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Snake Venom Hemorrhagins

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ABSTRACT: Viperine and crotaline snake venoms contain one or more hemorrhagic principles called hemorrhagins. These are zinc-containing metalloproteases characterized by the presence of a protease domain, with additional domains in some of them. They act essentially by degrading the component proteins of basement membrane underlying capillary endothelial cells. The toxins also act on these cells causing lysis or drifting apart, resulting in hemorrhage *per rhexis* or *per diapedesis*. Some of these toxins have been found to exert additional effects such as fibrinogenolysis and platelet aggregation that facilitate hemorrhage. The structural and functional features of this class of toxins have been discussed in this review in an attempt to get a better understanding of their toxicity. This can be of immense therapeutic value in the management of snake venom poisoning, as hemorrhagins are among the major lethal factors in snake venom.

KEY WORDS: viper, snake venom, metalloprotease, hemorrhage, endothelial cell, basement membrane, reprolysin.

I. INTRODUCTION

Snake venoms are known to possess a good number of enzymatic and nonenzymatic components that act in concert to produce the morbidity and mortality in the envenomated subject. They have been broadly classified into two groups depending on the principal target system, neurotoxic (venom of elapid and hydrophid snakes) and hemotoxic (venom of viperid snakes, including the subfamilies Viperinae and Crotalinae[1]). However, the delineation is not always very sharp, as a predominantly neurotoxic venom may contain a very potent hemotoxic component (e.g., the hemorrhagic toxin from the venom of *Ophiophagus hannah*[2]) and vice versa (e.g., the neurotoxic component of *Vipera palestinae* venom[3]).

Viperid venoms induce a vast hemostatic disturbance, several manifestations of which may lead to fatal consequences. The principal ones out of them are coagulation defects, disturbance in platelet aggregation, fibrinogen depletion, and hemorrhage (summarized in Table 1). However, not all venoms show all these pathophysiological effects.

True hemorrhagic toxins, often termed as the hemorrhagins, comprise a major group of active principles in viperid venom. These toxins act directly on the endothelial cells and the underlying basement membrane to induce local and systemic hemorrhage depending on the severity of envenomation. The massive extravasation of RBCs caused by these toxins alone can be responsible for the lethal action of the venom, and thus elucidation of their structure and mechanism of action has become a major area in snake venom research in the past 2 decades. Even if hemorrhage is not severe enough to cause mortality, it can lead to serious pathophysiological conditions, for example, hindrance in proper blood supply to extremities can be greatly debilitating for the envenomated subject.

With the increasing number of hemorrhagic principles being isolated and characterized, accumulation of data for comparison and grouping together for unifying concepts became vital for both academic interests and therapeutic purposes. Two excellent reviews (Bjarnason and Fox, 1988-1989, 1994) have been published in this area. However, much information has been accumu-

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TABLE 1
Hemotoxic Effects of Snake Venoms

Effect	Example	Species	
Decrease in blood pressure by liberation of bradykinin	Kallikrein-like serine proteases	Different viperid snakes	4, 5
Procoagulant Action			
Thrombin-like enzyme activity	Crotalase	*Crotalus adamenteus*	6
	Gabonase	*Bitis gabonica*	7
	Ancrod	*Calloselasma rhodostoma*	8
Activation of prothrombin	Ecarin	*Echis carinatus*	9
Activation of factor X & IX	RVV-X	*Daboia russelli*[c]	10
Activation of factor V	RVV-V	*Daboia russelli*	11
Activation of factor V, VIII, and XIII	Thrombocytin	*Bothrops atrox*	12
Activation of factor XI		*Bitis arietans*	13
Inactivation of antithrombin III		*Causus rhombeatus*	14
Anticoagulant Action			
Activation of protein C			
Thrombomodulin dependent	Batroxobin	*Bothrops atrox*	13
Thrombomodulin independent		*Agkistrodon contortrix*	14
Hydrolysis of phospholipids required for coagulation	Phospholipase A_2	*Vipera berus*	15
Inhibition of formation of prothrombinase complex		*Trimeresurus gramineus*	16
Direct fibrino(geno)lysis			
Digestion of α(A) chain[a]		*Agkistrodon acutus*	17
Digestion of β(b) chain	Protease II and III	*Crotalus atrox*	18
Digestion of γ chain	Atrolysin f[b]	*Crotalus atrox*	19
Stimulation of platelet aggregation			
Phospholipase action (releasing arachidonic acid)	Phospholipase A_2	*Trimeresurus mucrosquamatus*	20
Proteolysis	Crotalocytin	*Crotalus durissus terrificus*	21
Activation of endogenous PLA_2	Convulxin	*Crotalus horridus horridus*	22
	Aggregoserpentin	*Calloselasma rhodostoma*	23
Activation of von Willebrand factor	Botrocetin	*Bothrops atrox*	24
Inhibition of platelet aggregation			
Fibrinogen receptor antagonism	Echistatin	*Echis carinatus*	25
	Trigramin	*Trimeresurus gramineus*	26
5′-nucleotidase (ADPase) action		*Trimeresurus gramineus*	27
Biphasic effect on platelet aggregation		*Daboia russelli*	28
Hemorrhage (local and systemic)		Theme of Discussion of the Review	28

[a] Possess hemorrhagic activity, some are platelet aggregation inhibitor.
[b] A true hemorrhagic toxin.
[c] Formerly known as *Viperia russelli.*

lated since that time regarding the biological function, evolution, and relevance of these toxins in human envenomation. What we want to present in the current review is not just an extension of the understanding of these toxic principles causing hemorrhage. Throughout the whole course of the discussion, we have tried to capture the trend in this field of research and to suggest accordingly what needs to be done for a fuller understanding of hemorrhagins.

