemorrhagic toxin was performed using the Pharmacia Phastsystem and Pharmacia Phastgels with a pH gradient from pH 3.0-9.0 following the procedure described in the Pharmacia Technical Bulletin No. 100. Gels

were stained using a Pharmacia Phastsilver staining kit. Bio Rad IEF standards (pI 4.65-9.60) were used for determination of the isoelectric point of the hemorrhagic toxin.

Minimum Hemorrhagic Dose

The minimum hemorrhagic dose of the hemorrhagic toxin and crude venom was determined in female mice (CD-1, Charles River) weighing 25-30 grams, using a modification of the procedure described by Kondo et al. (1960). The hemorrhagic toxin in 10 mM MOPS buffer (pH 6.5) containing 100 mM NaCl was injected intradermally into the back of five mice in doses that ranged from 0.75-12.0 μg/mouse. Crude venom in phosphate-buffered saline (pH 7.4) was injected similarly in doses that ranged from 1.0-15.0 μg/mouse. Injection volumes were adjusted so that a 25 gram mouse would receive 50 μl of the toxin solution containing the final desired dose. The mice were killed by cervical dislocation after 2 hours, and the skins carefully removed and placed between two glass plates. The hemorrhagic lesions produced on the underside of the skins were measured across two diameters; the widest diameter and the diameter perpendicular to the first measurement and the mean of these two measurements calculated. The minimum hemorrhagic dose is defined as the lowest amount of toxin or venom required to produce a lesion with a mean diameter of 10 mm within 2 hours.

Protease Assay

Protease activity of the hemorrhagic toxin was assayed using N,N-dimethylcasein (Sigma Chemical Co, Inc) as substrate following the method of Lin et al. (1969). Twenty microliters of toxin solution (0.244 mg/ml) was added to 1.0 ml N,N-dimethycasein (0.1% in 5 mM MOPS buffer, pH 7.2) and incubated in a water bath at 38 C for 60 minutes. The

reaction was then stopped by immersing the test tubes in boiling water for 5 minutes. One milliliter of 4% sodium bicarbonate (pH 8.5) and 1.0 ml if 0.1% trinitrobenzenesulfonic acid were added and the solution incubated in a water bath at 50 C for 30 minutes. The reaction was stopped by the addition of 0.5 ml of 1 N HCl and 1.0 ml of 10% SDS and the absorbance determined at 340 nm. The protease activity was expressed as the molar concentration of amino terminals and was determined by dividing the absorbance at 340 nm by 1.0×10^4, the extinction coefficient of most trinitrophenyl alpha amino acids. One unit of activity is defined as μmoles amino terminals/minute/mg toxin. Inhibition of proteolytic activity was investigated by the addition of 5 mM EDTA to the 5 mM MOPS buffer containing 0.1% dimethylcasein. The remainder of the assay was carried out as described above.

Digestion of Fibrinogen

Fibrinogenolytic activity was determined using the method of Ouyang and Teng (1976). Ten micrograms of the hemorrhagic toxin were added to 1.0 ml of a 2.0 mg/ml aqueous solution of bovine fibrinogen and incubated at 37 C. The presence or absence of an insoluble fibrin clot was recorded. At various time intervals (5, 15, and 30 minutes; 1 and 4 hours) after addition of toxin, 50 μl of the solution was withdrawn and added to an equal volume of buffer containing 10 mM Tris-HCl (pH 8.0), 2.5% sodium dodecyl sulfate (SDS), and 5% β-mercaptoethanol. The samples were heated at 100 C for 3 minutes and 3 μl of the solution applied to SDS-PAGE gels as described above.

Defibrinating Activity

Crude venom was reconstituted in PBS (pH 7.4) and injected i.v. into female mice

(CD-1, Charles River) weighing 20-25 grams at four doses of 0.5, 1.0, 2.5, and 5.0 μg/g. The purified hemorrhagic toxin in 10 mM MOPS buffer (pH 6.5) was injected i.v. into mice at doses of 0.5 μg/g and 1.0 μg/g. The mice were bled from the infraorbital sinus after 1 hour using a 20 μl non-heparized glass microcapillary tube. The blood was allowed to stand for 1 hour at room temperature after which time the tube was broken and checked for the presence of clotted blood. Defibrinating activity was determined based on the presence of non-clotted blood in the tube.

Histological Examination

Hemorrhage induced by ACL hemorrhagic toxin I was studied using the method of Ownby et al. 1978). Fifty microliters of ACL hemorrhagic toxin in 10 mM MOPS buffer (pH 6.5) containing 100 mM NaCl were injected into the right thigh of female mice (CD-1; Charles River) weighing 25-30 grams. Tissue samples were taken at 30 minutes and 1 hour and fixed for 2 hours in 2.0% glutaraldehyde in 0.16 M cacodylate buffer (pH 7.4). Tissue was washed three times in 0.08 M cacodylate buffer containing 0.18 M sucrose and post-fixed for 1 hour in 1% aqueous osmium tetroxide. The tissue was dehydrated in ascending concentrations of acetone and embedded in Polybed 812 resin. Thick sections (1 μm) for light microscopic examination were cut with a Sorvall MT 5000 ultamicrotome and stained with Mallory's toludine blue-azure II dye.

Results

Isolation of Hemorrhagic Toxin.

The hemorrhagic toxin was isolated from the crude venom using liquid chromatography and a three column procedure. Fractionation of *A. c. laticinctus* venom by

Sephacryl S-200 HR gel filtration resulted in a total of ten fractions, designated A1-A10 (Figure 12). Hemorrhagic activity was present in fractions A4, A5, and A6 from this column. The hemorrhagic fraction, A6, contained a considerable amount of protein based on the absorbance at 280 nm and was chosen for further purification by HPLC. Figure 13 shows the elution profile of the separation of fraction A6 by a DEAE-anion exchange column. A total of 12 fractions, designated B1-B12, eluted from this column, with the hemorrhagic activity present in two fractions (B7 and B12) that eluted at a NaCl concentration of about 200 mM and 300 mM, respectively. Fraction B12 was then further purified using an HPLC gel filtration column. Figure 14 shows the elution profile of this column. One symmetrical peak with hemorrhagic activity was eluted from the column. The fraction was shown to be homogeneous by SDS-PAGE and is herein referred to as ACL hemorrhagic toxin I. At least four additional hemorrhagic toxins were identified in *A. c. laticinctus* venom but were not purified to homogeneity.

ACL hemorrhagic toxin I had a molecular weight of 29,000 as determined by SDS-PAGE (Figure 15). Figure 16 shows the results of isoelectric focusing electrophoresis of the hemorrhagic toxin. A total of 8-10 bands were present in the gel with isoelectric points that ranged from about 4.70-6.40 suggesting that ACL hemorrhagic toxin I exists as several isozymes.

Protease Activity

ACL hemorrhagic toxin I demonstrated proteolytic activity towards the substrate N,N-dimethylcasein. The proteolytic activity of the toxin, however, was less than that of the crude venom. The purified hemorrhagic toxin had a proteolytic activity of 817 units under experimental conditions used in this experiment whereas the crude venom had a proteolytic

Figure 12. Fractionation of *A. c. laticinctus* venom on a Sephacryl S-200 HR gel filtration column (2.5 cm x 100 cm). Venom was dissolved in 10 mM Tris buffer (pH 8.6) and applied to the column at a flow rate of 20.0 ml/hour. Hemorrhagic activity was in fractions A4, A5, and A6. Fraction A6 was chosen for further purification using a Waters DEAE-5PW HPLC anion exchange column.

