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Fibrinogenolytic Afibrinogenemia After Envenomation by Western Diamondback Rattlesnake (*Crotalus atrox*)

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The absence of fibrinogen and the presence of plasmic fragments X, Y, D, and E were demonstrated in a patient bitten by a western diamondback rattlesnake, *Crotalus atrox*. The factor VIII level and the platelet count were within normal limits. There were distinct changes of protease inhibitors in the patient's plasma. Alpha-1-protease inhibitor was elevated. Antithrombin-III was only slightly decreased after the envenomation, but α_2-antiplasmin and α_2-macroglobulin were initially significantly lowered, returning to normal values in 38 and 3 days, respectively. Plasmin-α_2-antiplasmin complex was present until day 10 after the envenomation. However, purified plasminogen was not activated in vitro by the venom. Cultured endothelial and smooth muscle cells from human blood vessels released an increased amount of plasminogen activator upon incubation with the venom. The release did not result from cell lysis. Platelets in normal human platelet-rich plasma were aggregated by 10 μg/ml of the venom, without serotonin secretion. The aggregation kinetics and serotonin secretion induced by adenosine diphosphate (ADP) or arachidonate were not significantly affected by the venom at 1–10 μg/ml. It is concluded that the predominant mechanism of afibrinogenemia in the patient after *Crotalus atrox* bite resulted from primary fibrinogenolysis and not from a consumptive coagulopathy. The lytic state seemed to be induced through an indirect activation of plasminogen by vascular plasminogen activator, which was probably released from endothelial cells and smooth muscle cells by the snake venom.

THE MAJORITY OF poisonous snake bites in the United States come from the venomous family Crotalidae, which includes rattlesnakes. The eastern (*Crotalus adamanteus*) and western (*Crotalus atrox*) diamondback rattlesnakes are the most dangerous and cause 80% of all snake bite fatalities, although these snakes are responsible for only one-tenth of the total number of bites. Adult *C. atrox*, a large snake, inflicts the most severe bites, which frequently result in tissue loss or amputation and occasionally in death.[1] The clinical symptoms depend on severity of envenomation and include local tissue damage, edema, ecchymosis and discoloration around the bite area, hemorrhagic vesicles and bullae, necrosis of the injured tissue, and pain immediately following the bite. The symptoms and treatment of patients bitten by *C. atrox* have been reviewed.[1,2] Clinical reports have addressed the use of antivenom or corticosteroids, cooling as first aid, and surgical debridement as forms of treatment.[3–5] The reports demonstrated that venom of this rattlesnake produced deleterious local tissue damage, decreased platelet count, and rendered blood incoagulable. Human envenomation by *C. atrox* has been studied in regard to the efficacy of antivenoms, cooling of the bitten area, surgical fasciotomy, or their combinations for limb salvage. However, systematic studies of coagulopathies following *C. atrox* bite in patients are not available, and the mechanism of hemostatic changes caused by the venom in humans remains controversial.

The venom of *C. atrox* contains fibrinolytic proteases[6–10] and hemorrhagic toxins.[11] Conflicting reports

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on the absence or presence of fibrinogen-clotting activity reflected the fact that the venom from young snakes does clot fibrinogen and plasma, but the venom from older snakes has no procoagulant activity.[12] These data are in contrast with the action of *C. adamanteus* venom, from a closely related rattlesnake, which contains crotalase, a potent fibrinogen-clotting enzyme.[6,13] In order to study the effect of *C. atrox* venom in vivo, commercial dried venom was dissolved and administered locally into rabbits. It was concluded that an induced consumptive coagulopathy, rather than a primary action of the venom, may be a major factor in bleeding states.[3] This view was disputed,[4,5] and it is not supported by this work.

We report the results of coagulation studies on a 38-yr-old female who developed afibrinogenemia following the bite of the western diamondback rattlesnake. The patient was not treated with antivenom or steroids and thus represents a case of the natural history of venom-induced coagulation pathophysiology and recovery. We demonstrate a systemic fibrinogenolytic state in the patient and provide laboratory data showing that *C. atrox* venom contains agents that mediate fibrinogen cleavage in vivo and in vitro. Experiments were performed to assess whether disseminated intravascular coagulation occurred in the patient, and studies were done to explain the mechanism of indirect activation of plasminogen after envenomation by the western diamondback rattlesnake.

CASE REPORT

A previously healthy 38-yr-old white female presented to the hospital 16 hr after having been bitten on the left foot by a western diamondback rattlesnake (*C. atrox*). The snake, about 1 m long, was identified by the accompanying person on the basis of colored diamondback patterns, and later the patient identified the snake from a picture. Initially, the patient felt well and showed no adverse effects from the bite. However, within 12 hr of being bitten, nausea, vomiting, and profound pain with swelling of the affected limb prompted her to seek medical care. Physical examination at the time of admission was normal except for the left lower extremity. The dorsum of the left foot revealed fang marks surrounded by a 10-cm hemorrhagic bulla. The circumference of the left lower extremity was several centimeters greater than the right, with nonpitting edema in its entirety, distally from the inguinal ligament. Petechiae and ecchymoses were present in the dependent areas of the limb. Neurologic examination was normal in the affected limb. Femoral, popliteal, and posterior tibial pulsations were intact in the affected limb and were symmetrical in comparison to the right leg. Laboratory examinations showed normal chest x-ray and electrocardiogram. The following assays in plasma were within normal limits: urea nitrogen, creatinine, glucose, bilirubin, calcium, phosphates, aspartate aminotransferase, alkaline phosphatase, sodium, potassium, chlorides, and bicarbonate. Initial clotting studies revealed incoagulable blood, characterized by markedly prolonged prothrombin time, activated partial thromboplastin time, thrombin time, and reptilase time (Table 1). This was accompanied by the absence of thrombin-coagulable fibrinogen (Fig. 1).

Due to the patient's delay in seeking treatment, no antivenom or steroids were administered. Initially, 4 U of fresh frozen plasma and 10 U of cryoprecipitate were administered in an attempt to support the coagulation system. Other than this initial treatment (which, as Fig. 1 demonstrates, had no effect), no specific therapy was used. Superficial debridement was performed on day 2, using sodium pentothal as an anesthetic. Daily blood samples were obtained over a period of 15 days for laboratory testing.

The patient showed slowly improving clinical condition and coagulation abnormalities during hospitalization. Bleeding time was normal 2 days after the bite. It should be noted, however, that in the first 7 days, tourniquet pressure for venipuncture resulted in breakdown of previously sealed venipuncture wounds. With this exception, no other hemorrhagic phenomena or complications were observed. She was discharged on day 21 in good health except for a slowly healing, postdebridement, granulating wound of the dorsum of the left foot in the area of the original snake bite. Her coagulation studies were all within normal limits at the time of discharge and on day 38, when another blood sample was obtained and tested.

Table 1. Coagulation Studies of Patient's Plasma

Time After Bite	Prothrombin Time (sec)	Partial Thrombo-plastin Time (sec)	Thrombin Time (sec)	Reptilase Time (sec)	Factor VIIIc* (%)	Platelet Count (per μl)
16 hr	†	†	†	†	102	162,000
1 Day	—	—	†	†	—	274,000
2 Days	‡	‡	‡	‡	—	234,000
3 Days	‡	‡	‡	‡	—	—
5 Days	16.0	34.7	29.4	22.9	—	455,000
8 Days	12.4	30.9	14.5	15.0	130	581,000
10 Days	9.7	28.4	9.4	12.4	—	—
12 Days	11.4	29.5	8.4	11.9	—	—
15 Days	11.1	30.8	8.4	12.2	90	—
38 Days	10.9	31.0	9.6	12.2	—	—
Normal value range	10–12	28–36	8–11	10–13	50–180	150,000–400,000

*Coagulant activity of factor VIIIc expressed as percent of normal mean.