Hemorrhagins (the term was introduced by Grotto L. *et al.*, 1967[29]) act directly on the capillary basement membrane and the endothelial cells to cause internal hemorrhage. In mild envenomation, their action is limited to the site of the bite; however, in severe cases hemorrhage can be wide-

spread, involving the whole extremity concerned and even organs distant from the site of the bite, such as heart, lung, kidney, intestine, and brain. In almost all cases, purified hemorrhagins are lethal at a sufficiently low dose, indicating that they can be one of the principal causes of lethality and they do not require synergy from other components of the venom. However, in snake bite, other venom factors may assist them to exert the lethal action and undoubtedly there can be other toxic components in the venom. A point to note here is the case of the snake *Bothrops asper*, the venom of which possesses two hemorrhagins, BH 2 and BH 3, which individually are only weakly hemorrhagic but exert a synergistic effect with the main hemorhagin BaH 1.[30]

Hemorrhagins have all been found to be metalloproteases that are rather specific in their action, both in terms of substrate preference (chief targets are basement membrane component proteins) and also in the peptide bond cleavage pattern. All but one (CVO protease V from *Crotalus viridis oreganus,* which appears to possess Ca^{++} [31]) have been found to contain 1 mol of zinc per mole of toxin. Although proteolytic degradation of basement membrane has been supposed to be instrumental in their ability to cause hemorrhage, the extent to which hemorrhagic and proteolytic potencies correlate is yet to be ascertained beyond doubt. The number of hemorrhagins in the venom of a particular species of snake may be one (e.g., bilitoxin from *Agkistrodon bilineatus*[32]) to many (e.g., seven in *Crotalus atrox*[33] — atrolysin a to g and four in *Bothrops jararaca*[34-36] — HF 1 to 3 and bothropasin). All of the hemorrhagins of a particular species are not equally hemorrhagic. The minimum hemorrhagic dose can be as little as 0.01 μg (HR-IB from *Trimeresurus flavoviridis*[37] venom) to as high as 200 μg (Cerastase from *Cerastes cerastes* venom [38]).

An interesting finding has been that several of the hemorrhagins have been absent in the venom of juvenile snakes and appear only in adult snakes.[39] A search for the signal that initiates the appearance of these toxins in the venom of snakes at a particular age in such species may be of considerable academic as well as practical interest.

As a final introductory point, it must be emphasized that although the hemorrhagins are the main causative agents of hemorrhage, several other factors residing in the crude venom can act as secondary factors that augment the process. Components that cause fibrinogenolysis render blood almost completely incoagulable. Anticoagulant factors directly block the clotting phenomenon. There are platelet aggregation inhibitors and enzymes that release kinin from kininogen. In the absence of blood coagulation and platelet aggregation, the two principal phenomena that occur following damage to blood vessels, hemorrhage initiated by hemorrhagins can go on unchecked, with massive extravasation of RBCs into surrounding tissues, giving rise to swelling, ecchymosis, blistering, cyanosis, and edema.[33] However, because the hemorrhagins are the essential principles responsible for hemorrhage and they alone can exert the lethal action, we have concentrated solely on them in the present review. In terms of biological activity, some hemorrhagins may exert effects other than hemorrhage, for example, myonecrosis (bilitoxin[40] and BaH 1[41]), fibrino(gen)olysis (atrolysin f[19], jararhagin[42]), inhibition of platelet aggregation (atrolysin a[43]), etc. However, only the main one, that is, production of hemorrhage, has been detailed here. One more point to be noted is that snake venoms may contain metalloproteases that are not hemorrhagic at all, a classic example of which is RVV-X from *Daboia russelli.*[10]

II. STRUCTURE AND CLASSIFICATION

All the hemorrhagins hitherto discovered appear to be metalloproteases containing a somewhat conserved proteinase domain. The fact responsible for the wide difference in molecular mass (22[44] to over 100 kDa[45]) is the presence of additional domains at the carboxy side of the proteinase domain, along with varying degree of glycosylation.[46,47] The toxins are synthesized in the venom gland in a latent zymogen form containing a signal (pre-) and a proenzyme (pro-) sequence. This is proteolytically processed, presumably by venom metalloproteases or by autoactivation to give rise to the active enzyme.[48] Thus, the pre-, pro-, and proteinase domain are common to all such toxins. Addition of other domains to this basic structure has resulted in

gradual increase in the molecular mass of the toxins, with alterations in the hemorrhagic potency, as additional domains are thought to modulate their activity.

The P-III class (to be clarified later in this section) of hemorrhagins is structurally related to the ADAMS[49] (*A Disintegrin-like And Metalloprotease-containing proteinS*)/MDCs (*Metalloprotease Disintegrin-like Cysteine-rich proteins*) group of type-1 integral membrane proteins. These hemorrhagic metalloproteases have been classified as members of the **reprolysin** subfamily of metalloproteases.[50]

In the last decade, there has been interesting revelations regarding the formation of hemorrhagins and a group of proteins called disintegrins from a common precursor. This has given rise to the recognition of the nucleotide sequence and the amino acid sequence of the putative precursor and has greatly aided in the classification of these hemorrhagins. The reader is referred to the appropriate articles (Refs. 46, 47, 51–55) for the details. The classification scheme of hemorrhagins emerging from such studies is presented here, which shows their arrangement in four groups based on structural considerations (Table 2).

It has been observed that the P-III class of hemorrhagins is the most potent group in terms of biological activity.These contain the pre- and pro-sequence, the proteinase domain, the disintegrin and the high cysteine domain. A consideration of each of these domains helps to give an idea of the structure of hemorrhagins. However, there still remain gaps in the understanding mainly because of the unavailability of sequence data of most of the hemorrhagins.