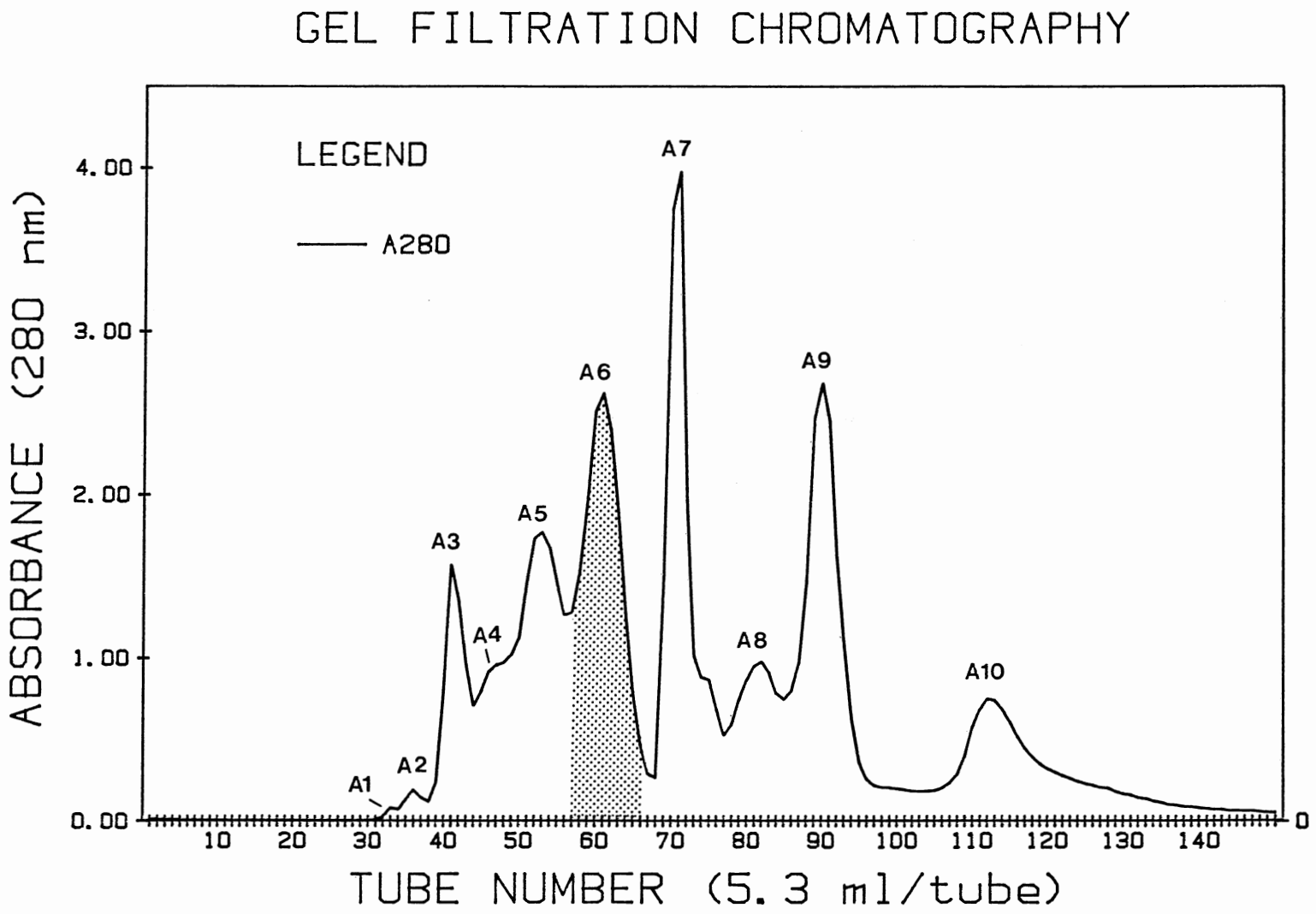

GEL FILTRATION CHROMATOGRAPHY
LEGEND
A280
ABSORBANCE (280 nm)
4.00
3.00
2.00
1.00
0.00
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
10
20
30
40
50
60
70
80
90
100
110
120
130
140
0
TUBE NUMBER (5.3 ml/tube)

Figure 14. Fractionation of fraction B12 from the DEAE column on a Waters Protein Pak 125 HPLC gel filtration column (7.5 mm x 30 cm). Fraction B12 in 10 mM MOPS buffer (pH 6.5) was applied at a flow rate of 0.5 ml/minute and eluted with the same buffer. Total elution time was 60 minutes.

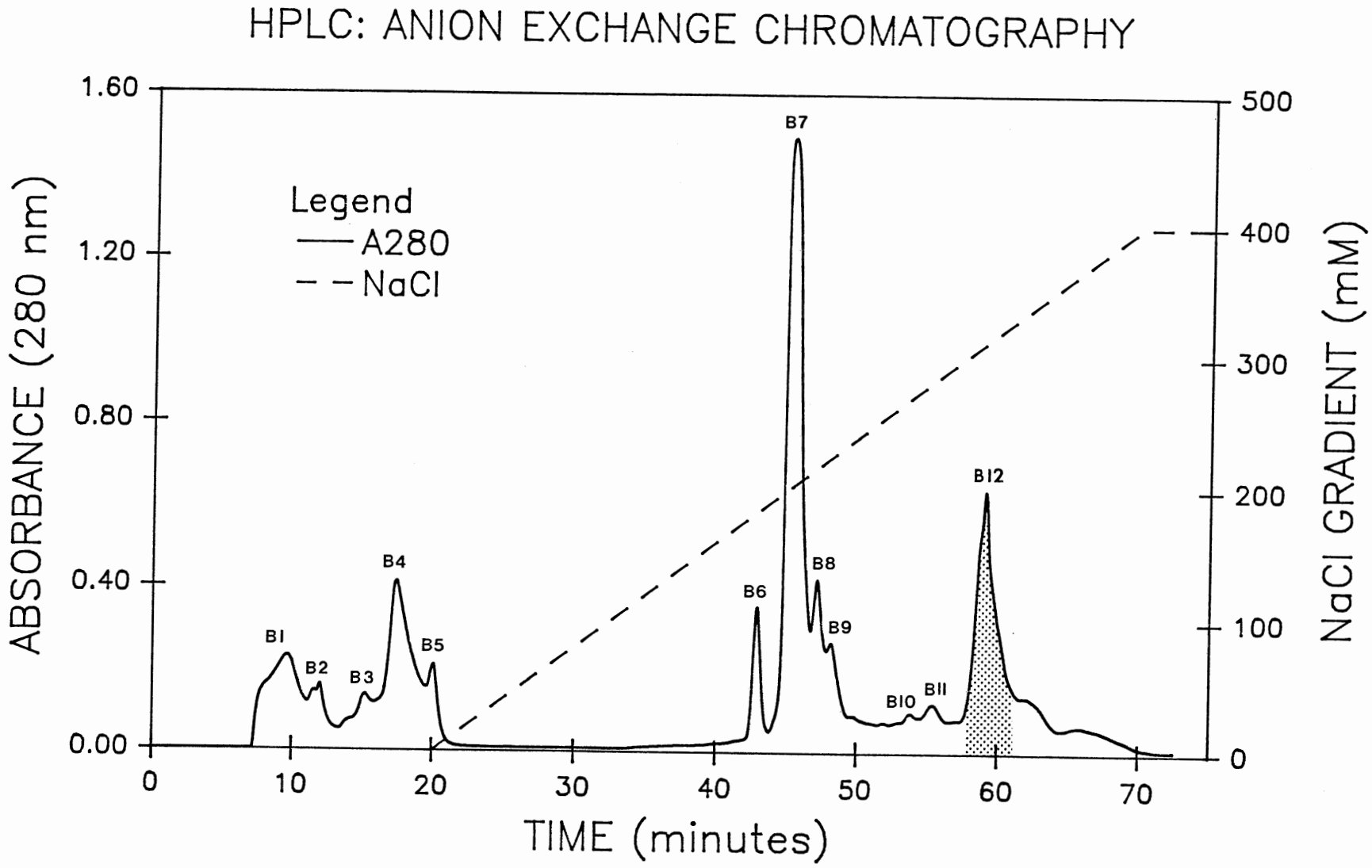

HPLC: ANION EXCHANGE CHROMATOGRAPHY
Legend
A280
NaCl
ABSORBANCE (280 nm)
1.60
1.20
0.80
0.40
0.00
NaCl GRADIENT (mM)
500
400
300
200
100
0
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
0
10
20
30
40
50
60
70
TIME (minutes)

Figure 13. Fractionation of fraction A6 from the Sephacryl S-200 column on a DEAE-5PW HPLC anion exchange column (21.5 mm x 15 cm). Fractions were eluted at flow rate of 3.6 ml/min with an isocratic gradient of 10 mM Tris buffer (pH 8.6) for 20 minutes after which time a 0.0-0.4 M NaCl gradient was applied over 50 minutes. Hemorrhagic activity was in fractions B7 and B12. Fraction B12 was further purified on a Waters Protein Pak 125 HPLC gel filtration column.

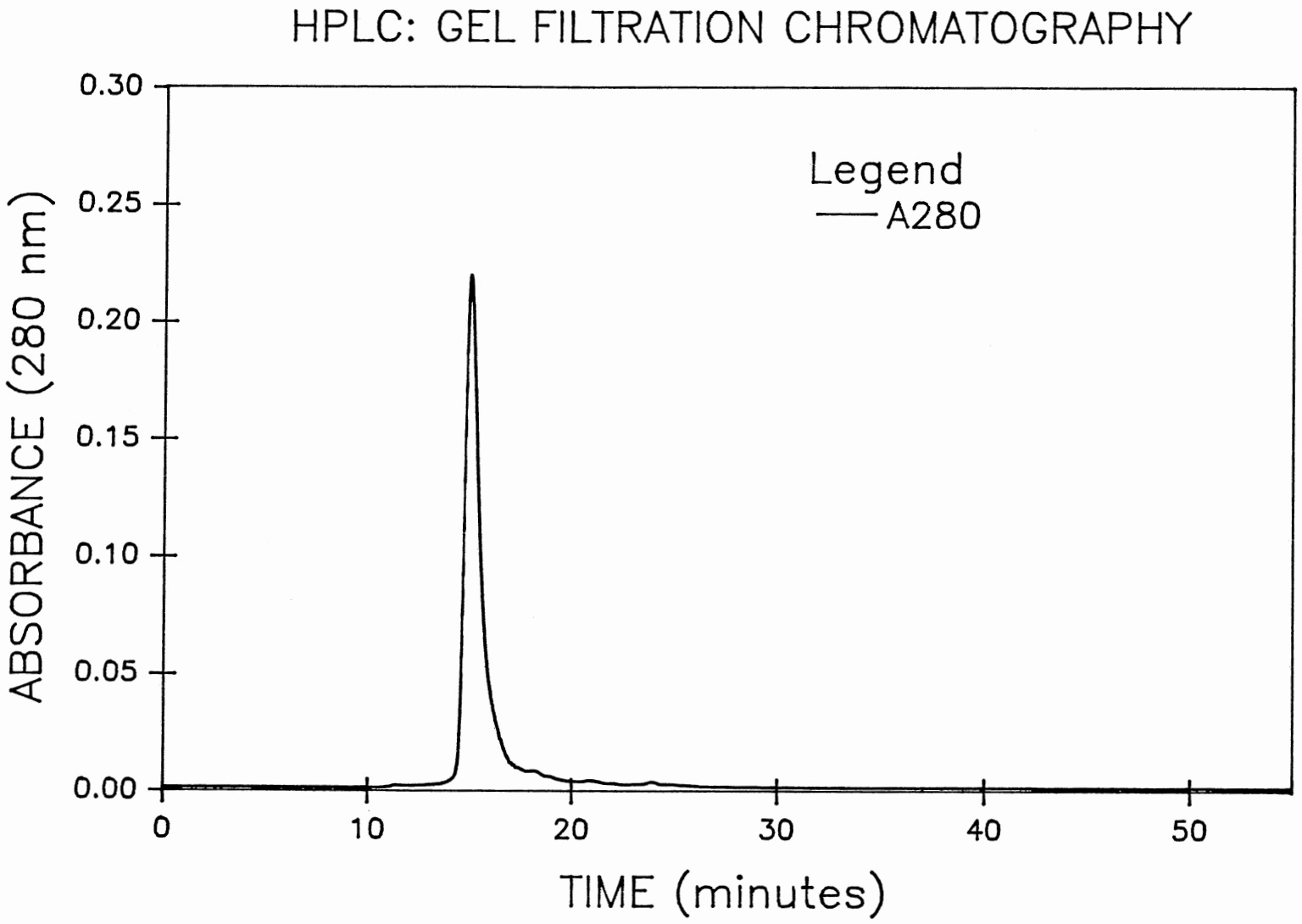

HPLC: GEL FILTRATION CHROMATOGRAPHY
Legend
A280
ABSORBANCE (280 nm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
TIME (minutes)

Figure 15. SDS-Polyacrylamide gel electrophoresis of ACL hemorrhagic toxin I. Lane 1: molecular weight standards, Lane 2; ACL hemorrhagic toxin I (3 μg).

