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THE PRESENCE OF PHARMACOLOGICAL SUBSTANCES MYOGLOBIN AND HISTAMINE IN VENOMS

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ABSTRACT: It is well documented that several pharmacological substances are released within the victim's body after snakebite. These substances are also believed to be endogenously present in animals, specifically levels of myoglobin and histamine that are reported to rise after envenomation. However, there is no published data regarding the presence of these substances in venoms *per se*.

This research reports the detection of myoglobin and histamine in snake, scorpion, honeybee, and toad venoms by immunological test. It is unlikely that the rise in levels of myoglobin and histamine is due to that added from the bite, since a single toxin devoid of such components is capable of elevating levels of these substances. Nonetheless, it is likely that the rise in levels of myoglobin and histamine after envenomation is due to the venom or toxin reacting with cells of various organs of the victim. Therefore, this phenomenon can be compared to cancer markers, which are endogeneously present in humans at low levels and elevated in cancerous states.
KEY WORDS: Myoglobin, histamine, snake, honeybee, scorpion, toad, venom, *Crotalus atrox*, *Crotalus polystictus*, *Naja kaouthia*, *Ophiophagus hannah*, *Daboia russelli, Oxyuranus s. scutellatus, Actinopyga agassizi, Bufo arenarum, Androcetonus australis, Apis mellifera*.

INTRODUCTION

Plasma, urea, creatine, and potassium levels are usually elevated in severe envenoming, and become progressively higher in terminal stages of fatal cases. An increase has recently been reported in the circulating levels of blood sugar, insulin, glucagon, and cortisol following envenomation in experimental dogs by the scorpion *Mesobuthus tamulus concanesis* (11). Several investigators have proved that citrate is a major component of snake venoms (5,6,12).

As early as 1961, Reid (16) demonstrated that myoglobinuria is one of the characteristic symptoms in human snakebite victims, and hemoglobinuria is frequently found after envenomation caused by *Pseudechic* venoms and Australian copperhead *Austrelaps superba* bites. The presence of myoglobin in serum and urine was demonstrated as an early marker for kidney dysfunction (17). In mouse animal model, myoglobinuria due to envenomation from *Pseudechis australis* snake venom was developed 60 minutes after venom injection as indicated by red or dark-brown urine (14).

Procamine, a polypeptide found in *A. mellifera* honeybee venom, contains a histamine residue at the C terminal. This was the first histamine-containing peptide to be isolated from a natural source (13). It was demonstrated that monoclonal antibodies to immunoglobin G4 induce histamine release from human basophils *in vitro* (4). Evidence of histamine release was demonstrated as a principal pharmacological component of venom from Australian wolf spider (15). Also, histamine release as a consequence of honeybee and yellow jacket venom allergy has been reported (10).

The above-mentioned references illustrate that myoglobin and histamine are released as a consequence of envenomation, showing increased levels. However, the presence of these parameters in venom is not yet documented or published except for honeybee venom containing histamine. Thus, this is a firsthand report showing the presence of the pharmacological substances myoglobin and histamine in venoms. The possible functions of these substances will require further research.

MATERIALS AND METHODS

The venoms used in these studies: *Crotalus atrox* (Western diamondback), *Crotalus polystictus* (Mexican rattlesnake), *Naja kaouthia* (Thailand cobra), *Ophiophagus hannah* (king cobra), *Daboia russelli* (Asian viper), *Oxyuranus s. scutellatus* (Australian taipan), Astrotia stokesii (sea snake), *Actinopyga agassizi* (sea cucumber), *Bufo arenarum* (Colorado river toad), *Androcetonus australis* (scorpion), *Apis mellifera* (honeybee) were purchased from Sigma.

Production of Polyclonal antibodies in mice

The animals for this research were used in compliance with US Public Health Service Policy on human care and use of animals. Myoglobin was purchased from Scripps Laboratory and histamine from Sigma. Adult Balb/C mice were injected intramuscularly (IM). The first injection consisted of a mixture of antigen and Freund's complete adjuvant (FCA). The subsequent injections consisted of antigen and Freund's incomplete adjuvant (FICA). For the production of anti-myoglobin, a dose of 20 µg/mouse was given in 0.2 ml volume three times, two weeks apart. Finally, the mice were bled and sera were tested by ELISA showing 1:24300/100 µl.