A. Pre-Sequence

The hemorrhagic toxins are translated with a pre- or signal sequence, which is highly conserved[46,47] and, in atrolysins, comprises of 18 amino acid residues. In them, processing is thought to occur between Gly18 and Ser19 to release the sequence.[60]

B. Pro-Sequence

All the snake venom metalloproteinases are translated as pre-, pro- proteinases. The cDNA

TABLE 2
Classification of Hemorrhagins

Class	Characteristic features
I	Designated as P-I class (Protein-I) and correspond to N-I class of nucleotide structures. They have only a proteinase domain apart from the pre- and pro- sequences. Their molecular masses vary from 20 to 30 kDa. These are mostly weakly acidic proteins Depending on the hemorrhagins' potency, this class has again been subdivided into I-A and I-B.[33] The hemorrhagic potency is very high and very feeble, respectively. Examples are atrolysin e (IA) and atrolysin b, c, and d (IB), all from Crotalus atrox[56] *Note:* In terms of nucleotide sequence, atrolysin e falls under N-II class, but the disintegrin-like domain coded for by the N-II is absent in the mature protein, making it come under P-I class of hemorrhagins
II	Designated as P-II class corresponding to N-II class of nucleotide structures. They have a proteinase domain and a disintegrin-like domain separated by a spacer region. The molecular mass is 30 to 60 kDa. Their hemorrhagic potency is low. Example is HP-I (*Calloselasma rhodostoma*)[57]
III	Designated as P-III class with correspondence to N-III class. These are highly potent toxins, varying from 60 to 90 kDa in molecular mass. They have a proteinase, a disintegrin-like and a high cysteine domain in succession Examples are atrolysin a (Crotalus atrox),[56] HR-IB (Trimeresurus flavoviridis),[58] jararhagin (Bothrops jararaca),[36] hannahtoxin (Ophiophagus hannah),[2] etc.
IV	Designated as P-IV class, although nucleotide sequence of any such is yet to be obtained. These are very large hemorrhagic toxins with rather low potency and comprise of a proteinase, a disintegrin-like, a high cysteine, and a lectin domain Example is mucrotoxin A (Trimeresurus mucrosquamatus)[59]

sequences of atrolysins show that a 169-residue domain is present between pre- and proteinase domains. This pro-sequence is also highly conserved. One interesting observation is the presence of a seven-residue-long consensus sequence PKMCGVT approximately 20 residues from the amino terminal to the start of the mature protein sequence. This sequence is very similar to the mammalian matrix metalloprotease consensus sequence PRCGV/NPV/LA/G. It has been hypothesized that the venom sequence with the cysteinyl residue acts in a manner similar to[62] the "Cysteine switch"[61] in matrix metalloproteinases (MMP). In MMPs the cysteinyl residue is thought to bind to the zinc at the active site of the enzyme preventing the access to the substrate. The enzyme is active only after proteolytic removal of the cysteinyl residue. The seven-residue-long sequence in the hemorrhagin pro-sequence can also act similarly. This conjecture has been supported by the observation that this sequence can inhibit the proteolytic ability of the enzyme through the interaction of cysteinyl residue with zinc, as has been found in case of atrolysin d.[33] Also, a synthetic peptide corresponding to this segment inhibits the metalloprotease.[63]

C. Proteinase Domain

This is approximately 200 residues long and contains the signature consensus sequence **HELGHNLGXXH**D (bold signifies strict conservation). The histidines seem to be absolutely vital, as the active site zinc is coordinated by them along with a molecule of water in a tetrahedral complex. The histidines are positioned at 142, 146, and 152. The domain has two (e.g., atrolysin b and c at cysteine 308-388 and cysteine 348-355) or three (e.g., in atrolysin a and e at cys 308-388, cys 348-372 and cys 350-355) disulfide bonds.[49] However, there appears to be little structural disturbance by the introduction of the additional disulfide bond where present. Hence, its structural (and functional) role is unclear.

The substrate specificity is thought to be conferred by the substrate binding site and is likely to be modulated by other domains. There appears to be a general preference for a hydrophobic residue in the substrate binding site of the hemorrhagins. Thus, there should be more or less conserved hydrophobic sequence(s) present in the proteinase domains of them. A likely candidate is the stretch of residues 355-361 (XXCI/VMXX) in atrolysins. This structure may act as a hydrophobic pocket for the substrate.[33]

D. Disintegrin-Like Domain

Disintegrins are a family of low molecular mass (49 to 89 amino acids) **cysteine-rich peptides[64] found in viperid venoms[65].** They contain a conserved sequence RGD in a 13-residue β-loop structure (replaced by KGD in barbourin from *Sistrurus miliarus barbouri* [65]) called the RGD loop that is the critical structural moity responsible for their biological activity.[66] These peptides have got a characteristic functional feature of being inhibitors of platelet aggregation by specifically binding to the platelet surface fibrinogen receptor glycoprotein IIb/IIIa (α_{IIb}/β_{III} integrin) complex, thereby blocking fibrinogen-induced platelet aggregation. In kistrin, a disintegrin, the aspartate residue of RGD has been shown to be crucial for this high-affinity binding.[67] It was also observed that two highly conserved disulfide bridges form a motif with respect to the RGD sequence, and all disintegrins lose their activity after reduction of these bridges that destroy this motif.[68,69]

Several high molecular mass (P-III and P-IV) hemorrhagic metalloproteases and some non-hemorrhagic metalloproteases from snake venom possess a domain that has a high sequence similarity to the disintegrins but differ in two important aspects — absence of RGD sequence (replaced by a far less conserved ECD sequence in hemorrhagins like HR-IB and jararhagin) and possession of two additional cysteinyl residues compared with disintegrins (thereby making the -S-S- bond pattern different). Therefore, the domain is called as disintegrin-like domain. It has been speculated that the two "extra" cysteines form disulfide bonds with cysteinyl residues residing within a region amino terminal to the disintegrin-like domain (called the spacer region that joins the metalloprotease and the disintegrin-like domains) and a cysteinyl residue in the cysteine-rich domain, respectively.