†No clot after 120 sec.

‡Clot absent, but small amount of flocculated material noticed.

Clotting time and factor VIIIc activity are mean values of three determinations.

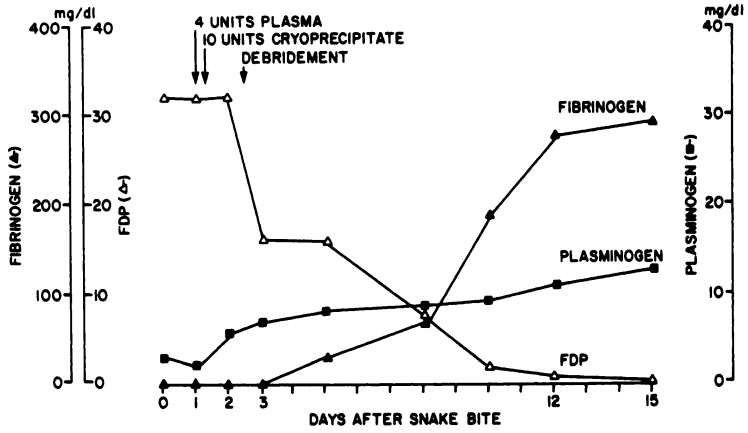

Fig. 1. Clinical course of patient envenomated by *Crotalus atrox*. The first specimen was obtained 16 hr after snake bite, and it is plotted as day 0. The tests for thrombin-coagulable fibrinogen (▲) and plasminogen (■) were done using citrated plasma. Fibrinogen/fibrin degradation products (FDP, △) were measured by a latex particles agglutination assay in a supernate after centrifugation of the whole blood, incubated at room temperature for 2 hr.

MATERIALS AND METHODS

Reagents

Freeze-dried *C. atrox* venom was obtained from Sigma Chemical Co., St. Louis, MO; human fibrinogen (grade L) from A.B. Kabi, Greenwich, CT; streptokinase (Varidase) from Lederle Laboratories, Pearl River, NY; thrombin (topical) from Parke, Davis & Co., Detroit, MI; human α-thrombin was kindly provided by Dr. John W. Fenton II, New York Department of Health, Albany, NY. Reptilase, purified from *Bothrops atrox* venom, was purchased from Pentapharm Ltd., Basel, Switzerland. Plasminogen, in a Glu-form, was purified from fresh normal citrated human plasma by affinity chromatography on lysine-Sepharose.[14] Purified protease inhibitors of human plasma were obtained from the following sources: α_2-antiplasmin and antithrombin-III from Dr. M. Wickerhauser, American Red Cross, Bethesda, MD; α_2-macroglobulin from Dr. F. Kueppers, Temple University, School of Medicine, Philadelphia, PA; α_1-protease inhibitor (α_1-antitrypsin) from Sigma Chemical Co.

Determination of Clotting Factors

To obtain platelet-poor plasma, the patient's blood was mixed either with 1/10 volume of 3.8% sodium citrate containing 1 *M* ϵ-aminocaproic acid, or with sodium citrate only, for determination of plasminogen activity, and centrifuged at 2,000 g at room temperature for 20 min. Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), reptilase time (RT), and recalcification time were performed according to standard procedures[15] using a fibrometer (BBL, Division of Becton, Dickinson and Co., Cockeysville, MD). Simplastin (General Diagnostics, Morris Plains, NJ) was used for PT and Inosithin (Associated Concentrates Inc., Woodside, NY) for APTT. Factor VIII was assayed for coagulant activity using a modified two-stage method.[16] Fibrinogen in plasma was measured as coagulable material formed upon addition of thrombin;[17] since very low levels of fibrinogen were found shortly after the snake bite, its concentration was also tested by the determination of the material precipitable at 56°C for 3 min.[18] Fibrinogen/fibrin degradation products were measured in serum as incoagulable fibrinogen-related antigen by the Thrombo-Wellco (Burroughs Wellcome Co., Research Triangle Park, NC) agglutination test, using latex particles coated with antibodies against human fragments D and E.[19] The latex agglutination test was done in a supernate of patient's blood, which was collected without anticoagulants, incubated at room temperature for 2 hr, and centrifuged at 2,000 *g*.

Plasminogen and Protease Inhibitors

The concentration of plasminogen and protease inhibitors, α_2-antiplasmin, α_2-macroglobulin, α_1-protease inhibitor (α_1-antitrypsin), and antithrombin-III, was determined in plasma by a radial immunodiffusion technique.[20] Monospecific antisera were obtained from commercial sources: anti-α_2-antiplasmin (Nordic Immunological Laboratories, Tilburg, The Netherlands), anti-α_2-macroglobulin (Hyland, Costa Mesa, CA), anti-α_1-protease inhibitor (Cappel Laboratories, Cochranville, PA), and anti-antithrombin-III (Behring Diagnostics, Somerville, NJ). Antiserum against human Glu-plasminogen was prepared in rabbits immunized intracutaneously with the antigen mixed in equal volumes of complete Freund's adjuvant. This antiserum was absorbed with plasminogen-free human plasma obtained by column chromatography on Lysine-Sepharose.[14] Antiserum against human fibrinogen was also prepared by immunization of rabbits and absorption with normal human serum.[21]

The activity of antithrombin-III was assayed by inhibition of thrombin using a two-stage method.[22] Plasminogen activity was determined from the rate of amidolysis of a chromogenic substrate, D-Val-Leu-LysNH-C_6H_4-NO_2·2HCl (S-2251, Ortho Diagnostics Inc., Raritan, NJ) after activation with streptokinase.[23]

Plasmin Complexes

Crossed-immunoelectrophoresis[24] was used for the detection of plasmin complexes with the four plasma protease inhibitors listed in the previous paragraph. Patient's plasma was separated in the first direction in 1% (w/v) agarose (SeaKem, Bausch and Lomb, Inc., Rochester, NY) in 0.05 *M* sodium veronal buffer, pH 8.6. The second direction was performed in 1% (w/v) agarose containing a 1:50 dilution of monospecific antiserum against the tested human plasma protease inhibitor. The washed agarose gels were stained with Coomassie Brilliant Blue R-250.

Gel Electrophoresis

Polyacrylamide slab gel electrophoresis in the presence of SDS[25,26] was used for the separation of plasma proteins. A linear concentration gradient of 3.5%–9% (w/v) of acrylamide gave good resolution of fibrinogen plasmic fragments X, Y, D, and E. The protein bands separated in 0.1 × 10 × 14 cm slab gels were transferred from the polyacrylamide gels to nitrocellulose paper sheets (Trans-Blot, Bio-Rad, Richmond, CA)[27] in a transverse electric field at 4°C (Electroblotter, BioRad, Richmond, CA) at 200 mA for 30 hr. Nitrocellulose paper containing the transferred plasma proteins was shaken gently at room temperature for 1 hr in a buffer containing 1% (w/v) bovine

serum albumin, 0.01 *M* Tris-HCl, 0.15 *M* NaCl, pH 7.5, to block any reactive sites of the nitrocellulose. The sheet was incubated for 2 hr in the same buffer containing rabbit antiserum against human fibrinogen diluted 1:100. After washing the sheets with the buffer, the nitrocellulose paper was shaken in the buffer containing 0.1 mg/ml of IgG fraction of goat antiserum against rabbit IgG, coupled with horseradish peroxidase (Cappel Laboratories, Cochranville, PA). After extensive washing, fragments with fibrinogen-related epitopes were demonstrated by the reaction of peroxidase with o-dianisidine (3,3'-dimethoxybenzidine, Sigma Chemical Co.), resulting in staining of the corresponding bands.[28]

Interaction With Fibrinogen

The changes of purified or plasma fibrinogen coagulability by *C. atrox* venom were followed by thrombin time. The results were calculated as a ratio of sample clotting time to that of control, where the latter contained buffer instead of the venom. In some experiments, after completion of the digestion, venom proteases were inhibited by treatment 3 times, 15 min each, with phenylmethylsulfonyl fluoride (PMSF) at 1 m*M* final concentration.