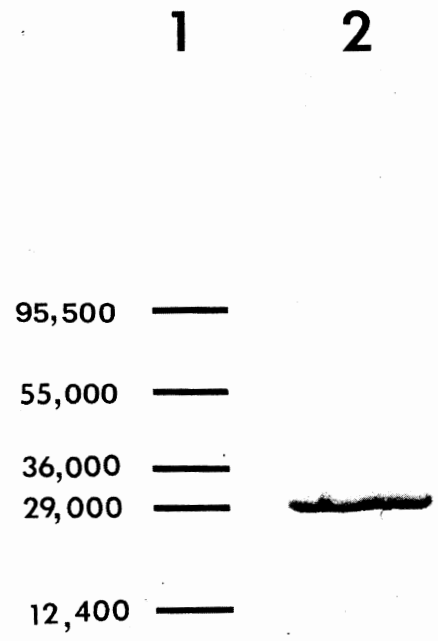
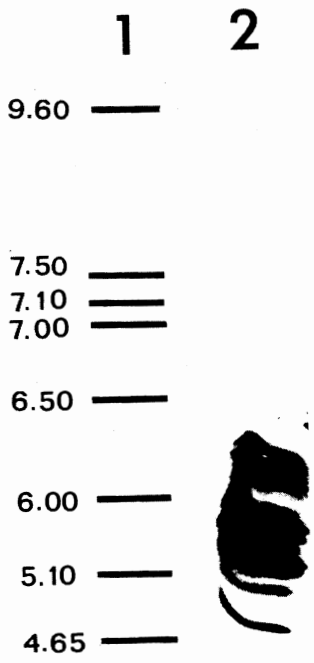

1
2
95,500
55,000
36,000
29,000
12,400

Figure 16. Isoelectric focusing electrophoresis of ACL hemorrhagic toxin I using Pharmacia Phastgels with a pH gradient from pH 3.0 to pH 9.0. Lane 1: IEF standards, Lane 2: ACL hemorrhagic toxin I (4 μg).


1
2
9.60
7.50
7.10
7.00
6.50
6.00
5.10
4.65

activity of 1,400 units. The proteolytic and hemorrhagic activity of ACL hemorrhagic toxin I were stable to freezing as the toxin was still active after being frozen one year at -20 C. Addition of 5 mM EDTA to ACL hemorrhagic toxin I resulted in a complete inhibition of enzymatic activity.

Minimum Hemorrhagic Dose

Minimum hemorrhagic dose (MHD) was used as a measure of hemorrhagic activity of the toxin and crude venom; the lower the MHD, the greater the hemorrhagic activity of the toxin or venom. The MHD of ACL hemorrhagic toxin I was 8.5 μg/mouse whereas the MHD of crude *A. c. laticinctus* venom was 6.7 μg/mouse.

Digestion of Fibrinogen

Fibrinogen reduced with β-mercaptoethanol was separated in Aα, Bβ, and gamma chains by SDS-PAGE (figure 17). When fibrinogen was incubated with ACL hemorrhagic toxin I, the Aα chain of fibrinogen was the most sensitive to hydrolysis and was totally hydrolysed within 5 minutes. The Bβ chain of fibrinogen was also hydrolyzed by ACL hemorrhagic toxin, although prolonged incubations (4 hours) were required before it was completely digested. The gamma chain of fibrinogen was resistant to hydrolysis and remained unchanged at incubation times up to 4 hours. Digestion of fibrinogen by ACL hemorrhagic toxin I resulted in several degradation products with molecular weights that ranged from 49,000 to 25,000, although two fragments with molecular weights of about 49,000 and 37,000 appear to be major products of digestion.

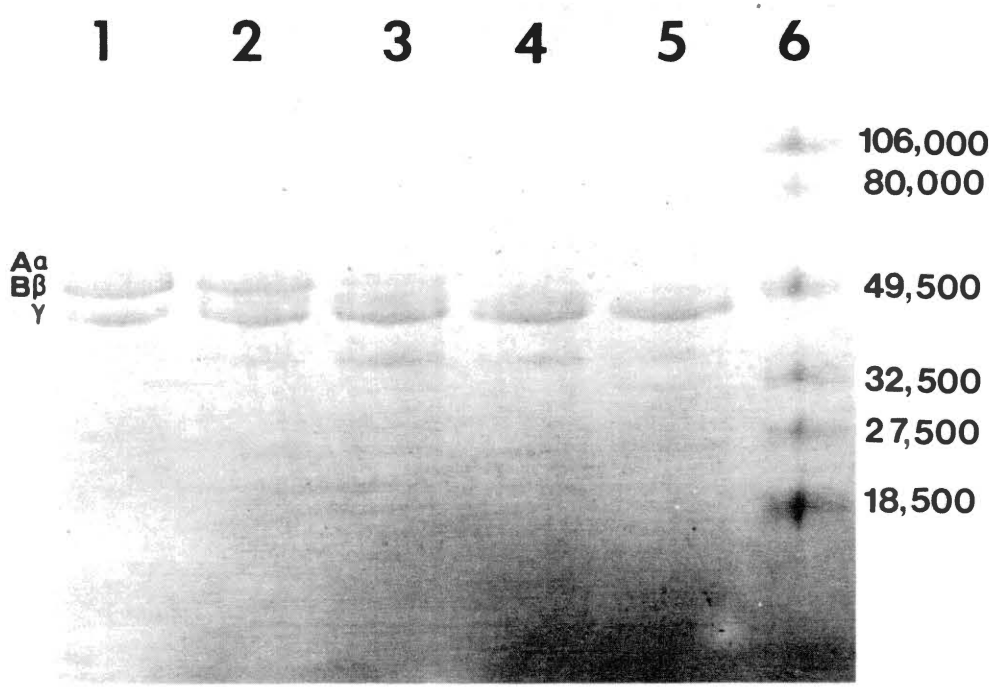

1
2
3
4
5
6
106,000
80,000
Aα
Bβ
γ
49,500
32,500
27,500
18,500

Figure 17. SDS-Polyacrylamide gel electrophoresis of fibrinogen after incubation with ACL hemorrhagic toxin I for various time periods. Lane 1: bovine fibrinogen, Lane 2: 5 minutes, Lane 3: 30 minutes, Lane 4: 1 hour, Lane 5: 4 hours, Lane 6: molecular weight standards.

Defibrinating Activity

ACL hemorrhagic toxin I did not demonstrate any defibrinating activity *in vivo* at doses up to 1.0 μg/g in mice. Crude *A. c. laticinctus* venom had two different effects on blood coagulation that were dose dependent. At a dose of 1.0 μg/g, the crude venom demonstrated defibrinating activity whereas at a higher dose (5.0 μg/g) the venom appeared to have a coagulant effect as determined by the presence of clotted blood in the microcapillary tubes.

Histological Examination

Gross examination of the tissue following i.m. injections of ACL hemorrhagic toxin I revealed considerable hemorrhage in the muscle. The hemorrhage had a rapid onset being present as early as 5 minutes after an injection. At a dose of 0.5 μg/g, the hemorrhage covered the majority of the lateral thigh 30 minutes after an i.m. injection. By 60 minutes, the hemorrhage had spread throughout most of the lateral aspect of the thigh as well as extending into many of the deep muscles.

At the light microscopic level a large number of extravasated erythrocytes were observed in the connective tissue between muscle cells (figure 18a). Many of the capillaries in the tissue appeared to be damaged and were congested with erythrocytes and platelets (figure 18b). In sections that contained part of the epimysium, a large number of extravasated erythrocytes were usually located in this region of the muscle (figure 18c).

Discussion

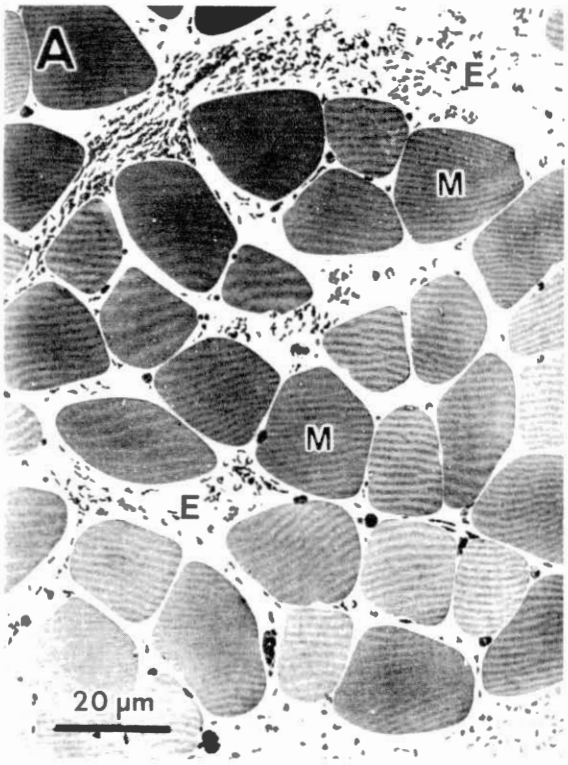
A toxin capable of inducing hemorrhage in mice was isolated from the venom of *A. c. laticinctus*. The toxin, designated ACL hemorrhagic toxin I, was isolated from the crude

Figure 18. Light micrographs of mouse skeletal muscle taken 30 minutes after injection of ACL hemorrhagic toxin I.