The biological function of the disintegrin-like domain remains obscure because of some reasons. Catrocollastatin, a protein isolated from *Crotalus atrox* venom, has been observed to inhibit platelet aggregation, although it lacks the typical sequence RGD.[70] Thus, the target of action seems to be different from GP IIb/IIIa on platelet surface. Again atrolysin a, a very potent hemorrhagin, inhibits platelet aggregation despite the absence of a RGD sequence. To investigate the role of disintegrin-like domain in this phenomenon, Jia et al.[43] tried to express a recombinant disintegrin-like domain. When the expression failed to produce monomer product, a recombinant protein comprising the spacer region, disintegrin-like, and the cysteine-rich domains of atrolysin a was expressed. This protein had the ability to inhibit both collagen- and ADP-stimulated platelet aggregation, suggesting the action of the recombinant protein on $\alpha_2\beta_1$ collagen integrin and/or $\alpha_{IIb}\beta_{III}$ integrin on platelets. Jararhagin, a structural homolgue of atrolysin a, binds to α_2 subunit of $\alpha_2\beta_1$ integrin to inhibit collagen-induced platelet aggregation.[71] IC_{50} values (concentration causing 50% inhibition) of atrolysin a and the recombinant protein fall well within the limit of IC_{50} values for ADP-stimulated platelet aggregation inhibition by the disintegrins, indicating similarity of potency. Kunicki et al. demonstrated that the RGD sequence could be replaced by RYD without loss of biological activity.[72] All these suggest that the presence of RGD is not an absolute criterion for platelet aggregation inhibitory action. The disintegrin-like domain can have a disintegrin-like activity, potentiating hemorrhage.

In the context of hemorrhage production, the disintegrin-like domain may have roles that are yet to be defined. It may modulate the metalloprotease activity, although the results so far obtained support the possibility that this domain renders the hemorrhagin more potent by inhibiting platelet aggregation and not by any effect on the enzymatic potency itself. Zhou et al.[70] hypothesized that "high molecular mass metallo-proteinases of hemorrhagic snake venoms are targeted to a specific site on collagen by the sequence aligned with RGD in the disintegrin-like domain. In this way the disintegrin-like domain could not only restrict the specificity of the metalloproteinase, but also play a synergistic role by attaching the enzyme to the substrate, and thereby increase the local concentration of the enzyme." The domain may also play a role in targeting the hemorrhagin to a particular site of action, for example, a platelet or endothelial cell where the protease action can occur on relevant substrates such as integrins, matrix proteins, or other system pro-proteases. Again, it is quite possible that the inhibition of platelet aggregation is by a totally different mechanism. Many hemorrhagins degrade fibrinogen.[73] Because fibrinogen is an important co-factor in platelet aggregation,[74-76] this depletion of fibrinogen can effectively inhibit the phenomenon,[77-78] leading to unhindered hemorrhage. Proteolytic degradation of platelet receptors (e.g., the collagen receptor $\alpha_2\beta_1$ by jararhagin)[79,80] may also be a major causative factor.

E. High Cysteine Domain

The P-III and P-IV hemorrhagins have a domain carboxy to the disintegrin-like domain that has an unusually high cysteine content. The characteristic sequence pattern ($CX_6CX_4CX_6CX_{14}CX_{12}CX_9CX_6CX_4X_{4/6/10}CX_{15/18/19}CX_5CX_4CX_{7/9/16}$) shows that this domain can be subdivided into two subdomains. The first one has highly conserved cysteine residue positions and the second is more flexible in that regard.[33] The first subdomain, because of its rigid conservation, is thought to be of fundamental significance in the context of the function of the domain.

As this domain is always present in conjunction with the disintegrin-like domain, its function has been thought to be aiding in correct alignment of the disintegrin-like domain. The domain may also be involved in protein-protein interaction.[33,49]

Evidently, the most potent P-III class of hemorrhagins have a multidomain structure. In this context it is important to study the individual function of the domains as well as their overall integrated role. Site-directed mutants can be tried in such studies to identify crucial functional moieties. Classic approaches such as side-chain modifications can help in both elucidation of the particular moieties as well as in detoxification and toxoidation programs. It is also important to find the role for zinc in structural and functional as-

pects. The Zn^{++} can be replaced by a series of divalent cations (both transitional like Cu^{++}, Fe^{++}, Co^{++}, Ni^{++} etc. and non-transitional ones like Ca^{++} and Mg^{++}), and alteration of biological activity, if any, may be monitored. Such a study has been performed with atrolysin a from *Crotalus atrox*, where Zn^{++} has been exchanged with Co^{++} without a major loss of biological activity.[81]

III. *IN VITRO* PROTEOLYTIC ACTIVITY

As the hemorrhagins are metalloproteases, their proteolytic prowess has been observed using a number of substrates. It has been well documented that the hemorrhagins are very specific with respect to the site of action and substrate preference. They cleave peptide bonds at specific sites of specific substrates both *in vitro* and *in vivo*. Hence, for assessing their proteolytic action it was imperative to develop sensitive assay systems using carefully designed substrates rather than employing general nonspecific protease substrates. Table 3 summarizes the different substrates that have been employed to detect the proyeolytic action *in vitro*. In fact, there are certain pre-requisites for the hemorrhagins for the optimal and the most effective action in the victim. As a protease it should inflict maximum damage with specific bond cleavage that makes the action very rapid and effective. This probably explains the high degree of specificity of bond cleavage and the strong substrate preference of these toxins. To produce fatal hemorrhage, they have to act quickly and to specifically attack those components crucial for the integrity of basement membranes.

Both protein and peptide substrates have been employed. The protein substrates include classic nonspecific protease substrates (such as casein, fibrinogen) and the basement membrane components such as collagen, laminin, and nidogen. The original casein digestion method[82] posed some problems in the assessment of the proteolytic action of hemorrhagins — only a small amount of soluble peptides were produced because of the specificity of hemorrhagins. It is only after the development of carefully designed sensitive assay systems that most of the toxins could be tested for their proteolytic activity.