Platelet Aggregation and Release

The platelets were counted in blood or platelet-rich plasma by phase microscopy using a standard Neubauer hemacytometer.[29] Platelet aggregation and serotonin release were measured over the same time period on the same platelet preparations. The serotonin in the platelet granules was labeled by incubation of platelet-rich plasma with 0.25 μM ^{14}C-serotonin (New England Nuclear, Boston, MA) at 37°C for 30 min prior to aggregation. Platelet aggregation was done, as described previously,[30] in a Chronolog aggregometer (Chronolog Corp., Havertown, PA) at 37°C, using as aggregating agent either adenosine diphosphate (ADP; 8 μM final concentration) or sodium arachidonate (1 m*M* final concentration). After 3 min aggregation, subsamples were withdrawn, treated with formaldehyde-EDTA mixture, and the percent of ^{14}C-serotonin release was determined in the supernate after centrifugation.[31]

Cell Cultures

Human endothelial cells (EC) were obtained from umbilical cord veins by collagenase (Type II, Sigma Chemical Co.) treatment.[32] Human smooth muscle cells (SMC) were derived from the remaining veins after additional collagenase and elastase (Type II, Sigma Chemical Co.) digestion.[33] The cells were cultured in 60-mm plastic tissue culture dishes at 37°C in a 95% air and 5% CO_2 atmosphere in 3 ml of Dulbecco's Minimum Essential Medium supplemented with 10% (v/v) fetal calf serum (Reher's Chemical Co., Phoenix, AZ). The medium was replaced with a fresh one every 2 days. EC formed confluent monolayers 6 days after planting. SMC grew in typical hill and valley patterns and were also cultured for 6 days. The cultures of EC and SMC contained 10^6 cells/dish. Six-day-old cell cultures were held without feeding for another 4 days to minimize the suppression of cell-associated plasminogen activator activity by serum inhibitors.

Preparation of Cell Releasates and Extracts

The cells were washed 3 times with serum-free medium and incubated in 3 ml of the same medium at 37°C in a 95% air and 5% CO_2 atmosphere for various times, either in the absence or presence of the snake venom, to release plasminogen activator into the medium (releasate). The extent of cell lysis was assessed by protein determination in the medium and by measurement of lactic dehydrogenase (LDH) in the medium and in the remaining cells.[34] The cells remaining after incubation were lysed with 2 ml of 0.015 *M* Tris-HCl, 0.15 *M* NaCl buffer, pH 7.4, containing 0.01 *M* EDTA and 0.025% (w/v) Triton X-100 (Rohm and Haas Co., Philadelphia, PA). The treatment was sufficient to release intracellular membrane-bound plasminogen activator. After a 15-min incubation at room temperature, the mixture was centrifuged at 8,500 *g* for 3 min in an Eppendorf centrifuge (Model 5412, Brinkman Instruments Inc., Westbury, NY) and the supernate was collected (extract). Plasminogen activator was determined both in releasates and extracts. In addition, protein concentration was determined in both solutions by the method of Bradford[35] using human serum albumin as a standard protein.

Determination of Plasminogen Activator

Plasminogen activator activity was measured by a solid-phase fibrinolytic assay using polystyrene test tubes 12 × 75 mm (no. 55,476, Sarstedt Inc., Princeton, NJ) coated with ^{125}I-human fibrin.[36] The substrate for tube coating was labeled by the ^{125}I-iodine monochloride technique.[37] Each tube contained 2 μg of fibrin, 40,000 cpm, and 2 μg of human Glu-plasminogen. The total amount of solubilized radioactivity was expressed in micrograms of lysed fibrin and was a measure of plasminogen activator activity. Radioactivity measured in liquid phase, in the absence of cell-derived material, was subtracted during calculations. In the absence of plasminogen, there was no lysis of fibrin by releasates; however, extracts had some endogenous proteolytic activity, which was subtracted from the corresponding samples.

RESULTS

Studies With Patient's Plasma

The patient's clinical course showed three periods on the basis of coagulable fibrinogen concentration in plasma (Fig. 1): (1) acute, days 0–3, characterized by the absence of coagulable fibrinogen; (2) recovery, days 4–11, associated with steadily improving hypofibrinogenemia; (3) normal state, commencing with day 12, with plasma fibrinogen concentration in the normal range.

An absence of coagulable fibrinogen in plasma in the acute period was supported by precipitation of only a small amount of heat-labile protein from plasma in the same period. An attempt to measure incoagulable fibrinogen by radial immunodiffusion did not furnish quantitative results, since two concentric rings were observed, but this fact demonstrated the presence of fibrinogen degradation products in the patient's plasma at high concentration (Fig. 1). The platelet count was 162,000/cu mm at 16 hr after the bite (Table 1), which is within the normal range.

In the recovery period, thrombin-coagulable fibrinogen appeared, and its concentration steadily increased, reaching the normal range on day 10 (Fig. 1); at the same time, incoagulable fibrinogen antigen in serum, corresponding to fibrinogen/fibrin degradation products, decreased to 2 mg/dl. In this period, the amount of heat-precipitable protein in plasma paralleled the amount of thrombin-coagulable fibrinogen, and the concentration of fibrinogen antigen in plasma determined by radial immunodiffusion corresponded to that

measured as thrombin-coagulable material. The platelet count increased during the recovery period, and thrombocytosis developed after 5 days. Since the patient was asymptomatic and doing well, the platelet count was not determined in the subsequent days.

Factor VIII coagulant activity was normal at the time of admission and remained within the normal range during hospitalization. Since she was neither pregnant nor on oral contraceptive pills, and was in good health prior to envenomation, it was assumed that her factor VIII level was in normal range before the snake bite.

Types of Fibrinogen Degradation Products in Patient's Plasma

In order to assess which enzymes were operative in the patient after the snake bite, plasma proteins were separated according to molecular weights in SDS-polyacrylamide gel electrophoresis and fibrinogen fragments detected using monospecific antibodies (Fig. 2). Fibrinogen in normal human plasma (NHP) consisted of two major populations of mol wt 340,000 and 320,000; occasionally, a faint third band of mol wt 290,000 was seen. In vitro, fibrinogen in plasma is cleaved by the venom to form a mol wt 325,000 product;[38] plasmin degrades fibrinogen into fragments X (mol wt 250,000), Y (150,000), D (103,000), and E (50,000).[39] Patient's plasma obtained on the day of the snake bite contained large amounts of fragments Y and D, small quantities of fragments X and E, and a small amount of fibrinogen of mol wt 340,000. The electrophoretic band of fragment D appeared as a doublet. There were no bands of mol wt 325,000 corresponding to fibrinogen degradation products formed by the venom. The same immunoelectrophoretic pattern was found on day 1. During days 2–5, the intensity of bands of all plasmic fragments decreased. These data indicated that the predominant mechanism of fibrinogen degradation in the patient's circulation may have been mediated by plasmin. On day 8, fibrinogen of mol wt 340,000 appeared in the circulation, in agreement with results in Fig. 1 and Table 1. The population of the fibrinogen molecules of mol wt 320,000 was evident on day 15. After reappearance of fibrinogen, another band was noticed with slower electrophoretic mobility, corresponding to mol wt 350,000. This fibrinogen-related band, present also on day 38, was accompanied by two even slower moving, very faint bands.