Production of Polyclonal antibodies for Histamine in Mice

There is a perception that small synthetic peptides do not generate antibodies when injected into animals. However, synthetic peptide can generate antibodies if it is tagged with a complete protein before injection. Landsteiner coined the term hapten for a chemically defined compound of low molecular weight, which would induce antibody production only when coupled to a larger carrier protein molecule before injection, thus making the injected animal produce antibodies to both the hapten and the carrier protein. For our other projects, we have successfully generated antibodies in mice against synthetic peptide as small as consisting of five amino acids using adjuvant only.

Adult Balb/C mice were injected IM with histamine mixed with FCA, 200 µg per mouse. The subsequent injections were given with similar concentration of histamine mixed with FICA. No detectable antibodies were revealed by ELISA after three injections in mice serum. Therefore, the mice continued to be immunized with 200 µg of histamine for three more times, two weeks apart. At the end of six injections, the mice sera showed the ELISA titer of 1:1800/100 µl, which was used for this study. This may be the first report of generating antibodies against a chemical, such as histamine without the use of carrier protein.

Purification of IgG

Isolation and purification of IgG from anti-myoglobin and anti-histamine was done on HPLC, using an ionic exchange column and gradient Trizma-HCl buffer, pH 7.4, as described by Lipps (7.8) for venoms (Figure 1). Fraction #5 was identified for IgG. The IgG fraction from the first run on HPLC was concentrated and re-fractionated under identical conditions. Purified IgG showed a single peak (Figure 2).

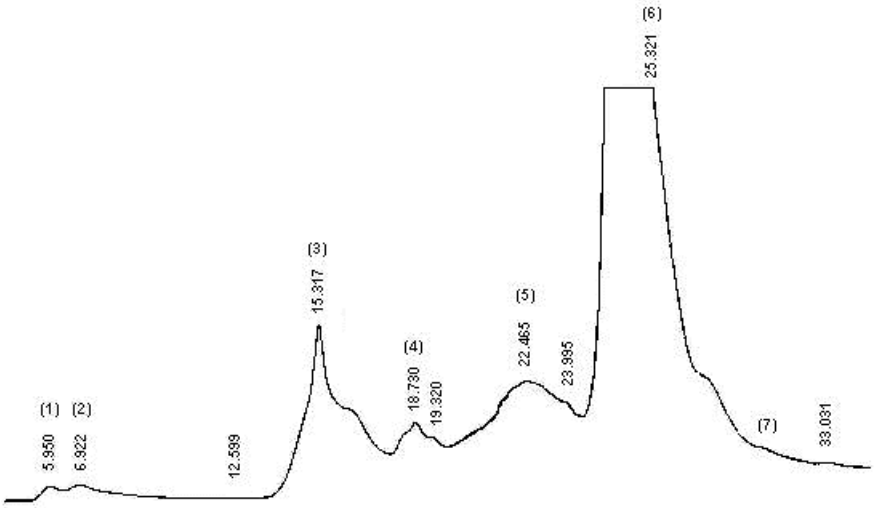

Figure 1. HPLC profile of mouse antiserum. Peak 5 represent IgG.

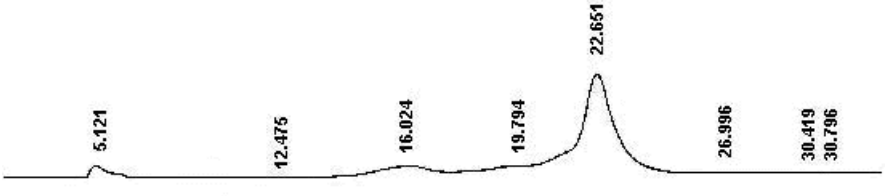

Figure 2. Single peak of purified IgG from anti-myoglobin or anti-histamine.

Enzyme-linked immunosorbent assay (ELISA)

ELISA tests were carried out in two different versions, and the reagents were purchased from Sigma Co.

A. Antibody was diluted and reacted with constant concentration of antigen 100µl/well. The presence of myoglobin and histamine in venoms were detected by ELISA test using anti-myoglobin and anti-histamine raised in mice. ELISA tests were carried out in 96-well microtiter plates (8,9). Venoms were diluted in carbonate-bicarbonate, pH 9.6, to give concentration 10 µg/ml. The wells of the plate were coated with antigen, each well receiving 100 µl. The plate was left at room temperature overnight, after which it was emptied and washed three times with PBS. The wells of the plate were blocked with 3% fish skin gelatin and washed 30 min later. After pilot testing, antisera diluted threefold from 1: 100 to 1: 72900 in gelatin and 100 µl of each dilution were added to three wells. The antigen controls that were without antibody and the highest concentration of antiserum that was 1:100 without antigen were incorporated. After 1-hour incubation at 37°C, the plate was washed and reacted with horseradish peroxidase conjugated with mouse IgG made in goat. The plate was incubated for 30 min, washed, and reacted with O-phenylenediamine-HCl for color development.