TABLE III
Proteolytic Activity of Hemorrhagins *In Vitro*

Protein Substrates
- *Nonspecific substrates*
  - Casein
  - Azo-conjugated proteins[83,84] (azocasein, azocollagen, azoalbumin, hide powder azure)
  - Fibrinogen[85]
  - Dimethylated casein, dimethylhemoglobin[56,81] (by the method of Lin et al.[86])
  - Muscle proteins (actin, troponin, and tropomyosin)
- *Specific substates (Basement membrane component proteins)*[87,88]
  - Collagen type IV[a]
  - Laminin
  - Nidogen (Entactin)
  - Laminin–nidogen (1:1 complex)
  - Fibronectin
  - Gelatin

Peptide Substrates
- Oxidized B chain of insulin
- Fluorogenic peptide (e.g,. 2-aminobenzoyl-Ala-Gly-Leu-Ala-4-nitrobenzylamide)
- Bee venom mellitin[85]
- Lutenizing hormone releasing hormone (LHRH)[84]
- Insulin analogues (truncated insulin B primary cleavage site peptides)[91]
- Dipeptides produced by solid-phase synthesis[91]

[a] All but two hemorrhagins are incapable of cleaving collagen type I. These two are BaP I (*Bothrops asper*)[89] and moojeni protease A (*Bothrops moojeni*).[90]

One anomaly arose when it was found that some hemorrhagins are very slow in exerting the proteolytic action (detectable peptide bond cleavage occurred only with prolonged incubation) *in vitro* but can induce hemorrhage in experimental animals very quickly after injection. Hemorrhagins from *Crotalus atrox* venom were capable of hydrolyzing basement membrane preparations *in vitro* completely only with prolonged incubation (up to 90 h at 37°C), whereas these toxins are able to produce a hemorrhagic lesion within 5 min of injection. This led to suspicion regarding the correlation between the hemorrhagic and proteolytic activity. We can speculate certain possible reasons for the prompt action *in vivo*. The hemorrhagins may activate endogenous proteases, or can effectively inhibit platelet aggregation. They can cause fibrinogen depletion and thereby render the blood incoagulable. There can, of course, be synergistic action, either by venom contaminants or by endogenous factors that get activated after exposure to the hemorrhagins. These possibilities are open to explore.

Regarding the specificity of bond cleavage, use of insulin B chain (oxidized) has provided great insights. It has been observed that the hemorrhagins have a tendency to cleave X-Leu bond (Table 4). Almost all of them cleave the His(10)–Leu(11), Ala(14)–Leu(15), and Tyr(16)–Leu(17) bonds of oxidized B chain of insulin, along with additional bonds, depending on the particular hemorrhagin.

However, the bond cleavage pattern and specificity should be verified using hemorrhagins' natural substrates (e.g., collagen). A study of such kind has been performed in atrolysin e, but in the absence of similar studies using hemorrhagins from other snake species this can only be considered as a sporadic data and no generalized concept can be deduced from this.

IV. MECHANISM OF HEMORRHAGE

The mechanism of action of the hemorrhagins, that is, how they cause the hemorrhage, has drawn the attention of researchers even before their structure could be resolved. As soon as it was established that the hemorrhagins are metalloproteases, the enzymatic action was given primary emphasis in elucidating the factor(s) responsible for the leakage of blood from the vessels. Subsequently, it became evident that the enzymatic disruption of the basement membrane (BM) underlying the endothelial cells of the capillaries (which have been found to be the prime target of the hemorrhagins) is the main factor responsible for hemorrhage. However, in-depth studies reveal certain other factors that may or actually do facilitate the process. We discuss all these factors sequentially here and try to describe a unifying principle that can be applied as a general mechanism of hemorrhage production.

A. Enzymatic Disruption of Basement Membrane (BM)

Both *in vitro* biochemical studies as well as *in vivo* microscopic observations have confirmed that hemorrhagins cause local hemorrhage by proteolytic digestion of the BM proteins. A brief description of the BM architecture is presented here to explain the nature of attack on it.

Basement membranes are extracellular sheets consisting of certain proteins such as type IV collagen, laminin, nidogen (entactin), fibronectin, and heparan sulfate proteoglycans.[105,106] BMs, also known as basal lamina, are placed beneath the epithelia (thus, obviously under capillary endothelium also).

The chief constituent is type IV collagen, the structure of which is more flexible compared with the fibrillar form. The specialized orientation pattern of these molecules results in the formation of a basic frame-like meshwork to which the other constituents bind by means of specific associations. Laminin is a flexible complex of three long polypeptide chains and assumes the shape of an asymmetric cross.[107] The short arms of laminin bind to collagen. Particular domains of laminin can also bind to heparan sulfate proteoglycan and nidogen.

The molecules of nidogen are of special interest regarding the assembly and degradation of BM. It binds to laminin with very high affinity via the G3 domain and can bind to collagen type IV (and also the proteoglycan) via the G2 domain.[108] Thus, it is thought to act as a bridge between the collagen type IV and laminin networks.[109] Sec-

TABLE 4
Sites of Cleavage of Snake Venom Protease on Oxidized B-Chain of Insulin

	1 5 | 10 15 | 20 25 30 *Phe-Val-Asn-Glu-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala*	Ref.
Hemorrhagic Proteinases		
Rhodostoxin (*Calloselasma rhodostoma*)	↑ ↑ ↑ ↑ ↑ ↑	92
Ac3 (*Agkistrodon acutus*)	↑ ↑ ↑ ↑	93
HR-I (*A.halys blomhoffi*)	↑ ↑ ↑ ↑ ↑	94
HR-II (*A.halys blomhoffi*)	↑ ↑ ↑ ↑ ↑	94
HF2 (*Bothrops jararaca*)	↑ ↑ ↑ ↑	95
Bothropasin (*B. jararaca*)	↑ ↑ ↑ ↑ ↑	96
Proteinase II (*Crotalus adamanteus*)	↑ ↑ ↑ ↑ ↑ ↑ ↑	97
Protease IV (*C.horridus horridus*)	↑	85
Atrolysin A (*C. atrox*)	↑ ↑ ↑ ↑ ↑	98
Atrolysin B (*C.atrox*)	↑ ↑ ↑ ↑ ↑	87
Atrolysin c,d (*C atrox*)	↑ ↑ ↑ ↑ ↑	91
Atolysin *e*(*C.atrox*)	↑ ↑ ↑	81
Atrolysin f (*C. atrox*)	↑ ↑ ↑ ↑ ↑	33
Mucrotoxin a (*Trimeresurus mucrosqumatus*)	↑ ↑ ↑ ↑ ↑ ↑	99
HR a (*T. mucrosquamauts*)	↑ ↑	100
HR b (*T. mucrosquamatus*)	↑ ↑	100
BIlitoxin (*Agkistradon bilineatus*)	↑ ↑ ↑ ↑ ↑ ↑	32
Moojeni protease A (*Bothrops moojeni*)	↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑	104
Non-hemorrhagic proteases		
H2- proteinase (*T. flavoviridis*)	↑ ↑ ↑	101
α -Protease (*C.atrox*)	↑ ↑ ↑	102
Protease A (*B. jararaca*)	↑ ↑ ↑ ↑	95
Peptidase A (*A. piscivorus leucostoma*)	↑ ↑ ↑ ↑ ↑ ↑ ↑	103
Proteinase a (*A. halys blomhoffi*)	↑ ↑ ↑	94