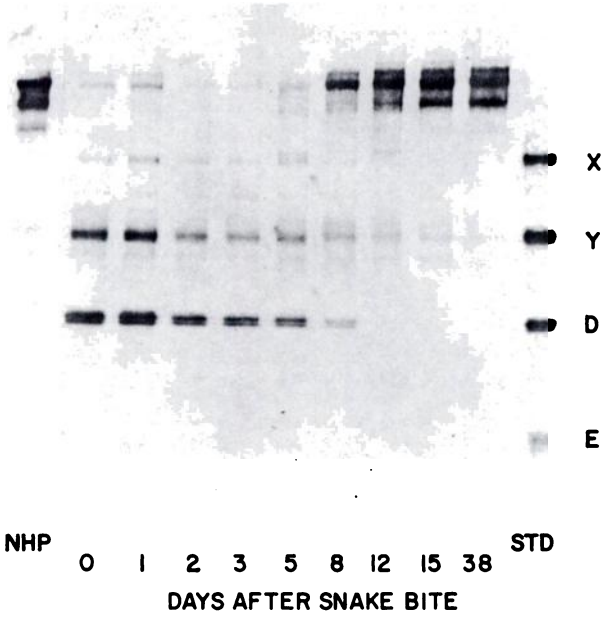

Fig. 2. Fibrinogen derivatives in patient's plasma. Electrophoresis was done in SDS-polyacrylamide slab gel in a 3.5%–9% concentration gradient of acrylamide and using 10-μg samples. The separated plasma proteins were transferred from the gel to nitrocellulose sheet. Fibrinogen and its derivatives were detected by a double-antibody technique, using IgG fraction of goat antiserum against rabbit IgG in the second step, coupled with peroxidase. Fibrinogen-related bands were stained by colored product of the enzyme-catalyzed reaction. NHP, normal human plasma; STD, standard mixture of plasmic degradation products of human fibrinogen: fragments X (mol wt 250,000), Y (150,000), D (103,000), and E (50,000).

Plasminogen and Protease Inhibitors

The striking observation was a very low plasminogen concentration in plasma; 2.8 mg/dl (14% of normal) on the day of the bite (Fig. 1). The return of plasminogen was slow, since it reached only about 50% of the normal concentration during the recovery period. Fifteen days after the snake bite, the plasminogen level was still 13 mg/dl, as compared to its normal value of

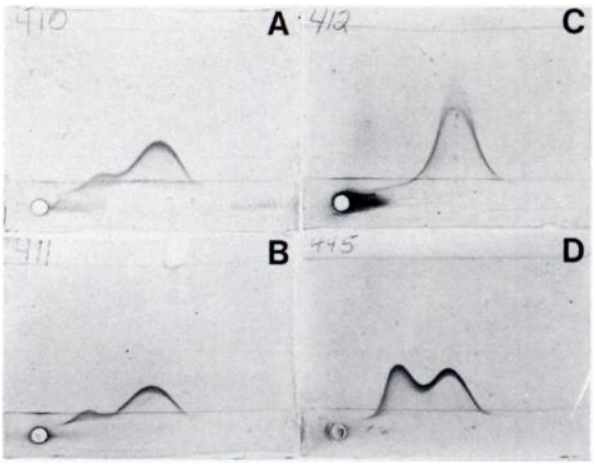

Fig. 3. Demonstration of plasmin–α_2-antiplasmin complex in patient's plasma. Crossed-immunoelectrophoretic patterns were obtained using, in the second direction, an antiserum against human α_2-antiplasmin. (A) day 0, (B) day 3, (C) day 38, (D) normal human plasma + 1 μg/ml of human plasmin.

20 mg/dl. Plasminogen amidolytic activity changed in parallel with the concentration of the zymogen. The search for plasmin-α_2-antiplasmin complex in patient's plasma demonstrated its presence in the first sample collected after the bite (Fig. 3A). The complex was evident until day 10 and disappeared in subsequent samples (Fig. 3, B and C).

Four protease inhibitors, α_2-antiplasmin, α_2-macroglobulin, α_1-protease inhibitor, and antithrombin-III, were affected in patient's plasma to different extents (Fig. 4). The most depressed was α_2-antiplasmin, which fell to 3.7 mg/dl (53% of the normal value) between days 2 and 5 and was still low (5.7 mg/dl) on day 15. The result is consistent with the presence of plasmin-α_2-antiplasmin complex demonstrated by crossed-immunoelectrophoresis. A decreased level of α_2-macroglobulin, 190 mg/dl (72% of normal), was found only during the first 2 days after the snake bite; however, attempts to detect enzyme–α_2-macroglobulin complexes by crossed-immunoelectrophoresis were unsuccessful. α_1-Protease inhibitor was significantly increased, reaching 450 mg/dl (155% of normal) between days 1 and 3; complexes with this inhibitor have not been found. Antithrombin-III concentration and activity both decreased to 75% of the normal values on the day of the bite and recovered in 5 days; complexes have not been found.

Estimation of C. atrox Venom in the Patient's Plasma

In order to reproduce in vitro the effects observed in the patient, experiments were done with unfractionated venom. Three different preparations from the same supplier were used, and similar results were obtained. The authors are aware that *C. atrox* venom from young snakes, less than 8 mo of age, contains a fibrinogen-clotting activity, which disappears by 10 mo of age.[12] Since, in our experiments, the venom did not clot purified fibrinogen (Fig. 4), it was most likely collected from adult snakes. The following considerations were taken into account in the estimation of the possible range of *C. atrox* venom concentration in the

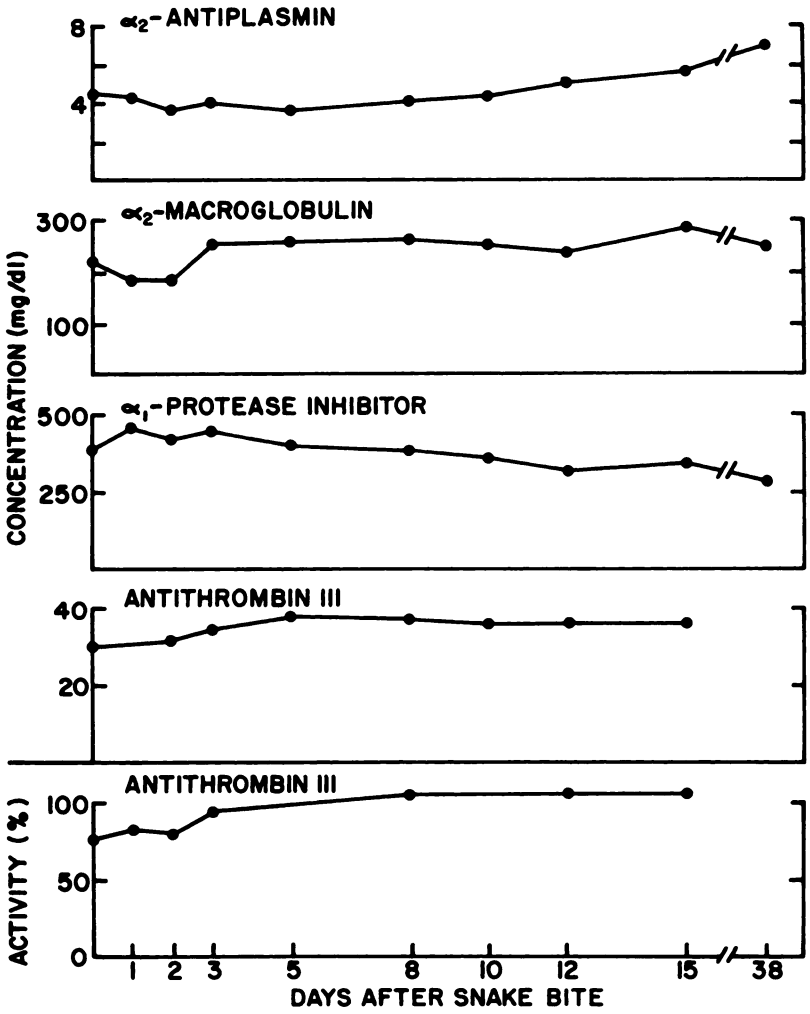

Fig. 4. Concentration of protease inhibitors in patient's plasma. Determination of specific inhibitors was done using radial immunodiffusion. Antithrombin-III activity was measured by a thrombin inhibition test.