A. Note extravasated erythrocytes (E) in endomysium.

B. Note capillaries congested with erythrocytes and platelets (arrows)

C. Note large number of extravasated erythrocytes (E) in epimysium.


A
E
M
M
E
20 µm


B
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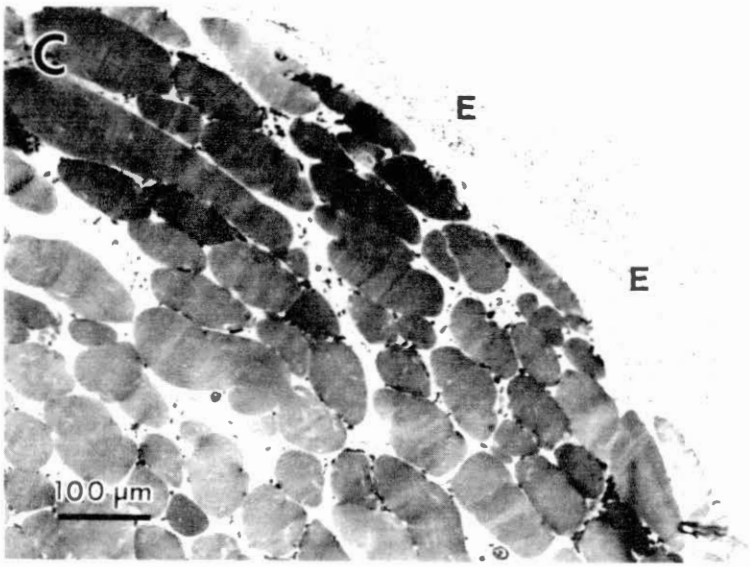
E
10 µm


C
E
E
100 µm

venom by low pressure liquid chromatography and HPLC using a three column procedure. ACL hemorrhagic toxin I was present as a single band on SDS-PAGE when stained with silver which strongly suggests it was a homogeneous protein. In addition, ACL hemorrhagic toxin I eluted as a single, symmetrical peak from a HPLC gel filtration column which is further evidence that the molecule is homogeneous. ACL hemorrhagic toxin I appears to be present in very low amounts in the venom as only 0.473 mg was recovered from 500 mg of crude venom. This represents approximately 0.1% of the total starting venom protein.

The molecular weight of ACL hemorrhagic toxin I was estimated to be 29,000 by SDS-PAGE. The size of snake venom hemorrhagic toxins varies considerably and ranges from 22,000 daltons for hemorrhagic toxins isolated from *A. acutus* venom (Xu et al., 1981) to approximately 100,000 daltons for viriditoxin, a hemorrhagic toxin isolated from *Crotalus viridis viridis* venom (Gleason et al., 1983) and LHF-1, a hemorrhagic toxin isolated from *Lachesis muta muta* venom (Sanchez et al., 1987). In general, however, snake venom hemorrhagic toxins can be grouped into three distinct classes based on molecular weight. These include a low molecular weight group which have molecular weights from 22,000-35,000, an intermediate molecular weight group with molecular weights from 45,000-65,000, and a group of high molecular weight toxins which have molecular weights between 80,000-100,000 (Ownby, 1990). ACL hemorrhagic toxin I with a molecular weight of 29,000 belongs to the group of low molecular weight snake venom hemorrhagic toxins. The majority of snake venom hemorrhagic toxins so far isolated fall into the low and intermediate molecular weight groups.

Although ACL hemorrhagic toxin I was present as a single band with SDS-PAGE, the results of isoelectric focusing suggest that the toxin exists in several isoforms with isoelectic points that range from 4.70-6.40. The presence of different charged forms of toxins in snake

venoms, including hemorrhagic toxins, has been reported. Bjarnason and Tu (1978) reported the isolation of five hemorrhagic toxins from *C. atrox* venom. Two of the toxins, hemorrhagic toxin *c* and hemorrhagic toxin *d*, had identical molecular weights yet varied slightly in isoelectric point. Since these toxins had similar proteolytic and hemorrhagic activities and the same zinc content, it was concluded that these two hemorrhagic toxins were isoenzymes. In a more complete study of these toxins, Bjarnason and Fox (1987) showed that hemorrhagic toxin *c* and hemorrhagic toxin *d* had virtually identical solution conformations and differed only by a substituion of asparatate for alanine in hemorrhagic toxin *c*. Apparently, the additional aspartate in hemorrhagic toxin *c* resulted in the slightly more acidic isoelectric point (6.1 vs 6.2) of this toxin. It is possibile is that the different isoforms of ACL hemorrhagic toxin I could result in part from substitutions of charged amino acids or from a heterogeneity in the post-translational modifications of amino acids such as the amidation of glutamic acid and/or aspartic acid. These changes would not produce large enough changes in molecular weight so that the different forms could be separated by SDS-PAGE although the variation in charged groups present on the molecules could allow their separation by the isoelectric focusing gels.

Hemorrhagic toxin *c* and hemorrhagic toxin *d* from *C. atrox* venom varied only slightly in isoelectric point. However, the isoforms of ACL hemorrhagic toxin I had large differences in isoelectric points. Silveira et al. (1989) isolated a thrombin-like enzyme from *L. m. muta* venom that existed in several isoenzyme forms with isoelectric points that ranged from 3.0-5.0. The toxin was found to be a glycoprotein that had a high content of sialic acid. Isoelectric focusing of the toxin after removal of the sialic acid by neuramidase showed a reduction in this heterogeneity, and it was concluded that the different charged forms were due in part to variations in the content of sialic acid between toxin molecules. Although in

the present investigation it was not determined if ACL hemorrhagic toxin I is a glycoprotein, this is another possibility that could explain the different isoforms.

ACL hemorrhagic toxin I demonstrated enzymatic activity towards the general protease substrate N,N,-dimethylcasein. The enzymatic activity of ACL hemorrhagic toxin I was completely inhibited by the addition of 5 mM EDTA which suggests that the toxin is a metalloprotease. In this regard ACL hemorrhagic toxin I is similar to other snake venom hemorrhagic toxins which also have been shown to be metalloenzymes, most of which require zinc for enzymatic activity (Bjarnason and Fox, 1988). The proteolytic activity of ACL hemorrhagic toxin I was less than that of the crude venom which suggests that there are additional proteases present in the venom. Fractionation of *A. c. laticinctus* venom revealed at least four additional fractions that showed enzymatic activity towards N,N,-dimethylcasein (data not shown). Since the proteolytic activity of some of these semi-pure fractions was higher than that of ACL hemorrhagic toxin I, the higher enzymatic activity of the crude venom most likely results from the sum effect of these additional proteases in the venom.

ACL hemorrhagic toxin I was able to hydrolyze bovine fibrinogen *in vitro*. The activity of ACL hemorrhagic toxin I on fibrinogen resulted in the rapid hydrolysis of the Aα chain of fibrinogen whereas incubations of up to 4 hours were required before the Bβ chain was lost. The hydrolysis of fibrinogen by ACL hemorrhagic toxin I was very similar to that produced by other snake venom hemorrhagic toxins. In a study of bilitoxin, a hemorrhagic toxin isolated from *A. bilineatus bilineatus*, Imai et al. (1989) showed that the Aα chain of fibrinogen was also the most sensitive to hydrolysis and that it was completely digested within 5 minutes after addition of the toxin. Similarly, Mori et al. (1987) reported that hemorrhagic toxins from *C. ruber ruber* venom acted to rapidly hydrolyze the Aα chain of fibrinogen. The Bβ chain of fibrinogen was digested by these hemorrhagic toxins, although prolonged

incubations (1-3 hours) were required before it was completely hydolyzed. The gamma chain of fibrinogen was not digested by either ACL hemorrhagic toxin I, bilitoxin, or hemorrhagic toxins from *C. r. ruber* venom. Although ACL hemorrhagic toxin I was able to hydrolyze bovine fibrinogen, incubation of the toxin with fibrinogen *in vitro* did not produce an insoluble fibrin clot.

Many viperid snake venoms possess defibrinating activity which is due to the disruption of blood coagulation. This can occur through several different mechanisms which include 1) the inactivation of plasma clotting factors, 2) the activation of plasma inhibitors of coagulation such as plasminogen or protein C, and 3) the consumption of fibrinogen by either thrombin-like enzymes or by fibrinogenolytic/fibrinolytic enzymes in the venom (Russell, 1980). Although ACL hemorrhagic toxin I was able to hydrolyze the Aα and Bβ chains of fibrinogen *in vitro*, it did not possess any defibrinating activity *in vivo* at doses up to 1.0 μg/g. Apparently, at this dose the toxin does not degrade sufficient quantities of fibrinogen *in vivo* to compromise the clotting of blood. Crude *A. c. laticinctus* venom did demonstrate defibrinating activity *in vivo*, however, this activity appears to be due to toxins other than ACL hemorrhagic toxin I.

The digestion of fibrinogen by ACL hemorrhagic toxin I resulted in several degradation fragments with molecular weights that ranged from 25,000-49,000. A fragment with a molecular weight of about 37,000 appears to be a major product of the digestion of fibrinogen by ACL hemorrhagic toxin I. A fragment with a similar molecular weight was also produced by the action of hemorrhagic toxin-3 from *C. r. ruber* venom on fibrinogen (Mori et al., 1989) which suggests that ACL hemorrhagic toxin I and this other hemorrhagic toxin have similar cleavage specificities. This is important because it suggests that these toxins may be chemically similar even though they were isolated from the venoms of two

different snake genera. It is possible that the hemorrhagic toxins from a large number of venomous snake species may be similar and that they have the same mechanism of action. Therefore, it may be possible to determine the mechanism of hemorrhagic action of a large number, or perhaps all, snake venom hemorrhagic toxins by studying only a small number of toxins from a few species. Once the mechanism of action of snake venom hemorrhagic toxins is determined then it may be possible to develop an effective treatment to antagonize this action which can be a serious complication in treating snakebite. The ability to use only a few purified hemorrhagic toxins to determine this has many advantages since these toxins are often quite difficult to isolate to homogeniety and are unstable and quickly lose their activity. ACL hemorrhagic toxin I, which was relatively easy to isolate and appears to be somewhat stable since it was still active after one year at -20 C, may be a good choice as a model toxin to study the hemorrhage induced by snake venoms.