ond, nidogen has been found to be highly influenced by Zn^{++}. Zn^{++} modulates the affinity of nidogen for laminin, collagen IV, and heparan sulfate proteoglycans in a complex fashion. Zn^{++}, at 4°C to 37°C, inhibits binding of nidogen to laminin.[108] The functional importance of Zn^{++} binding to nidogen is not clear, as little is known regarding the role of Zn^{++} in extracellular matrices. The only, and in this connection interesting, point to note is that snake venom hemorrhagic metalloproteases (as well as matrix metalloproteases) contain Zn^{++}. Third, nidogen is highly susceptible to proteolytic degradation, which can allow a rapid disruption of the BM structure.[109]

Thus, the most effective way to degrade BM is to attack either type IV collagen, "the scaffolding structure", or nidogen, "the bridging molecule". In fact, hemorrhagins can effectively degrade both, as confirmed by *in vitro* studies. In addition, they can hydrolyze laminin and fibronectin but not the proteoglycans. These capabilities have made them very effective toxins, mediating disruption of BMs to cause the hemorrhage.

Certain observations, both *in vivo* and *in vitro,* have raised some questions regarding the proteolytic degradation of BM as the sole mediator of hemorrhage. The discrepancy between the complete hydrolysis time of BM *in vitro* and the onset of hemorrhage on toxin administration *in vivo* is the first. Next comes the observation that endothelial cells damaged by atrolysins[89] and ACl-1[110] often had much of their BM intact. In hemorrhagins such as atrolysins the relation between the hemorrhagic and general proteolytic potency is not always parallel and may even be inverse. All these observations have necessitated a search for mechanisms of hemorrhage other than BM degradation. Certain factors that have been suspected to be involved but not yet been conclusively documented are degradation of platelet receptors and adhesive proteins vital for platelet aggregation and fibrinogenolysis, preventing both fibrinogen-induced platelet aggregation as well as coagulation. These are the two main lines of defense against extravasation following injury to blood vessels. Platelet receptors may also be blocked by disintegrin-like domains of P-II and P-III class of hemorrhagins. Proteolytic action may also be manifested in the activation of endogenous proteases. Limited studies have been performed to explore these possibilities. Jararhagin has been found to degrade the main collagen receptor $\alpha_2\beta_1$ integrin and the adhesive ligand von Willebrand factor.

However, despite the observations and possibilities, we consider the BM degradation as the most direct and main, if not the sole, mechanism of induction of hemorrhage by these toxins. There are certain reasons behind the proposition. All these metalloproteases tested so far can specifically cleave BM component proteins. Blockage of proteolytic activity by metal chelators such as EDTA and *o*-phenanthroline inhibits hemorrhage. Indirect proof comes from the fact that collagenases from the bacterium *Clostridium histolyticum* is known to induce hemorrhage in experimental animals.[115] The afore mentions anomalies can be explained by assuming that the hemorrhagic potency of the toxins is related to the action on specific substrates such as BM proteins rather than general nonspecific substrates.[88] This is nicely demonstrated in atrolysins. Atrolysin a and e are much more active on BM preparations than atrolysin b, which is much more potent than the first two in cleaving insulin B chain. In producing hemorrhage atrolysin a and e are much more active than atrolysin b. The appearence of intact BM in atrolysin and ACl-1–treated tissues may be due to the fact that these toxins need not always produce microscopically detectable digestion of BM. The attack on the BM in these cases might have been enough for effective extravasation, although they escaped detection by EM by being physically minute.

B. Effect on Capillary Endothelial Cells

Capillaries, with a single-cell thick wall, are the main targets of the hemorrhagic toxins. Exposure to these toxins induce a disturbance in the endothelial cells (ECs), the degree of which varies from a simple fall-off from the substratum (BM) to complete lysis. Once this was established, investigations turned to explore whether the extravasation is by a *per rhexis*(through the cell by disrupting the plasma membrane and the integrity of the cell) or a *per diapedesis*(through the gaps between the cells, keeping them viable and intact) mechanism. Interestingly, hemorrhagins have adopted both of them — some act

through the former, and the others through the latter (Table 5).

Because of the basic differences in the way endothelial cells are affected (*per rhexis* vs. *per diapedesis*), no unifying principle regarding the action of hemorrhagins could emerge. Cultured endothelial cells (normal and transformed) as well as experimental animals were exposed to crude venoms, and the purified toxins followed by monitoring the effects over an adequate period of time by electron microscopy, cinematography, and vital microscopy. Despite that, the data still remain scattered and incomplete, as systematic and detailed study has not yet been performed in most of these toxins. To complicate the situation, hemorrhagins have often been found to exert additional toxic effects, such as myotoxicity and fibrinogen depletion. In our opinion, formulation of a well-defined investigative approach comprising sequential steps that can be routinely applied to the purified hemorragins is what is needed. Investigators should try to answer certain queries in order to fully elucidate the mechanism of action and to corrborate the data to the real phenomenon of envenomation — how far BM degradation occurs, to what extent this degradation as such affects the endothelial cells, the biochemical phenomena behind the lysis of cells (*per rhexis*) or formation of gaps between cells (*per diapedesis*), whether proteolytic activity other than BM degradation are involved in these processes, whether the cellular effects are direct or an indirect consequence of activation of some other system(s), and the involvement of other factors present in the venom in these processes.