Table 2. The Effect of *Crotalus atrox* Venom on Coagulation Tests

Venom Concentration (μg/ml)	PT (sec)	TT (sec)	APTT (sec)	RCT (sec)
0	11.0	10.0	32.0	122
1	11.4	10.2	32.0	133
10	11.5	10.8	32.8	181
100	11.8	10.3	210	>300

PT, prothrombin time; TT, thrombin time; APTT, activated partial thromboplastin time; RCT, recalcification time. The data are mean values of three determinations.

patient's blood after envenomation. The average venom volume injected in a single bite is 0.1–0.5 ml, and its protein concentration varies from 100 to 300 mg/ml.[40] Thus, a snake bite delivers between 10 and 150 mg of venom proteins. The patient's plasma volume, calculated from her weight of 70 kg, was 2,700 ml. If the entire amount of the injected venom would be in the circulating blood, it would correspond to a concentration range of about 3–60 μg/ml. Envenomation of a 70-kg person with 150 mg of venom proteins gives a dose of 2.14 mg/kg, a figure comparable to LD_{50} dose for mice determined to be between 1 and 5 mg/kg.[12] Since part of the venom must have been retained in tissue, causing necrosis, the concentration in the patient was probably at the lower range end. Consequently, in experiments done in vitro, the *C. atrox* venom was used in a concentration range between 1 and 100 μg/ml.

Interference With Coagulation Tests

The effect of the venom on standard coagulation tests was tested using normal human citrated plasma. In these experiments, the venom was added to plasma simultaneously with reagents to a final concentration of 1, 10, or 100 μg/ml. Prothrombin time and thrombin time (Table 2) were unaffected. However, activated partial thromboplastin time and recalcification time were prolonged at the highest venom concentration. Thus, coagulation tests characterized by a short clotting time were not influenced by the venom, but those with long clotting times were significantly lengthened. A shortened clotting time was not observed in any of the four coagulation tests under various experimental conditions; an acceleration of clot formation would result from activation of blood coagulation by the venom.

Interaction With Fibrinogen In Vitro

Thrombin clotting time of purified human fibrinogen was prolonged upon incubation with the venom (Fig. 5). The effect was very potent; for example, at a final venom concentration of 1.6 μg/ml, fibrinogen lost its coagulability after 1 hr of incubation. The initial reaction rate for each curve in Fig. 5 plotted versus venom concentration (not shown) gave a straight line, demonstrating saturation of venom enzymes with fibrinogen as a substrate and implying high affinity of these enzymes for the substrate. There was no evidence of a shortened clotting time, which is consistent with the absence of a thrombin-like enzyme in the venom. Normal human plasma also became incoagulable after incubation with the venom. However, 2.5 times more venom was required in plasma, as compared to purified fibrinogen, in order to achieve the same rate of anticoagulation. This observation indicated that some fibrinogenolytic proteases in *C. atrox* venom were inhibited by human plasma.

Venom-treated plasma prolonged the thrombin clotting time of normal plasma by 50% when equal amounts of these two plasmas were in the test mixture. The venom not only directly rendered fibrinogen incoagulable, but also, fibrinogen degradation products, generated in plasma by the venom, decreased the rate of clot formation in normal plasma.

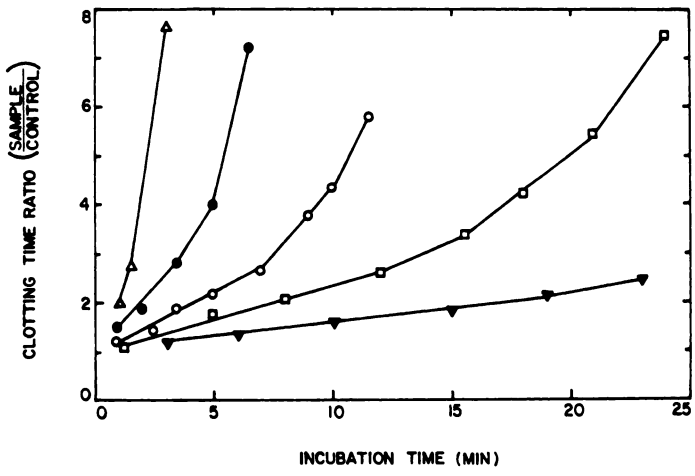

Fig. 5. Dose-dependent effect of *Crotalus atrox* venom on fibrinogen coagulability by thrombin. Equal volumes of purified human fibrinogen (2 mg/ml) and *Crotalus atrox* venom at the concentration of 50 μg/ml (△), 25 μg/ml (●), 12.5 μg/ml (○), 6.3 μg/ml (□), and 3.1 μg/ml (▼) were incubated at 37°C. At different times (abscissa), the thrombin clotting time was recorded, and it is shown as a ratio of sample clotting time to that of control (ordinate).

Interference With Platelets

The aggregation of normal platelet-rich plasma was unaffected by low concentration (1 μg/ml) of the venom; intermediate concentration (10 μg/ml) caused aggregation without ^{14}C-serotonin secretion (Fig. 6). The extent of aggregation induced after 3 min in the presence of these two concentrations of the venom was 3% and 29%, respectively, as compared with 83% caused by 8 μ*M* ADP alone. Small oscillations recorded with the venom correlated with the presence of small aggregates observed by phase-contrast microscopy. ADP- or arachidonate-induced platelet aggregation and ^{14}C-serotonin secretion were unaffected by either venom concentration. The results indicated that the venom, at an intermediate concentration on the order of 10 μg/ml, can aggregate platelets to a small extent. Experiments done with the venom at 100 μg/ml final concentration demonstrated some lysis of platelets and have not been further pursued.

Lack of Plasminogen Activation In Vitro

In order to resolve the question of whether the venom can directly activate the human fibrinolytic system, experiments were performed using purified human Glu-plasminogen. This proenzyme was fully activated by streptokinase, as shown by the hydrolysis of the chromogenic substrate (Table 3). On the other hand, mixtures of plasminogen and the venom hydrolyzed the substrate with the same rates as those observed with the venom alone. These results established that the venom did not contain direct activators of human plasminogen. The low concentration of plasminogen determined in the patient's plasma could be secondary to the action of the venom in vivo on cells of the blood vessel wall, affecting release of activators.