In summary, a hemorrhagic toxin was isolated from the venom of *A. c. laticinctus* and designated ACL hemorrhagic toxin I. The toxin has a molecular weight of about 29,000 and is slightly acidic although it appears to exist in several isoforms. The hemorrhage produced by ACL hemorrhagic toxin I has a rapid onset and is present as early as 5 minutes after injection of the toxin. ACL hemorrhagic toxin I is a metalloprotease with activity towards the substrates N,N-dimethylcasein and bovine fibrinogen. Although the toxin is able to hydrolyze fibrinogen *in vitro*, it does not possess any defibrinating activity *in vivo* whereas the crude venom does show this activity. The results of the digestion of fibrinogen indicates that ACL hemorrhagic toxin I has similar cleavage specificities to other snake venom hemorrhagic toxins which suggests that these toxins may be chemically related. The broader implication of this is that these toxins may have similar mechanisms of hemorrhagic action so that it may be possible to determine the mechanism of action of a large number of snake venom hemorrhagic

toxins by studying only a few representive toxins.

CHAPTER V

PATHOGENESIS OF HEMORRHAGE INDUCED BY ACL HEMORRHAGIC TOXIN I AND CRUDE VENOM OF *AGKISTRODON CONTORTRIX LATICINCTUS* (BROAD-BANDED COPPERHEAD)

Introduction

Hemorrhage is a common and often serious sequela of bites by many species of venomous snakes, although this type of damage is especially prominent following bites by snakes in the subfamily Crotalinae (pit vipers). Hemorrhage induced by crotaline snake venoms contributes to the local tissue damage which occurs following many bites by these snakes. This tissue damage is a serious complication in the treatment of snakebite since it often produces severe necrosis of tissues surrounding the bite site. In many cases, this necrosis leads to a loss of tissue or in severe bites to loss of function of the bitten region.

Hemorrhage can occur through two different mechanisms that result in a loss of blood from the vascular system. These include 1) hemorrhage *per rhexis*, in which there is a loss of blood through areas of lysed or disrupted endothelial cells, and 2) hemorrhage *per diapedesis*, in which there is a loss of blood through widened intercellular junctions between intact endothelial cells. Ownby et al. (1978) studied the hemorrhage produced by three purified hemorrhagic toxins isolated from *Crotalus atrox* venom and found that these toxins caused hemorrhage *per rhexis*. A hemorrhagic toxin from *C. horridus horridus* venom (Ownby and

Geren, 1987), as well as bilitoxin, a hemorrhagic toxin isolated from *Agkistrodon bilineatus bilineatus* venom also caused hemorrhage *per rhexis* (Ownby et al., 1990). The only report of a snake venom hemorrhagic toxin that produced hemorrhage *per diapedesis* is that of Ohsaka et al. (1975), in which a toxin from *Trimeresurus flavoviridis* venom was reported to cause a loss of blood through widened intercellular junctions.

Because the hemorrhage caused by many venomous snakebites is important in the pathogenesis of these bites, there has been considerable research on the hemorrhagic toxins in snake venoms. Although much has been learned about the chemical nature of these toxins, their mechanism of hemorrhagic action remains unknown. Virtually all snake venom hemorrhagic toxins have been shown to be metalloproteases with activities toward general protease substrates such as the B chain of insulin, casein, and collagen (Bjarnason and Fox, 1988). It has been proprosed that these toxins act by hydrolyzing the basal lamina of capillary endothelial cells which results in a loss of integrity of the endothelium (Bjarnason et al., 1988). However, it is not clear if this is indeed the mechanism since in many cases endothelial cells have much of their basal lamina intact after injection of snake venom hemorrhagic toxins (Ownby et al., 1978; Ownby and Geren, 1987; Ownby et al., 1990). The purpose of the following experiment was to study the hemorrhage induced by both crude *A. contortrix laticinctus* venom and ACL hemorrhagic toxin I, a purified hemorrhagic toxin isolated from this venom.

Materials and Methods

Venom

A. c. laticinctus venom was obtained from 15 snakes collected in central Texas, U.S.A., and maintained at the Venom Research Serpentarium, Department of Physiological

Sciences, Oklahoma State University, U.S.A. The snakes were extracted approximately every two weeks during 1988-1989. The venom was extracted by allowing the snakes to bite through parafilm which covered a glass funnel that emptied into a polycarbonate centrifuge tube resting in an ice bath. Immediately after extraction, the venom was centrifuged at 28,000 x *g* for 20 minutes using a Sorvall RCR5 refrigerated centrifuge (4 C) to remove insoluble material, frozen, and then lyophilized. Lyophilized venom was stored over dessicant at -20 C until needed.

Isolation of Toxin

ACL hemorrhagic toxin I was isolated from crude *A. c. laticinctus* venom as described in chapter IV. A three step procedure using both low pressure liquid chromatography and HPLC was used to purify the toxin from the venom. Briefly, 500 mg of crude venom was dissolved in 10 mM Tris buffer (pH 8.6) containing 100 mM NaCl and applied to a Sephacryl S-200 HR gel filtration column (2.6 cm x 100 cm) at a flow rate of 20 ml/hour. Fractions eluting from this column were checked for hemorrhagic activity and the fraction of interest concentrated using a Millipore Minitan concentrating unit with 1000 MW exlusion limit membrane and then lyophilized. The fraction from the Sephacryl column was reconstituted in 10 mM Tris buffer (pH 8.6) and applied to a Waters DEAE-5PW HPLC anion exchange column (21.5 mm x 15 cm) at a flow rate of 3.6 ml/minute. The hemorrhagic fraction of interest from the DEAE column was similarly concentrated and lyophilized. The DEAE fraction was reconstituted in 10 mM MOPS buffer (pH 6.5) containing 100 mM NaCl and further purified using a Waters Protein Pak 125 HPLC gel filtration column (7.5 mm x 30 cm). This column was eluted for a total of 60 minutes at a flow rate of 0.5 ml/minute. One symmetrical peak was obtained from this final column and

was shown to be homogeneous by SDS-PAGE. The fraction, designated ACL hemorrhagic toxin I, was stored in 10 mM MOPS buffer at -20 C until needed.

Histological Examination

Hemorrhage induced by crude *A. c. laticinctus* venom and ACL hemorrhagic toxin I was studied using the method of Ownby et al. (1978). Crude venom was reconstituted in phosphate-buffered saline to a concentration of 1.5 μg/μl and injected into the right thigh of female mice (CD-1, Charles River) weighing 25-30 grams. Injection volumes were adjusted so that a 25 g mouse would receive 50 μl containing a final dose of 2.5 μg/g body weight. ACL hemorrhagic toxin I in 10 mM MOPS buffer (pH 6.5) was similarly injected into mice at a dose of 0.5 μg/g body weight. Tissue samples were taken at 5 and 30 minutes and 1, 3, 6, and 12 hours after injection of the crude venom and 30 minutes and 1 hour after injection of ACL hemorrhagic toxin I. Experiments consisting of injecting two mice per time period with venom and one mouse with 50 μl of phosphate-buffered saline as a control and experiments consisting of injecting two mice per time period with ACL hemorrhagic toxin I and one mouse with 50 μl of 10 mM MOPS buffer as a control were performed in duplicate. Samples were fixed for 2 hours in 2.0% glutaraldehyde in 0.16 M cacodylate buffer (pH 7.4). The tissue was washed three times in 0.08 M cacodylate buffer containing 0.18 M sucrose and post-fixed for 1 hour in 1% aqueous osmium tetroxide. The tissue was dehydrated in ascending concentrations of acetone and embedded in Polybed 812 resin. Thick sections (1 μm) for light microscopic examination were obtained with a Sorvall MT 5000 ultramicrotome and stained with Mallory's toludine blue-azure II dye. A total of about 50 thick sections for the crude venom experiments and about 20 thick sections for the hemorrhagic toxin experiments were examined. Thin sections (silver to light gray) for electron microscopic

examination were cut with glass or diamond knives and stained with 4% methanolic uranyl acetate and 4% aqueous lead citrate. The thin sections were observed using a JEOL 100 CX scanning-transmission electron microscope. A total of about 50 thin sections for the crude venom experiments and 10 thin sections for hemorrhagic toxin experiments were examined.

Results

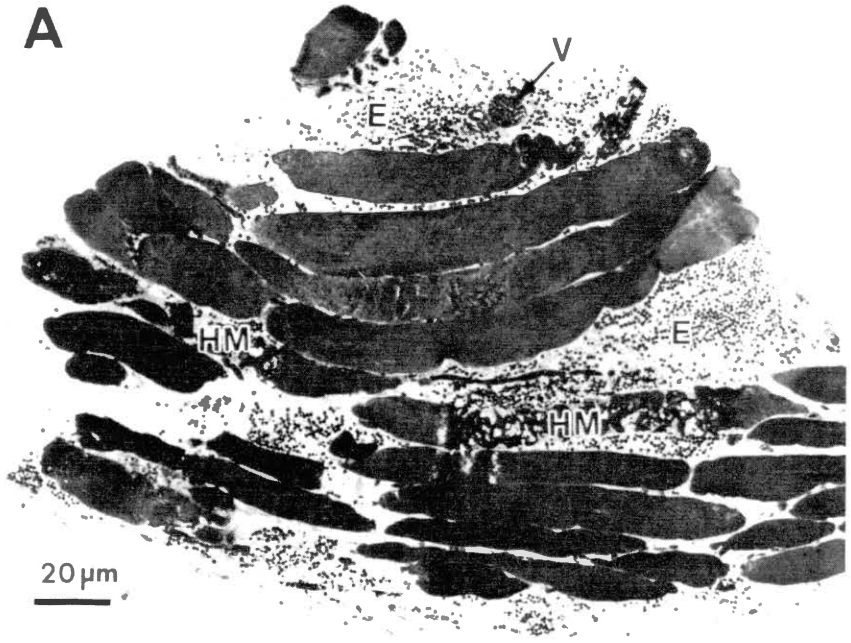
Gross examination showed that considerable hemorrhage was present following injections of crude *A. c. laticinctus* venom. Hemorrhage produced by the venom had a rapid onset and was present as early as 5 minutes after injection. The amount of hemorrhage continued to increase until approximately 3-6 hours after the injection after which time it covered the majority of the lateral thigh. The size of the lesion usually did not increase significantly after 3-6 hours, however, necrosis of the tissue caused by the venom continued to develop and often produced an ulceration of the skin surrounding the injection site.