Here we present the observation in a generalized manner so as to draw analogies among the actions of different hemorrhagins acting in a similar fashion and to recognize the fundamental differences in the effects of two different groups of hemorrhagins. This may be important from a therapeutic point of view also, as two different mechanisms may indicate the necessity of separate approaches for treatment.

1. Hemorrhage per rhexis

This is characterized by an initial swelling of cells. Formation of blebs from the luminal plasma membrane occurs within a short interval. Transmission electron microscopic observations frequently depict swollen mitochondria, but intercellular junctions remain unaltered. The cells get detached from the substratum with subsequent or prior rupture of the plasma membrane, allowing the blood to pass through the damaged cells into the surrounding tissue space. Capillary BM, at the same time, gets disorganized and is often wholly or partially absent.

Many larger vessels in most such cases have been found to be congested with erythrocytes and platelets. Persistent hemorrhage occurs in capillaries in the form of extravasated and hemolyzed erythrocytes. Capillaries are also congested with platelets. With advancement of time they become very obscure due to extensive damage to the cells. In some capillaries platelets appear outside the lumen, again suggesting direct damage to the plasma membrane. A large amount of

TABLE 5
Mechanism of Extravasation by Hemorrhagins

Toxin	Species	Mechanism	Ref.
ACI–1	*Agkistrodon contortrix laticinctus*	*Per rhexis*	110
Bilitoxin	*Agkistrodon bilineatus bilineatus*	*Per rhexis*	40
HT-1 and -2	*Crotalus ruber ruber*	*Per rhexis*	111
Atrolysin a	*Crotalus atrox*	*Per rhexis*	111,112
Proteinase IV	*Crotalus horridus horridus*	*Per rhexis*	113
Proteinase H	*Crotalus adamenteus*	*Per rhexis*	114
HR-1,-2a,-2b	*Trimeresurus flavoviridis*	*Per diapedesis*	115
BaH 1	*Bothrops asper*	*Per diapedesis*	116

intravascular as well as extravascular fibrin is also presnt.

Vital microscopy,[89] in case of exposure of the capillaries to BaP1, reveals that hemorrhagic events occur in capillaries and small vennules and are of an explosive nature. RBCs escape to the interstitial space intermittently giving rise to "burst-shaped microhematomas". No micro-hemorrhage originated from large vessels or arterioles. The hemorrhage, at least in this case, has been found to be "an explosive and intermittent event rather than a slow and sustained process".

The sequence in which endothelial cell damage and BM degradation occurs or whether both occur concomitantly has not yet been determined conclusively. Apart from the direct mechanism of cell damage, some indirect ones have also been suggested. BaH1 and BaP1 (*Bothrops asper*) have been studied in detail, and Rucavado et al. (1995) have suggested that EC degeneration *in vivo* is only a secondary event resulting from disturbance in the interaction between these cells and the surrounding BM. Thoumine et al. (1995)[117] have observed that hemodynamic shear stress modulates the composition of the extracellular matrix (ECM) of endothelium indirectly inducing alterations in these cells. When BM starts getting degraded, tangential fluid shear stress and hydrostatic pressure in capillaries may have substantial effect on the functional anatomy of ECs. BaP1 and moojeni protease A (*Bothrops moojeni*), unlike other hemorrhagins, can degrade collagen type I, which can induce widespread ECM degradation. As cell morphology is greatly influenced by the matrix, ECM damage may well prove to be enough to alter different cells, including ECs even if the toxin does not exert cytotoxicity as such.

Direct cytotoxicity *in vitro* has been demonstrated in the hemorrhagic toxin from *Crotalus ruber ruber*(HT-1 and HT-2) and *Crotalus atrox*(atrolysin a and d).[111] However, such studies have not been conducted in other hemorrhagins, and it is too early to comment on this aspect of toxin action in a generalized mode.

2. Hemorrhage per diapedesis

In contrast to the mechanism described, this process does not render the ECs nonviable. The cells *in vivo* get detached from the substratum, get rounded, and fall off into the lumen following exposure to the toxins that cause hemorrhage *per diapedesis*. However, they are not ruptured and do not become leaky. Extravasation occurs from widened cell-cell junctions. If the toxins are thoroughly washed off of the detached cells they can be recultivated with normal viability and monolayer formation just like the normal (untreated) cells. Their viability can be confirmed by trypan blue incorporation studies following toxin exposure. The ECs, in such cases (as with the pericytes) appear to undergo some functional and organic changes rather than morphological ones. However, as also in case of hemorrhage *per rhexis* the molecular details behind the changes are yet to be characterized.

In the absence of any kind of study regarding the details of molecular phenomena, we are to suspect the proteolytic prowess of the hemorrhagins once again as the chief reason. This may disrupt the assembly and organization of membrane proteins resulting in lysis (*per rhexis* mechanism) or drifting apart of cells (*per diapedesis* mechanism). The hemorrhagins may directly attack the subcellular organization or they may initiate some signaling phenomena culminating through a cascade of events, resulting in hemorrhage. It is quite possible that different hemorrhagins adopt different strategies to cause hemorrhage. It is also unclear whether the different mechanism of hemorrhage reflect real differences in the mode of action or are these merely due to differences in time, dose, or route of administration. Future studies on the action of hemorrhagins on the ECs can be directed toward elucidation of the biochemical phenomena and thereby exploration of these possibilities. In this context it should be mentioned that there may be factors in the venom that indirectly assist in hemorrhage, for example, the vascular apoptosis-inducing factor (VAP) from *Crotalus atrox* venom induces slow apoptosis in vascular endothelial cells, thereby making the way for widespread systemic hemorrhage in the envenomated subject hours after bite.[118]