Cellular Plasminogen Activators

Incubation of cultured human EC or human SMC with the venom resulted in a dose-dependent detachment of cells from the dishes. EC were more sensitive

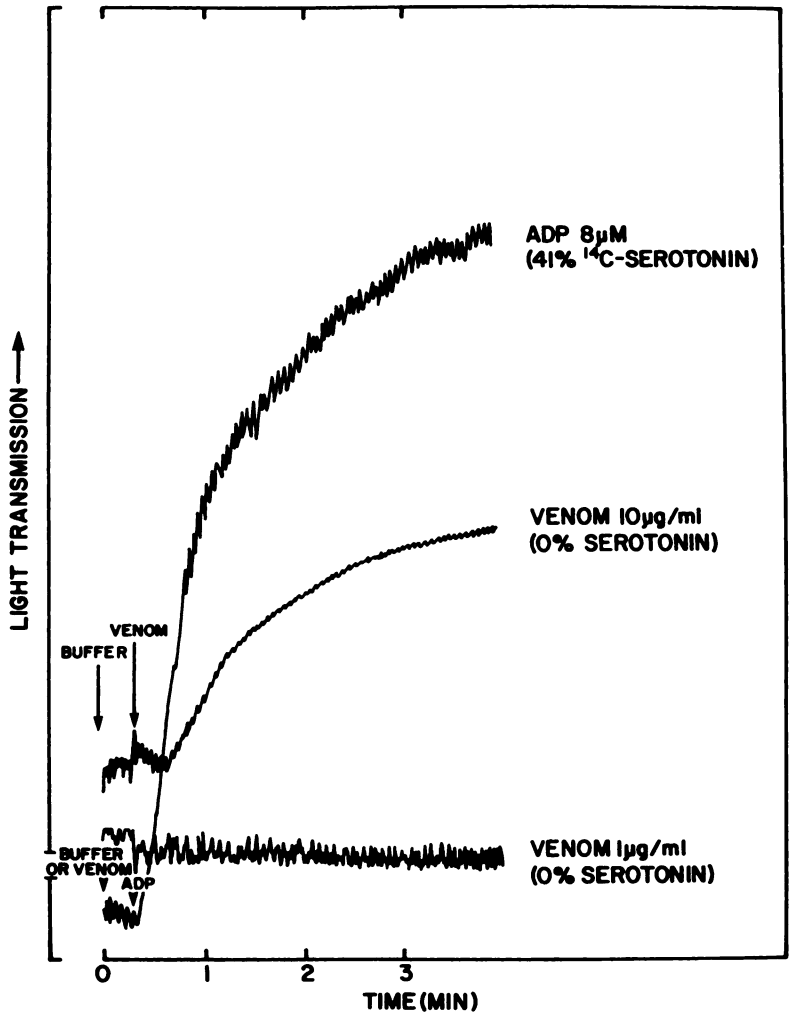

Fig. 6. Aggregation of normal human platelet-rich plasma by *Crotalus atrox* venom. Platelet count 278,000/μl. Addition of reagents was at the beginning (first arrow) and after 15 sec (second arrow). Platelet shape change is indicated by a decrease in the amplitude of the oscillations or a decrease of light transmission; aggregation is indicated by an increase of light transmission. Final concentrations of ADP and venom are shown at each curve. ^{14}C-serotonin secretion after 3 min is given in parentheses as a percent of total uptake.

Table 3. The Effect of *Crotalus atrox* Venom on Amidolytic Activity of Plasminogen

Sample	Activator	Amidolytic Activity (pNA μmole/min)	Specific Activity (pNA μmole/min) per milligram of Snake Venom
Plasminogen (2.5 μg)	Buffer	0	—
Plasminogen (2.5 μg)	Streptokinase (500 U)	13×10^{-3}	—
Buffer	Venom (0.75 μg)	0.24×10^{-3}	0.32
Buffer	Venom (7.5 μg)	4.0×10^{-3}	0.54
Buffer	Venom (50 μg)	13.1×10^{-3}	0.26
Plasminogen (2.5 μg)	Venom (0.75 μg)	0.17×10^{-3}	0.23
Plasminogen (2.5 μg)	Venom (7.5 μg)	3.9×10^{-3}	0.52
Plasminogen (2.5 μg)	Venom (50 μg)	13.3×10^{-3}	0.27

pNA, paranitroaniline. The data are mean values of six determinations.

than SMC, since a similar extent of detachment was attained after 30 min of incubation at a venom concentration of 2 μg/ml with EC (Fig. 7, left B) and of 25 μg/ml with SMC (Fig. 7, right D'). At a venom concentration of 25 μg/ml and 4 hr incubation (Fig. 7, E and E'), both EC and SMC were completely detached, forming aggregates of various sizes. The initial detachment was not associated with cell lysis, as indicated by LDH measurements. Even after incubation for 4 hr with 25 μg/ml of the venom, the LDH distribution between the intracellular (measured in extracts) and extracellular (in releasates) pools was 84% and 16% in EC, and 90% and 10% in SMC. Incubation of cells for the same time without the venom resulted in the respective LDH distribution of 88% and 12% in EC, and 92% and 8% in SMC.

Plasminogen activator in releasates or in extracts from either EC or SMC was inactivated by the venom in a dose-dependent manner according to the equation: $y = 100 - 4x$, where y was percent of plasminogen activator activity remaining after 2 hr incubation at 37°C with the venom at a final concentration of x μg/ml. For example, at the venom concentration of 12.5 μg/ml, 50% of the activator activity disappeared after 2 hr.

The inactivation of plasminogen activator by the venom complicated the experiments, since the measured activity reflected a dynamic equilibrium between its synthesis plus release and degradation. The venom stimulated release of plasminogen activator from both types of cells. Qualitative and quantitative aspects were different for EC and SMC (Fig. 8). EC released maximal activator activity after 2 hr incubation, either without or with the venom. The released activity was inversely proportional to the venom concentration and decreased during 4 hr incubation. The venom at 2 μg/ml was most effective, releasing 6.2 and 5.1 times more activator after 1 and 2 hr, respectively, than was released from untreated cells. The protein concentration in EC releasates was proportional to venom concentration and increased upon extended incubation. In contrast, plasminogen activator activity released from SMC after 2 and 4 hr was directly proportional to the venom concentration between 2 and 10 μg/ml. At maximum stimulation (10 μg/ml of the venom), SMC released 11.1 and 13.7 times more activator after 2 and 4 hr, respectively, than control SMC. The protein concentration was also increased in SMC releasates, but it did not parallel the activator. The content of plasminogen activator and protein in extracts from EC and SMC was little affected by the venom; both values only slightly decreased as concentrations of the venom increased.

DISCUSSION

Thrombin-like enzymes that clot fibrinogen have been recognized and purified from most of the Crotalidae group venoms.[41] Our data suggest that the venom of the western diamondback rattlesnake (*C. atrox*) does not possess a potent fibrinogen clotting enzyme (Fig. 5), corroborating the data of other investigators.[6,8-10,12] The venom was reported to contain fibrinolytic[8-10] and other proteolytic enzymes.[11] We found two different types of fibrinogenolytic enzymes in this venom.[42] Laboratory data from the patient presented in this work extended these observations and allowed us to suggest that the predominant mechanism of action of *C. atrox* venom was fibrinogenolytic. The afibrinogenemic state was attained in the patient by the degradation of circulating fibrinogen to incoagulable plasmic derivatives (Table 1, Figs. 1 and 2).

The direct degradation of plasma fibrinogen by the venom in vitro, on the other hand, was most likely responsible for the prolongation of coagulation tests of longer duration (Table 2). The venom-derived fibrinogen fragments were not only incoagulable by thrombin (Fig. 5), but also inhibited the clotting of normal plasma. The same inhibitory effects are well known for plasmic fragments Y and D.[39] A loss of fibrinogen clotting function on the action of *C. atrox* venom