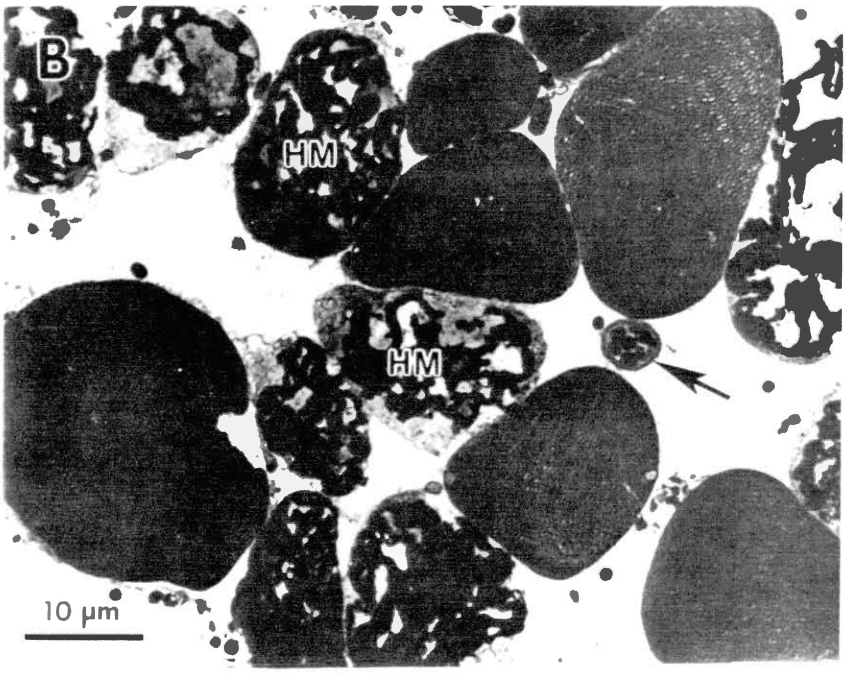
Hemorrhage produced by ACL hemorrhagic toxin I also had a rapid onset and was present as early as 5 minutes after an injection. At a dose of 0.5 μg/g, the hemorrhage induced by ACL hemorrhagic toxin I covered most of the lateral thigh as well as extending into many of the deep muscles by 30 minutes.

At the light microscopic level, a large number of extravasated erythrocytes were usually present in the connective tissue between muscle cells after injection of *A. c. laticinctus* venom (Figure 19a). In many sections, capillaries were difficult to locate in the tissue and appeared to be completely lysed by the venom. In other sections where the damage was not as extensive, many of the capillaries and larger venules were congested with erythrocytes (Figure 19b).

Similarly to the crude venom, a large number of extravasated erythrocytes were

Figure 19. Light micrographs of mouse skeletal muscle taken at various time periods after injection of *A. c. laticinctus* venom.
A. 5 minutes. Note extravasated erythrocytes (E), muscle cells with hypercontracted myofilaments (HM), and venules congested with erythrocytes (V).
B. 1 hour. Note capillaries congested with erythrocytes (arrow) and muscle cells with hypercontracted myofilaments (HM).


A
V
E
HM
E
HM
20 µm


B
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HM
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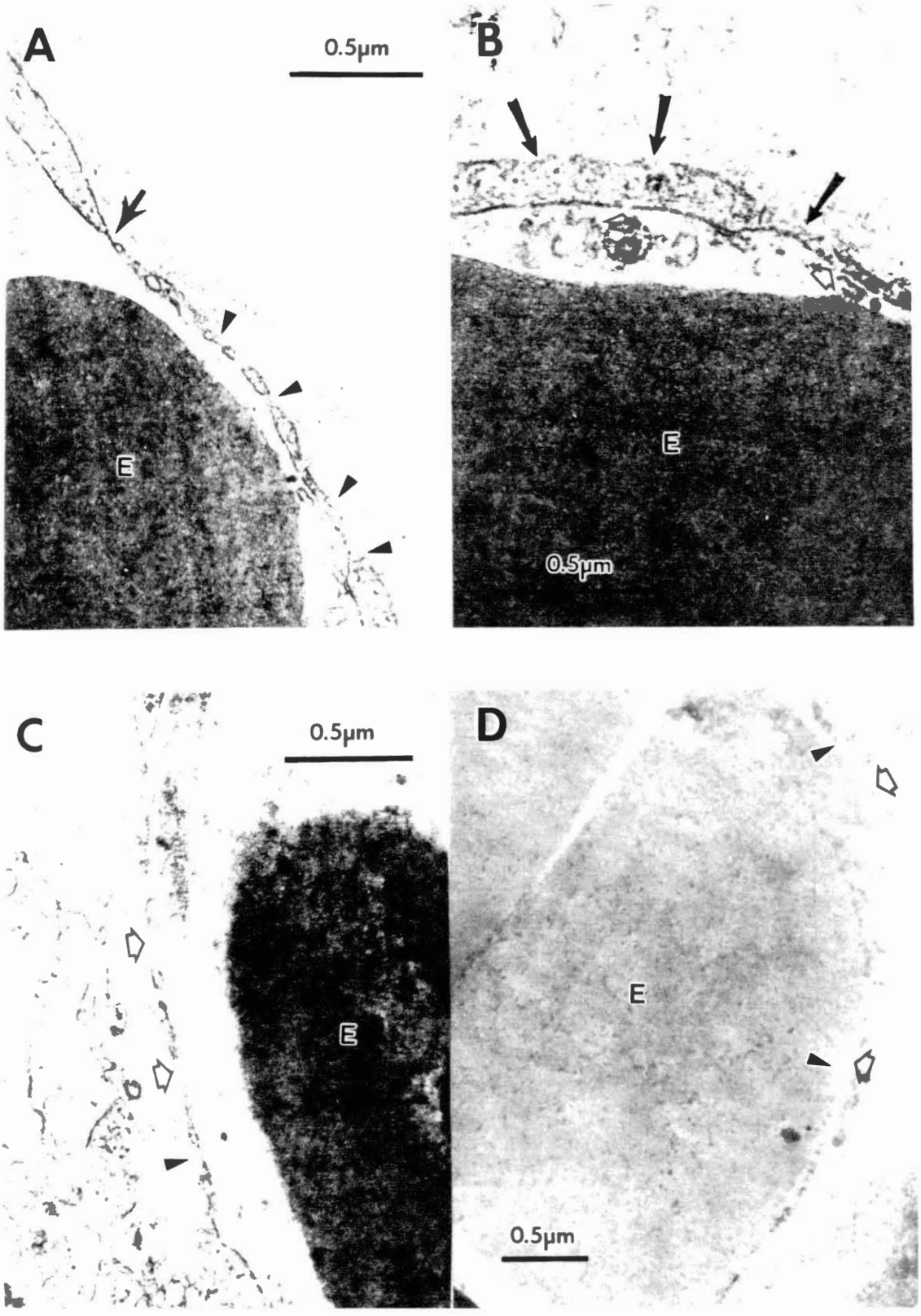
present in the connective tissue following injections of ACL hemorrhagic toxin I. Many capillaries damaged by ACL hemorrhagic toxin I were congested with erythrocytes and platelets. Capillaries congested with platelets appeared to be more prevalent in tissue from animals injected with ACL hemorrhagic toxin I than in tissue from animals injected with crude venom although this was not quantitated.

Capillaries showing three types of pathological changes were identified at the electron microscopic level in tissue injected with crude *A. c. laticinctus* venom. These changes included 1) capillary endothelial cells with segments that were very thin (Figure 20a), 2) capillary endothelial cells that had segments of disrupted plasma membrane (Figure 20b and 20c), and 3) endothelial cells that appeared to be completely lysed by the venom (Figure 20d). Figure 20b is a high magnification of a different portion of the endothelial cell shown in Figure 20a. The plasma membrane appears to be disrupted and is present only as a fuzzy material. The abluminal plasma membrane of the endothelial cell appears to be disrupted whereas the luminal plasma membrane is mostly intact. However, there were also segments where both the abluminal and luminal plasma membranes were disrupted which produced a discontinuity of the endothelial cell (Figure 20c). Thin regions and regions of discontinutity of endothelial cells were not observed in capillaries from controls. In some capillaries, large areas of damaged endothelial cells were missing whereas adjacent endothelial cells appeared normal. Intercellular junctions between damaged endothelial cells were intact. The basal lamina was often present around segments of damaged endothelial cells, however, it was difficult to visualize around some segments of heavily damaged endothelial cells.

ACL hemorrhagic toxin I induced changes in capillary endothelial cells similar to that of the crude venom. The plasma membrane of endothelial cells damaged by ACL hemorrhagic toxin I appeared to be disrupted. Many capillaries were congested with

Figure 20. Electron micrographs of capillaries taken 30 minutes after injection of *A. c. laticinctus* venom.
A. Note thin area of endothelial cell (arrow) and areas with disrupted plasma membrane (arrowheads); E = erythrocyte.
B. Note disrupted segments of abluminal plasma membrane (arrows) and luminal plasma membrane (open arrows); E= erythrocyte.
C. Note disrupted plasma membrane (arrowhead) and discontinuity of endothelial cell (open arrows); E = erythrocyte.
D. Note lysis of majority of endothelial cell (arrowheads) although a portion of basal lamina is present (open arrows). The lower contrast compared to 20A-20C is due to greater thickness of section.


A
0.5μm
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C
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D
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0.5μm

erythrocytes and showed aggregations of platelets that appeared to be plugging holes in ruptured endothelial cells. In some capillaries, platelets appeared to be outside the lumen of the capillary which suggests a discontinuity of the plasma membrane of the endothelial cells (Figure 21a). The basal lamina was often intact over focal regions of damaged plama membrane (Figure 21b). Large amounts of both intravascular and extravascular fibrin were present following injections of the toxin (Figure 21c and 21d). The intercellular junctions of endothelial cells adjacent to these aggregates of platelets were intact (Figure 21c). At 30 minutes, aggregates of platelets were usually restricted to small areas of the capillaries. However, other capillaries had large segments of their plasma membrane disrupted and these capillaries were completely occluded by platelets (Figure 21d).