V. INTERACTION WITH α_2-MACROGLOBULIN

The plasma protease inhibitors comprise the third largest group of functional proteins in

human plasma. These are associated with the control of a variety of events in which protease actions are involved. Of all these inhibitors, α_2-macroglobulin plays an unique role being an inhibitor of all four classes of endopeptidases viz. serine, cysteine, aspartate, and metalloproteases and backing the primary function of other inhibitors. α_2-macroglobulin is a large (720 kDa) tetrameric glycoprotein comprising two noncovalently linked pairs of identical disulfide-linked subunits. This can form 1:1 or 2:1 stoichiometric enzyme-inhibitor complexes with proteases of all four classes.[119,120] The inhibitory action of α_2-macroglobulin has been explained via the "Trap model".[119,121-126] A large variety of proteases have been found to form complexes with α_2-macroglobulin,[120,127,128] and the rate of complex formation appears to depend on the nature and the molecular mass of the protease.[136]

Where other proteases, by means of their strict specificity, are supposed to regulate the different physiological phenomena, the broad specificity of α_2-macroglobulin probably renders it active against proteases from exogenous sources also. Thus, α_2-macroglobulin can act against proteases secreted by invading organisms that reach the blood.[129] In this context, it becomes very important in cases of envenomation by viperid snakes in which a substantial amount of exogenous proteases is administered into different tissues of the victim and, ultimately, to the blood. Besides that, α_2-macroglobulin is the principal metalloprotease inhibitor in blood. These two reasons make it a perfectly suitable candidate for study regarding its interactions with venom proteases, including the hemorrhagins. The development of the pathological condition as a result of protease action, then, must be by overcoming the plasma protease inhibitors, the chief one of which, in this context, is α_2-macroglobulin. Studies have shown that α_2-macroglobulin is effective in inhibiting the proteolytic effects of certain crotalid venoms.[130] On the other hand, some cobra and viper venoms cause inhibition of α_2-macroglobulin in human serum at a particular concentration, probably by generating endogenous proteases that block α_2-macroglobulin.[131] Several snake venom proteases that are not hemorrhagic have been inhibited by α_2-macroglobulin: fibrolase from *Agkistrodon contortrix contortrix,*[132] habutobin from *Trimeresurus flavoviridis,*[133] and basilase from *Crotalus basiliscus basiliscus,*[134] whereas some inactivated α_2-macroglobulin, for example, proteinase-a, -b, -c from *Crotalus basiliscus* (they also inactivate $_2$-antiplasmin).[135] Thus, there seems to be a variety of interactions between venom proteases and α_2-macroglobulin.

The interaction between the hemorrhagins and α_2-macroglobulin has been worked out in some detail in *Crotalus atrox, Crotalus adamenteus,* and *Bothrops jararaca.* Proteinase H from *Crotalus adamenteus* is neither inhibited by α_2-macroglobulin nor does it inhibit α_2-macroglobulin.[132] Among the toxins that have been found to be inhibited are atrolysins c, d, and e (as evident from the loss of activity against gelatin type I and collagen type IV)[136] and protease II from *Crotalus adamenteus* (as evident from its inability to digest α_1 protease inhibitor).[137]

However, jararhagin was only incompletely inhibited by α_2-macroglobulin,[138] and atrolysin a showed almost no inhibition.[136] The inhibition of jararhagin is incomplete even in the presence of a large molar excess of α_2-macroglobulin despite detectable bait region cleavage by the toxin. It has been proposed that the factor responsible is the low ability of jararhagin to form a stable covalent complex with $_2$-macroglobulin on usual cleavage in the bait region. A considerable amount of cleavage occurs in the bait region, suggesting it to be a good substrate for the metalloprotease jararhagin, but it fails to form a stable covalent "trapped" complex with α_2-macroglobulin.[138] In contrast to jararhagin, the bait region of α_2-macroglobulin has been supposed to be a poor substrate for atrolysin a, which explains why it cannot cleave α_2-macroglobulin bait region effectively to be physically entrapped like other proteases that get inhibited.[136]

The inhibition of hemorrhagic (and also non-hemorrhagic proteolytic toxins by plasma protease inhibitors like α_2-macroglobulin is definitely an important factor in controlling the local and systemic effects following envenomation. Hemorhagins, if not effectively inhibited by plasma protease inhibitors, can go on unchecked and cause debilitating, often fatal hemorrhage. It is interesting to discover that all the hemorrhagins cause hemorrhage after envenomation, whereas some of them have been found to be inhibited by

α_2-macroglobulin *in vitro*. Hence, there remains a disparity between the *in vivo* and *in vitro* observations. There may be two reasons. First, there are possibly enzymes that interact more readily or in a higher stoichiometric ratio (thus out competing the hemorrhagins) with α_2-macroglobulin, and, second, there are factors in snake venoms that initiate the formation of endogenous proteases in the blood of the victim, most of which causes coagulation defects in blood. These proteases generated by venom action may preoccupy α_2-macroglobulin readily, rendering it unavailable for interaction with hemorrhagins.

CONCLUSION

Throughout the discussion on hemorrhagic toxin of snake venoms we have tried to weave the present data and reasonable speculations emerging therefrom. Our aim has been to arrange the discrete and somewhat sporadic data in order to clarify one of the major pathophysiological consequences of envenomation by snake venoms. This makes sense not only in the context of snake bite but also in understanding hemorrhage in a more general way. For the snakes, this class of powerful toxins are intimately related to predation as well as digestion of prey, and thereby is of immense survival value. The assault on the hemostatic system of the prey has been much perfected somewhat and sharpened with the appearance of hemorrhagins.

On practical grounds other than the serious pathological conditions, they also need in-depth studies. If they are found to be selectively cytotoxic (as atrolysin a and d, HT-1 and -2), these can have application potential in fields such as tumor biology. Although it is only too early to comment on these possibilities, future research can be directed to explore these areas.

Finally, we consider the potentiality of the hemorrhagins as potent candidates for toxoidation. Previous studies in our laboratory on toxoidation against the venom of Russell's viper (*Daboia russelli russelli*), a major poisonous snake of India, have yielded encouraging results.[139-142] γ-Radiated crude venom can be used effectively for active immunization, and this can successfully neutralize the hemorrhagic effect, one of the major causes of lethality of the venom. Current studies have been undertaken to isolate the causative agent(s) of hemorrhage and to utilize it (them) for toxoidation. If it succeeds, similar programs can be undertaken to effectively combat the problem of envenomation by hemotoxic snake venoms worldwide.

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