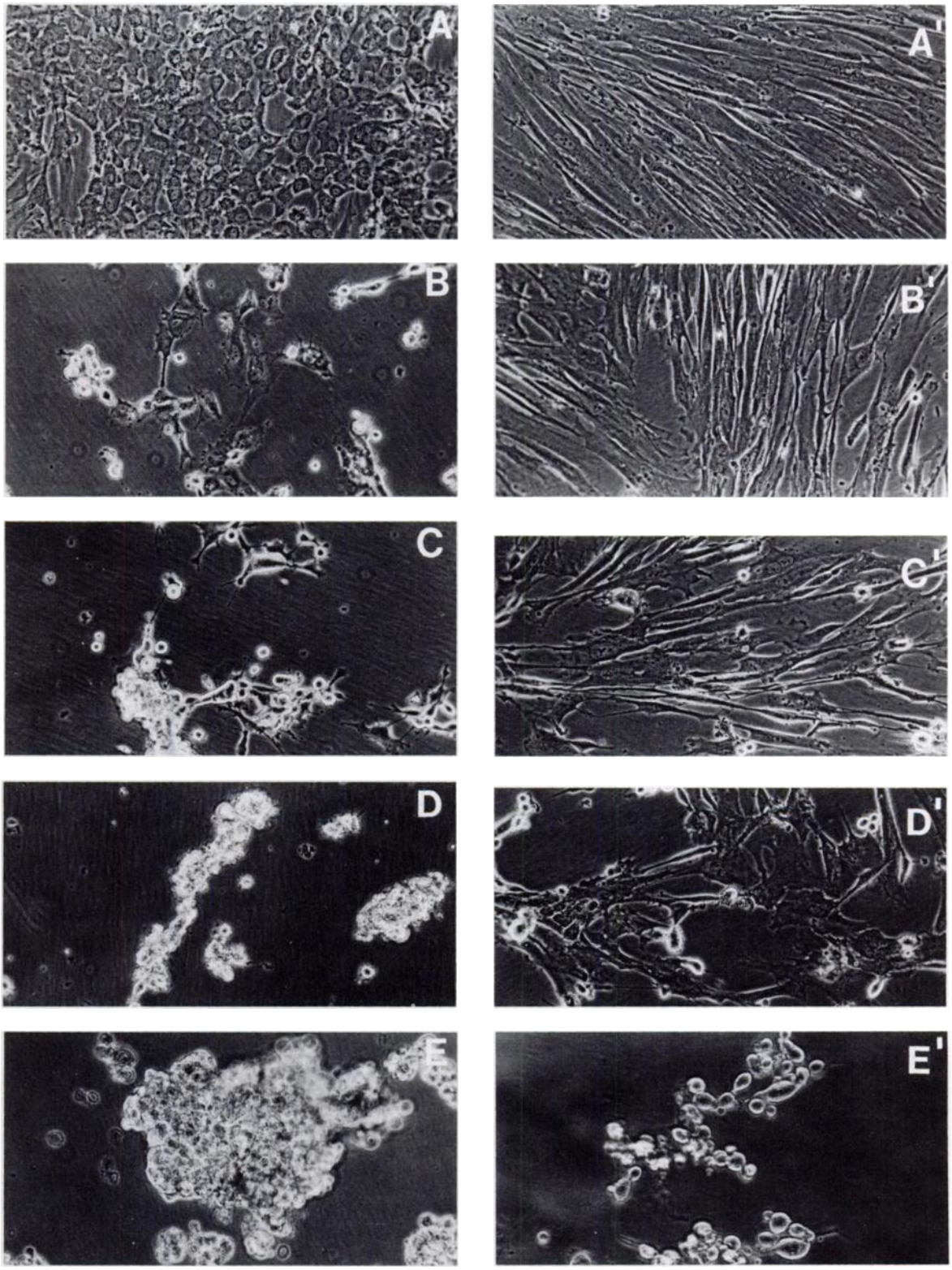

Fig. 7. Detachment of cells in primary cultures of human endothelial cells (left, A–E) and human smooth muscle cells (right, A^1–E^1) treated with *Crotalus atrox* venom. The respective patterns on the left and right side represent endothelial cells and smooth muscle cells exposed to the same venom concentration for the same time (magnification 400×). (A,A^1) No venom, 30 min incubation; (B,B^1) 2 μg/ml venom, 30 min; (C,C^1) 10 μg/ml venom, 30 min; (D,D^1) 25 μg/ml venom, 30 min; (E,E^1) 25 μg/ml venom, 4 hr.

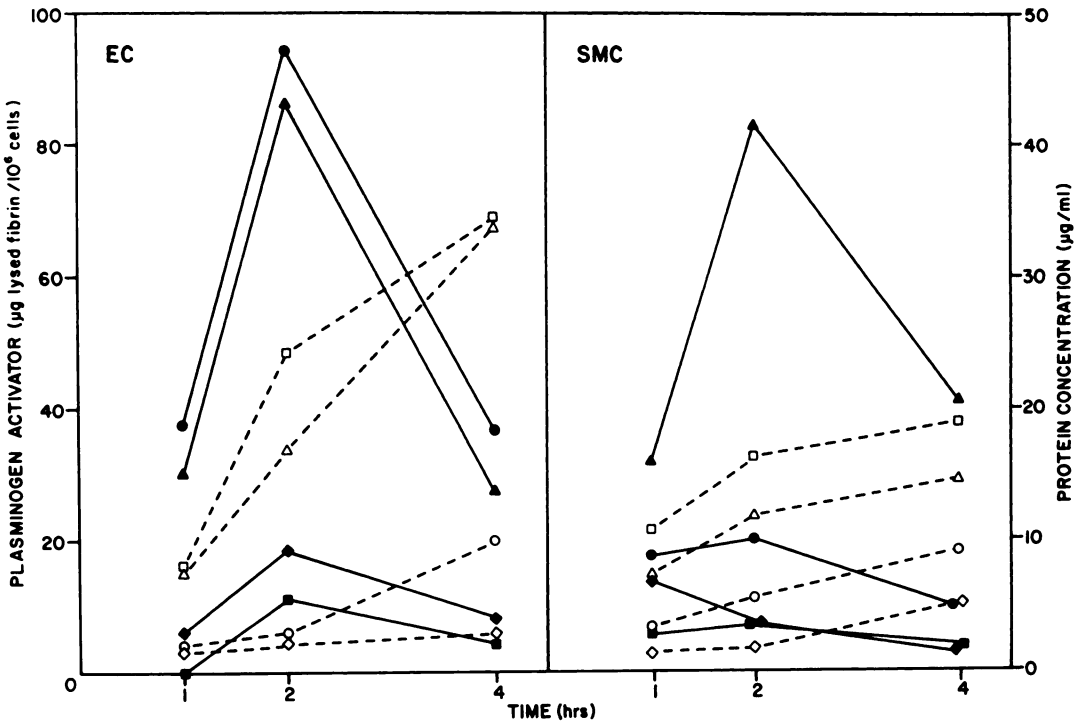

Fig. 8. Release of plasminogen activator (closed symbols) and protein (open symbols) from cultured human endothelial (EC) and smooth muscle (SMC) cells by *Crotalus atrox* venom. The cells were incubated with the venom for various times (abscissa) and the release of plasminogen activator activity (left ordinate) was measured in micrograms of ^{125}I-fibrin solubilized per 10^6 cells. The released protein concentration (right ordinate) was determined in micrograms per milliliter. The venom was used at a final concentration of 2 μg/ml (●, ○), 10 μg/ml (▲, △), and 25 μg/ml (■, □), and the data are mean values of 3 experiments. Control data in the absence of the venom (♦, ◇) show mean values of 6 experiments.

enzymes was probably associated with specific cleavages of polymerization sites in the fibrinogen molecule that are involved in fibrin clot formation.[38,43,44] Several other plasma proteins were affected in vivo after envenomation, so that fibrinogen was not the only susceptible species.[45]

Platelet-aggregating proteases were isolated from venoms of *Bothrops atrox*[46,47] and *Crotalus horridus horridus*.[48,49] However, their mechanism of action was different from that shown in this work (Fig. 6), since *C. atrox* venom aggregated platelets without serotonin release. It is possible that proteases of this venom degraded platelet membrane glycoproteins and exposed fibrinogen receptors, similar to that observed with α-chymotrypsin, since proteolytically altered platelets aggregated spontaneously in the presence of fibrinogen.[50] In 30 clinical cases of rattlesnake envenomation, including *C. atrox*, a dose-related platelet count decline was observed followed by a rebound thrombocytosis.[51] Since the platelet count in the patient before the snake bite was indeterminable we cannot comment on the immediate effects of the venom on the platelet count. However, there was a pronounced thrombocytosis in the patient by day 5. This observation, taken together with dose-dependent aggregation of platelet-rich plasma (Fig. 6), permits estimation of the venom concentration in the patient's plasma to be in the range of approximately 1–10 μg/ml.