B. ELISA determination of antigen detection level - In this version, the antigen was diluted and the various concentrations of antigen were reacted with the constant concentration of IgG. The microplate was coated with antigen diluted threefold in carbonate bicarbonate buffer from 100 µg/ml to 0.015 µg/ml. and 100 µl/well of each concentration was added to three wells. Next day the plate was washed 3 times with PBS and blocked with 3% gelatin. The coated antigens were reacted with 10 µg/ml of IgG from anti-myoglobin or anti-histamine. The rest of the procedure was similar to that described above.

RESULTS AND DISCUSSION

The lower the detection level, the higher the presence of substance. ELISA titer of 300 or greater was considered as positive.

The results illustrate that the venoms of various species of snakes, scorpion, honeybee, and toad showed a detectable presence of myoglobin in various degrees (Table 1). Honeybee venom showed the highest titers to both myoglobin and histamine. Honeybee venom showed ELISA titer for myoglobin 5400 and histamine 1800 per 100 µl. The presence of histamine was moderate in venoms from *O. scutellatus*, honeybee, toad, and scorpion but miniscule in other venoms. Detection levels of myoglobin and histamine in venoms were in accordance to the ELISA titers. Honeybee venom showed ELISA titer for myoglobin 1:5400/100 µl and the detection level 2.0 µg/100 µl, the lower the detection level, the higher the concentration of the antigen. The results clearly indicate that the presence of myoglobin and histamine can be detected in venoms by immunological tests in two versions of ELISA.

Table 1. ELISA binding affinity of venoms to anti-myoglobin and anti-histamine. Detection levels of myoglobin and histamine in venoms by IgGs from anti-myoglobin and anti-histamine.

	Anti-myoglobin		**Anti-histamine**	
Venom	ELISA titer/100 µl	Detection level in µg	ELISA titer/100 µl	Detection level in µg
Crotalus atrox Rattlesnake	1800	16.5	450	16.5
Crotalus polystictus Mexican rattlesnake	1800	16.5	300	16.5
Naja kaouthia Cobra snake	900	11.0	300	33.0
Ophiophagus hannah King cobra	900	11.0	300	33.0
Daboia russelli Viper snake	1800	16.5	600	16.5
Oxyuranus scutellatus Taipan snake	2700	5.5	900	5.5
Astrotia stokesii Sea snake	600	33.0	600	11.0
Actinopyga agassizi Sea cucumber	600	33.0	300	33.0
Bufo arenarum River toad	1800	5.5	900	5.5
Androcetonus australis Scorpion	1800	16.5	900	5.5
Apis mellifera Honeybee	5400	2.0	1800	3.0
Negative controls				
Buffer	---	---	---	---
Cobratoxin	---	---	---	---
Positive controls				
Myoglobin	24300	0.04	---	---
Histamine	---	---	1800	0.11

--- means less than 100 ELISA titer/100 µl.

Toxicologists for a long time have been interested in substances present in venoms. As early as 1965, Anton and Gennaro (1) showed the presence of minute amounts of serotonin and norepinephrine in venoms and various tissue organs of *Agkistrodon piscivorus*, the cotton mouth, and *Crotalus adamanteus*, a rattlesnake. These substances contribute significantly to profound tissue damage (1).

Nerve growth factor was discovered almost half a century ago, first in snake venom (2,3) and later in mouse submaxillary glands. However, the function of NGF in venom is not yet clear (7). Nevertheless, the presence of citrate in snake venom at least clarifies why snake blood does not clot (personal observation).

Myoglobin and histamine are present endogenously in animal sera and urine. Except for honeybee venom containing histamine, this is the first report to prove the presence of myoglobin and histamine in snake, scorpion, toad, and honeybee venoms. This research showed the presence of these substances in venoms. Peck and O'Connor (13) showed the presence of histamine in a single peptide, procamine, of honeybee venom. In our hands, cobratoxin, a single peptide from the venom of *Naja kaouthia*, failed to show detectable presence of myoglobin or histamine by immunological test. What is the function or role of these substances for them to be present in venomous animals? Future research is warranted to explore these questions. The levels of myoglobin and histamine as a consequence of injection of a venom or a single toxin in mice is under investigation in our laboratory.