Discussion

Crude *A. c. laticinctus* venom and ACL hemorrhagic toxin I, a purified hemorrhagic toxin from this venom, both produced considerable hemorrhage in mice. The hemorrhage had a rapid onset and was present as early as 5 minutes after injections of either toxin or venom. The crude venom and toxin both produced a disruption of capillary endothelial cells although the was no evidence that intercellular junctions between endothelial cells were disrupted. Therefore, the hemorrhage induced by *A. c. laticinctus* venom and ACL hemorrhagic toxin I was predominantly *per rhexis*.

Although the hemorrhage induced by ACL hemorrhagic toxin I and crude venom was *per rhexis*, the question still remains as to what their hemorrhagic mechanism of action is at the molecular level. Bjarnason et al. (1988) studied hemorrhagic toxins from *C. atrox* venom and proposed a possible mechanism of action that was related to their proteolytic activity. Because these toxins were able to hydrolyze basal lamina preparations *in vitro*, they suggested

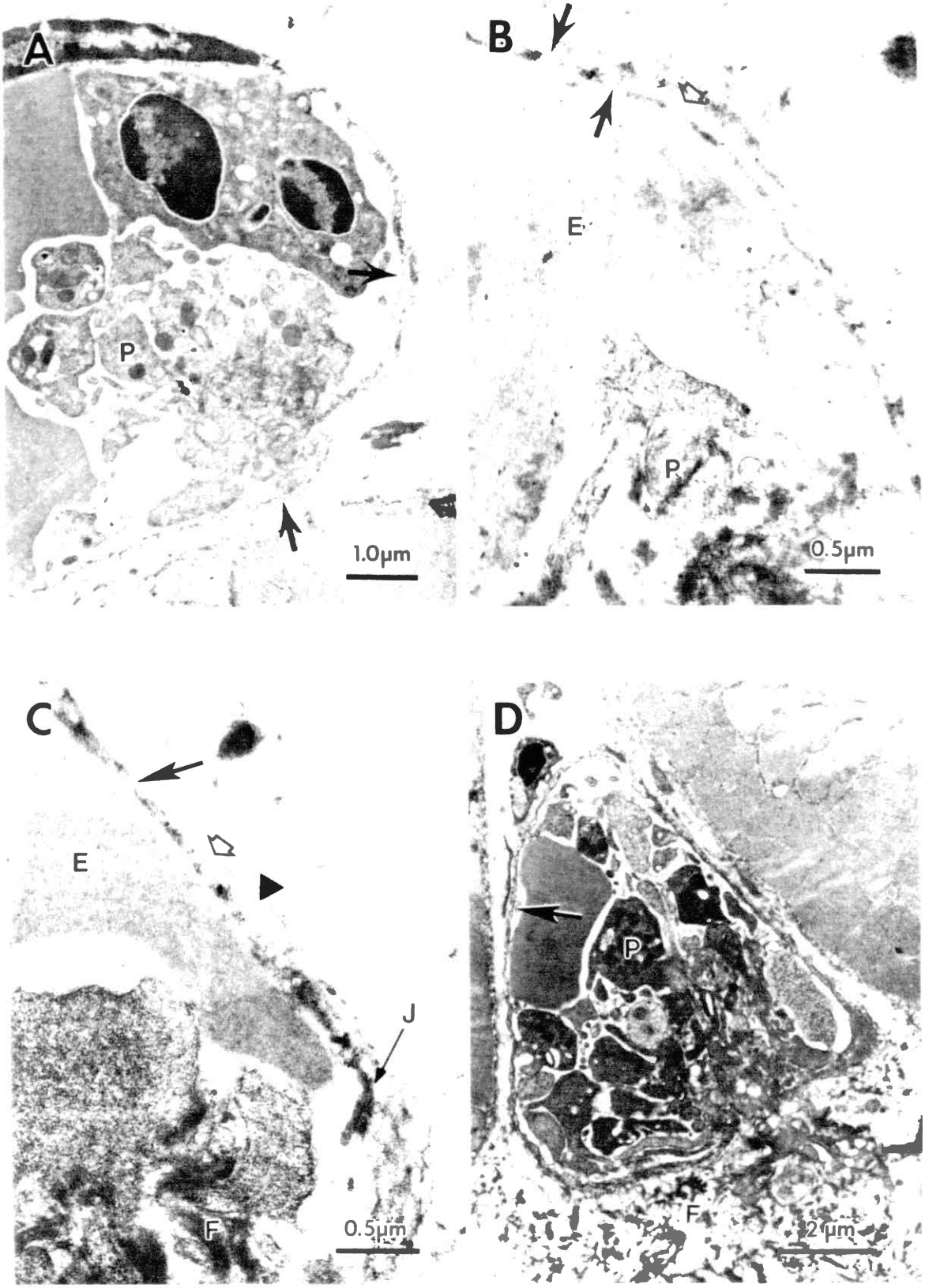
Figure 21. Electron micrographs of capillaries from tissue taken 30 minutes after injection of ACL hemorrhagic toxin I.

A. Note thin areas of capillary (arrows) and aggregate of platelets (P).

B. Note region of disrupted endothelial cell with intact basal lamina (open arrows), thin areas of endothelial cell (arrows), erythrocyte (E), and platelets (P).

C. Note area of disrupted endothelial cell (arrowhead) with intact basal lamina (open arrow), intact intercellular junction (J), and aggregates of platelets and fibrin (F).

D. Capillary that is completely occluded by platelets (P). Note thin region of capillary (arrow) and large amounts of extravascular fibrin (F).


A
P
1.0μm
B
E
P
0.5μm
C
E
J
F
0.5μm
D
P
F
2 μm

that the toxins could enzymatically digest the basal lamina of capillary endothelial cells resulting in a loss of integrity of the endothelium. According to the hypothesis, this would ultimately cause a disruption of capillaries which allows blood to escape from the vessels. Although hemorrhagic toxins from *C. atrox* venom were capable of hydrolysing basal lamina preparations *in vitro*, extremely long incubation times of up to 90 hours at 37 C were required for complete hydrolysis. It is difficult to understand how this could be the mechanism of action of snake venom hemorrhagic toxins since they typically produce a hemorrhagic lesion within 5 minutes of an injection (Ownby et al., 1978). In addition, there is no *in vivo* evidence to support that this mechanism results in a disruption of the endothelium (Ownby et al., 1978; Ownby and Geren, 1987; Ownby et al., 1990). Ownby et al. (1978) studied the hemorrhage induced by purified hemorrhagic toxins from *C. atrox* venom *in vivo* and found that endothelial cells damaged by these toxins often had much of their basal lamina intact. Capillaries damaged by *A. c. laticinctus* venom and ACL hemorrhagic toxin I also often had segments of intact basal lamina in areas where the plasma membrane was disrupted. This suggests that the enzymatic hydrolysis of basal lamina may not be the mechanism of hemorrhagic activity of certain snake venom hemorrhagic toxins. Indirect evidence that removal of the basal lamina may not necessarily lead to the loss of endothelial cell viability comes from the study of Koefoed (1987) which showed that insect midgut epithelial cells that had their basal lamina removed by elastase were still viable and formed a tube. This suggests that removal of the basal lamina does not always lead to a disruption of integrity of certain epithelial cell types.

In some capillaries that were severely damaged by *A. c. laticinctus* venom or ACL hemorrhagic toxin I, the basal lamina was difficult to visualize and appeared to be missing. However, it is not clear if the absence of the basal lamina is the cause of the damage or is a

result of the disruption of the endothelial cell. It is possible that once they were damaged, endothelial cells were unable to maintain the integrity of the basal lamina in areas of the disruption. Therefore, the absence of the basal lamina in these areas may be a consequence of a disruption of the capillaries by a mechanism unrelated to enzymatic hydrolysis of the basal lamina.

Endothelial cells damaged by *A. c. laticinctus* venom and ACL hemorrhagic toxin I often showed pathological changes that were at the level of the plasma membrane. Capillaries often had segments of endothelial cells where the plasma membrane appeared to be degraded. In some endothelial cells, segments of both the abluminal and luminal plasma membranes were missing which produced a discontinuity of the capillaries. Many of these endothelial cells had more than one region where the plasma membrane was discontinuous whereas a few larger capillaries appeared to be missing entire endothelial cells. This suggests that endothelial cells can be damaged at several sites along the plasma membrane, with each disruption resulting in the loss of a larger portion of the cell. Therefore, damage may initially be restricted to one or a few regions of the endothelial cell although it may continue to develop until entire cells are completely degraded. This would eventually result in the loss of large segments of capillaries thereby producing holes through which blood can escape into the surrounding connective tissue. This is supported by light microscopic studies which show that virtually no capillaries could be visualized in areas of muscle severely damaged by *A. c. laticinctus* venom although large numbers of erythrocytes were present in the endomysium.

The exact mechanism by which *A. c. laticinctus* venom and ACL hemorrhagic toxin I may induce a disruption of the plasma membrane of endothelial cells is not clear. In many endothelial cells damaged by the toxin and crude venom, segments of the plasma membrane appeared to be absent. This suggests that ACL hemorrhagic toxin I and crude venom induce

a degradation of the plasma membrane. One possible mechanism is that they may either directly or indirectly activate intracellular phospholipases which may hydrolyze membrane phospholipids (Trump et al., 1981). Such a hydrolysis could result in a loss of integrity of the phospholipids and a dissolution of the membrane. Another possibility is that ACL hemorrhagic toxin I may also possess phospholipase A activity and may act directly on the plasma membrane, although this was not determined in this investigation. Additional research is needed to conclusively determine the mechanism of action of ACL hemorrhagic toxin I and other hemorrhagic toxins in *A. c. laticinctus* venom.