REFERENCES

01 ANTON AH., GENNARO JF. Norepinephrine and serotonin in the tissue and venoms of two pit vipers. *Nature*, **1965, 208**, 1174-5. [Links]

02 COHEN S. Purification and metabolic effect of nerve growth-promoting protein from snake venom. *Proc. Natl Acad. Sci. USA*, **1959, 46**, 302-11. [Links]

03 COHEN S., LEVI-MONTALCINE, R. Nerve growth stimulating protein from snake venom. *Proc. Natl Acad. Sci. USA*, **1956, 42**, 571-4. [Links]

04 FAGAN DL., SLAUGHTER, CA., CAPRA, JD., SULIVAN, TJ. Monoclonal antibodies to immunoglobin G4 induce histamine release from human basophils in vitro. *J. Allergy Clin. Immunol.*, **1982, 70**, 399-404. [Links]

05 FENTON AW., WEST PR., ODELL GV., HUDIBERG SA., OWNBY CL., MILLS JN., SCROGGINS BT., SHANNON SB. Arthropod venom citrate inhibits phospholipase A2. *Toxicon*, **1995, 33**, 763-70. [Links]

06 FREITAS MA., GENO PW., SUMMER LW., COOKE ME., HUDIBERG SA., OWNBY CL., KAISER I., ODELL GV. Citrate is a major component of snake venoms. *Toxicon*, **1992, 30**, 461-4. [Links]

07 LIPPS BV. Biological and immunological properties of nerve growth factor from snake venoms. *J. Nat. Toxins*, **1998, 7**, 121-30. [Links]

08 LIPPS BV. Detection of nerve growth factor (NGF) in venoms of diverse source: isolation and characterization of NGF from venom of honey bee (*Apis mellifera*). *J. Nat. Toxins*, **2000, 9**, 13-9. [Links]

09 LIPPS BV., KHAN AA. Antigenic cross reactivity among the venoms and toxins from unrelated diverse sources. *Toxicon*, **2000, 38**, 973-80. [Links]

10 MALY FE., MARTI-WYSS S., BLUMBER S., CUHAT-STARK I., WUTHRICH B. Mononuclear blood cell sulfidoleukotriene generation in the presence of interleukin-3 and whole blood histamine release in honey bee and yellow jacket venom allergy. *J. Investig. Allergy Clin. Immunol.*, **1997, 7**, 217-24. [Links]

11 MURTHY KR., HAGHANAZARI L. The blood levels of glucagon, cortisol, and insulin following the injection of venom by the scorpion (*Mesobuthus tamulus concanesis*, Pocock) in dogs. *J. Venom. Anim. Toxins*, **1999, 5**, 48-53. [SciELO] [Links]

12 ODELL GV., FERRY PC., VICK MM., FENTON AW., DECKER LS., COWELL RL., OWNBY CL. GURIRREZ J.M. Citrate inhibition of snake venom proteases. *Toxicon*, **1998, 36**, 1801-6. [Links]

13 PECK ML., O'CONNOR R. Procamine and other basic peptides in venom of the honeybee (*Apis mellifera*). *J. Agric. Food Chem.*, **1974, 22**, 51-60. [Links]

14 PONRAJ D., GOPALKRISHNAKONE P. Establishment of an animal model for myoglobinuria by use of a myotoxin from *Pseudechis australis* (king cobra snake) venom in mice. *Lab Anim. Sci.*, **1996, 46**, 393-8. [Links]

15 RASH LD., KING RG., HODSON WC. Evidence that histamine is the principal pharmacological component of venom from an Australian wolf spider (*Lycosa godeffroyi*). *Toxicon*, **1998, 36**, 367-75. [Links]

16 REID HA. Myogloburia and sea-snake poisoning. *Br. Med. J.*, **1961, 1**, 1284-9. [Links]

17 WU AH., LAIOS I., GREEN S., GORNET TG., WONG SS., PARMLEY L., TONNESEN AS., PLAISIER B., ORLANDO R. Immunoassays for serum and urine myoglobin: myoglobin clearance assessed as a risk factor for acute renal failure. *Clin. Chem.*, **1994, 40**, 796-802. [Links]

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