In addition to regions of degraded plasma membrane, capillaries damaged by ACL hemorrhagic toxin I and crude venom also had segments of endothelial cells that were very thin. One explanation for these thin segments could be a localized hypercontraction of the cytoskeleton. It is possible that a contraction of cytoskeletal microfilaments in endothelial cells may pull the luminal and abluminal plasma membranes together thereby producing these very thin areas. Contraction of the cytoskeleton has been observed in many different cell types, including endothelial cells, following administration of various chemicals and toxins. For example, Morel et al. (1990) showed that pulmonary microvessel endothelial cells contracted upon addition of various inflammatory agents such as angiotension and bradykinin. Cytochalasin D, a toxin produced by molds, has been shown to cause a contraction of cytoskeletal microfilaments in HeLa cells resulting in a retraction of the cytoplasm (Miranda et al., 1974). In both these cases the contraction is beleived to result from elevated levels of free intracellular Ca^{2+} which produces a hypercontraction of actin and myosin myofilaments. In endothelial cells damaged by ACL hemorrhagic toxin I and crude venom, the thin segments were usually adjacent to areas where the plasma membrane was degraded. Therefore, it is possible that these thin segments of endothelial cells may result from a contraction of actin

and myosin microfilaments induced by high levels of Ca^{2+} entering the cells through these areas of disrupted plasma membrane.

In summary, *A. c. laticinctus* venom and ACL hemorrhagic toxin I, a purified hemorrhagic toxin from this venom, both induced considerable hemorrhage in mice. The data suggest that hemorrhage resulted from lysis of capillary endothelial cells which allowed escape of blood from the vascular system. Hemorrhage induced by both toxin and crude venom was predominantly *per rhexis* rather than *per diapedesis* because intercellular junctions between endothelial cells were intact. Endothelial cells damaged by ACL hemorrhagic toxin I and crude venom often had large segments of both luminal and abluminal plasma membrane that were completely degraded resulting in a discontinuity of the cells. Focal segments of ruptured endothelial cell damaged by ACL hemorrhagic toxin I were usually plugged by aggregates of platelets and fibrin although this was not as common with the crude venom. The basal lamina was often intact which suggests that a hydrolysis of the basal lamina by the proteolytic activity of the toxin and venom may not be the mechanism of hemorrhagic action. The results of this investigation suggest that ACL hemorrhagic toxin and other hemorrhagic toxins in *A. c. laticinctus* venom act directly on capillary endothelial cells to cause a degradation of the abluminal and luminal plasma membrane which results in holes in capillaries through which blood can escape.

CHAPTER VI

SUMMARY

Myonecrosis and hemorrhage induced by crude venom and venom toxins of *Agkistrodon contortrix laticinctus* (broad-banded copperhead) were studied. Crude *A. c. laticinctus* venom induced a rapid necrosis of mouse skeletal muscle cells with damaged cells present as early as 5 minutes after injection. A myotoxin, designated ACL myotoxin, was isolated from the crude venom using HPLC. The toxin was a protein consisting of two polypeptide chains, each with a molecular weight of approximately 14,500. ACL myotoxin is basic with an isoelectric point greater than 9.0. The type of myonecrosis induced by ACL myotoxin was similar to the crude venom. The myonecrosis was characterized by a hypercontraction of myofilaments which results in a pattern of densely clumped myofibrils in the cytoplasm. In addition, muscle cells with clear vacuoles and muscle cells with areas of disorganized myofibrils were observed after injection of the toxin.

Electron microscopic examination revealed that the sarcolemma appeared disrupted along focal regions of muscle cells damaged by the toxin at early time periods. Therefore, it is possible that an influx of ions through these disruptions may cause the pathological changes induced by ACL myotoxin. The hypercontraction of myofilaments suggest elevated levels of free intracellular Ca^{2+}. Results from *in vitro* studies using cut skeletal muscle preparations co-incubated in various ions showed that densely clumped myofibrils were produced by 150 and 300 mM NaCl but not by $CaCl_2$ concentrations as high as 600 mM. This suggests that an

influx of extracellular Ca^{2+} alone may not cause the densely clumped myofibrils and suggests that an influx of Na^{+} may ultimately be responsible for initiating this pathological change. One possible mechanism is that the influx of Na^{+} causes a release of Ca^{2+} from the sarcoplasmic reticulum, perhaps by a sustained depolarization of the membrane, which reaches a concentration high enough to produce hypercontracted myofilaments. The other pathological changes, including areas of disorganized myofibrils and muscle cells with clear vacuoles, could be explained by osmotic swelling due to the influx of Na^{+} and other solutes.

Although ACL myotoxin appears to disrupt the permeability barrier of the sarcolemma of skeletal muscle cells, the mechanism of action at the molecular level is not known. Gutierrez et al. (1989) proposed that a non-phospholipase myotoxin from the venom of *Bothrops nummifer* disrupted the sarcolemma of mouse skeletal muscle cells by a detergent-like action since the toxin contained a large number of hydrophobic amino acids. The results of this study show that ACL myotoxin also contains a large number of hydrophobic amino acids and produces a type of myonecrosis similar to that produced by the *B. nummifer* myotoxin. Therefore, it is possible that ACL myotoxin may have a similar mechanism of action. Future research on the sequence and conformation of ACL myotoxin may help in determining if this is correct. Data indicating possible hydrophobic and polar domains may allow for creation of a model that may explain the insertion of ACL myotoxin into the plasma membrane as well as predicting possible interactions at the lipid-water interphase. Additional research on the interaction of ACL myotoxin and artificial lipid bilayers may also prove useful in determining the mechanism of action. Studies using nuclear magnetic resonance (NMR) techniques may show possible perturbations of ester bonds in lipids of the bilayer which could then be compared with results from studies on other cytotoxins with a detergent-like action to see if any evidence exists for a similar mechanism of action.

A. c. laticinctus venom induced considerable hemorrhage in mice. Fractionation of the crude venom revealed at least five hemorrhagic fractions. In order to study hemorrhage induced by this venom, one of the hemorrhagic toxins was isolated to purity and characterized as to its chemical and biological activities. The hemorrhagic toxin, designated ACL hemorrhagic toxin I, was isolated from the venom using both low pressure liquid chromatography and HPLC. ACL hemorrhagic toxin I is a metalloprotease with a molecular weight of 29,000. The toxin appears to exist in several slightly acidic isoforms with isoelectric points that range from 4.70-6.40. The minimum hemorrhagic dose for ACL hemorrhagic toxin I is 6.7 μg/mouse. ACL hemorrhagic toxin I showed enzymatic activity towards bovine fibrinogen and hydrolyzed the A$\propto$ and Bβ chains but not the gamma chain. Although the toxin was able to hydrolyze bovine fibrinogen *in vitro*, it did not demonstrate any defibrinating activity *in vivo* at doses up to 1.0 μg/g whereas the crude venom showed this activity. This suggests that the defibrinating activity of the crude venom is due to toxins other than ACL hemorrhagic toxin I. In addition, incubation of the toxin with fibrinogen did not produce an insoluble fibrin clot which suggests that the fibrin observed in capillaries after injection of the toxin is most likely due to polymerization of fibrinogen in response to the disruption of the endothelium by the toxin and not by a direct action of the toxin on fibrinogen. ACL hemorrhagic toxin I did not cause a hypercontraction of myofilaments in skeletal muscle cells therefore its activity differed from ACL myotoxin, a myotoxin isolated from the same venom.

ACL hemorrhagic toxin I and crude venom both caused hemorrhage predominantly *per rhexis*. Endothelial cells damaged by toxin and crude venom had segments where both the abluminal and luminal plasma membrane were degraded. In some capillaries, the plasma membrane of entire endothelial cells was missing. The basal lamina was often intact around

smaller segments of damaged endothelial cell. This suggests that the mechanism of hemorrhagic activity of ACL hemorrhagic toxin I and crude venom may not involve the hydrolysis of the basal lamina due to enzymatic activity, which has been proposed to be the mechanism of other snake venom hemorrhagic toxins. The results of this study suggests that *A. c. laticinctus* venom and ACL hemorrhagic toxin I may have a direct effect on capillary endothelial cells resulting in the degradation of both the abluminal and luminal plasma membrane which produces holes in the capillaries. Future research on the possible role of membrane calcium channels may help determine the mechanism of action of ACL hemorrhagic toxin I. Endothelial cells damaged by ACL hemorrhagic toxin I and crude venom often had segments that were very thin. One explanation for this could be a contraction of the cytoskeleton due to excess calcium. One mechanism for this could be by by activating calcium channels which causes an influx of calcium from the extracellular fluid. An influx of calcium may also be linked to the degradation of the plasm membrane by the activation of calcium-activated phospholipases. Assuming this to be correct, future studies directed toward inhibiting these phospholipases, or possibly chelating the excess calcium, may help lead to a better treatment of the hemorrhage induced by snakebite.

Local tissue damage such as hemorrhage and myonecrosis are only part of the pathological changes induced by *A. c. laticinctus* venom. *A. c. laticinctus* venom contains a large number of different toxins with a wide variety of biological activities. These include toxins that produce hypotension, toxins that affect blood cogulation, including both procoagulant and anticoagulant toxins, and edema-inducing toxins. Clinically, the use of antivenom has been shown to be relatively effective in preventing many of these systemic pathologies, however, its use does not always prevent the hemorrhage and myonecrosis produce by snakebite. Therefore, studies on the hemorrhagic toxins and myotoxins in snake

venoms may be beneficial in developing a new treatment for snakebite. This study was undertaken as an initial investigation of the hemorrhage and myonecrosis induced by *A. c. laticinctus* and ACL hemorrhagic toxin I and ACL myotoxin, a purified hemorrhagic toxin and myotoxin from this venom. Although the hemorrhage induced by ACL hemorrhagic toxin I and the myonecrosis induced ACL myotoxin does not represent the total hemorrhage and myonecrosis produced by the crude venom, due to the presence of additional hemorrhagic toxins and myotoxins in the venom, the other hemorrhagic toxins and myotoxins may be similar to ACL hemorrhagic toxins I and ACL myotoxin so that by studying these purified toxins it may be possible to develop a better treatment that will prevent the damage caused by the crude venom.

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