The major mechanism contributing to blood incoagulability after *C. atrox* envenomation involved depletion of plasminogen (Fig. 1), formation of plasmin as evidenced by the presence of plasmin–α_2-antiplasmin complex (Fig. 3), and generation of plasmic degradation products of fibrinogen (Figs. 1 and 2). Since *C. atrox* venom did not directly activate human plasminogen (Table 3), the mechanism of its activation in humans would necessarily be indirect; for example, by the release of vascular activators from the blood cells or blood vessels or via activation of protein C with subsequent cellular releases. This event would cause a decrease of plasminogen in the circulation and an accompanying increase of plasmin. Preliminary data on the lytic state induced in dogs by *C. atrox* venom implied a possible mediation of cellular activators.[52] Snake venoms are known to contain agents that cause tissue hemorrhage. A purified hemorrhagin from *Vipera palestinae* venom induced swelling and necrosis

of EC, allowing the escape of erythrocytes through gaps in damaged capillaries.[53] *Echis coloratus* venom induced a leakage of an electron-dense tracer in brain capillaries by labilizing EC and opening intracellular junctions.[54] Injection of hemorrhagic toxins from *C. atrox* venom into mice resulted in extensive hemorrhage, accompanied by degeneration of capillaries, thinning of EC, and formation of gaps within the cells.[55] Our data showed that *C. atrox* venom did change the morphology of human vascular EC and SMC in tissue culture, even at the venom concentration of 2 μg/ml (Fig. 7). At this venom concentration, the released plasminogen activator was significantly increased in both types of cells (Fig. 8). The release did not result from cell lysis, as confirmed by LDH measurements. Some snake venoms have a potentiating effect on the rate of cellular protein biosynthesis. For instance, *Agkistrodon rhodostoma* venom increased biosynthesis of fibrinogen mRNA and fibrinogen in rat hepatocytes.[56] Similar effects may be induced by *C. atrox* venom regarding biosynthesis of plasminogen activator. The increased release of plasminogen activator from EC and SMC exposed to the venom may help explain the decreased plasminogen concentration in the patient's plasma, the formation of plasmin, and the appearance of plasmic degradation products of fibrinogen. Supportive evidence for the generation of plasmin is provided by the decreased concentrations of α_2-antiplasmin and α_2-macroglobulin in the patient's plasma (Fig. 3).

Our conclusions are at variance with the recent report suggesting consumption coagulopathy at the bite wound site as a possible result of coagulopathy after envenomation by *C. atrox*.[3] It is entirely possible that local tissue damage by the venom may have caused the leakage of thromboplastin and the exposure of collagen, both events leading to the platelet aggregation at the snake bite site as observed by these authors. The rabbit model differs from the human system, since species differences in fibrinogen response to other snake venoms have been observed. For instance, the clotting time of rabbit fibrinogen by reptilase (from *Bothrops atrox* venom) was 10 times longer than that of human fibrinogen.[57] Comparison of data on 56 patients with rattlesnake bites showed swelling and tissue necrosis to be the most frequent clinical complication.[58] One cannot exclude that tissue thromboplastin might have been released and entered the circulation. However, there was no convincing evidence that blood coagulation was activated via thrombin generation after the rattlesnake bite of the studied patient. Factor VIII level was within normal limits during hospitalization (Table 1). High molecular weight complexes, characteristic of soluble fibrin, were absent, and there was a lack of derivatives crosslinked by factor XIIIa (Fig. 2). Antithrombin-III antigen and activity decreased only slightly, recovered, and in 3 days were within the range of normal values (Fig. 4). The platelet count remained within normal range (Table 1). Interference with hemostasis can also be caused by plasmin in the patient's circulation; notably, plasmin is known to aggregate platelets.[59] Minor bleeding from the venipuncture sites of our patient in the first 7 days most likely resulted from absence of fibrinogen, a high concentration of degradation products, and perhaps also from the presence of hemorrhagic toxins[11] rather than from thrombocytopenia or altered platelet functions.

In the authors' opinion the most plausible mechanism of afibrinogenemia in the patient envenomated by *C. atrox* is a primary fibrinogenolysis. This state was brought about by (1) stimulation of release of vascular plasminogen activator by the venom, (2) activation of plasminogen by the released activator and formation of plasmin in the absence of a fibrin clot, (3) proteolysis of fibrinogen by plasmin and formation of incoagulable fragments X, Y, D, and E, and (4) inhibition of blood coagulability by fibrinogen derivatives with anticoagulant function. The evidence on venom-stimulated secretion of plasminogen activator has implication for a new mode of thrombolytic therapy. Since the stimulation apparently resulted from the increased biosynthesis of the activator, and not from a simple depletion of its intracellular pool, the use of such a stimulant of endogenous plasminogen activator, rather than administration of an exogenous one, would constitute a rationale for a therapy.

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REFERENCES

1. Glass TG Jr: Early debridement in pit viper bites. JAMA 235:2513, 1976

2. Russell F, Carlson R, Wainschel J: Snake venom poisoning in the United States—experiences with 550 cases. JAMA 233:341, 1975

3. Simon TL, Grace TG: Envenomation coagulopathy in wounds from pit vipers. N Engl J Med 305:443, 1981

4. Butner AN: Envenomation coagulopathy from snake bites. N Engl J Med 305:1347, 1981

5. Sullivan JB Jr: More on envenomation coagulopathy from snake bites. N Engl J Med 306:806, 1982

6. Didisheim P, Lewis JH: Fibrinolytic and coagulant activities of certain snake venoms and proteases. Proc Soc Exp Biol Med 93:10, 1956

7. Weiss HJ, Allan S, Davidson E, Kochwa S: Afibrinogenemia in man following the bite of a rattlesnake (*Crotalus adamanteus*). Am J Med 47:625, 1969

8. Denson KWE, Russell FE, Almagro D, Bishop RC: Characterization of the coagulant activity of some snake venoms. Toxicon 10:557, 1972

9. Bajwa SS, Markland FS, Russell FE: Fibrinolytic enzyme(s) in western diamondback rattlesnake (*Crotalus atrox*) venoms. Toxicon 18:285, 1980

10. Bajwa SS, Markland FS, Russell FE: Fibrinolytic and fibrinogen clotting enzymes present in the venoms of western diamondback rattlesnake, *Croatalus atrox*, eastern diamondback rattlesnake, *Crotalus adamanteus*, and southern Pacific rattlesnake, *Crotalus viridis helleri*. Toxicon 19:53, 1981

11. Bjarnason JB, Tu AT: Hemorrhagic toxins from western diamondback rattlesnake (*Crotalus atrox*) venom: Isolation and characterization of five toxins and the role of zinc in hemorrhagic toxin e. Biochemistry 17:3395, 1978

12. Reid HA, Theakston RDG: Changes in coagulation effects by venoms of *Crotalus atrox* as snakes age. Am J Trop Med Hyg 27:1053, 1978

13. Markland FS, Damus PS: Purification and properties of a thrombin-like enzyme from the venom of *Crotalus adamanteus* (eastern diamondback rattlesnake). J Biol Chem 246:6460, 1971

14. Deutsch DG, Mertz E: Plasminogen: Purification from human plasma by affinity chromatography. Science 170:1095, 1970

15. Bowie EJW, Thompson JH Jr, Didisheim P, Owen CA Jr: Mayo Clinic Laboratory Manual of Hemostasis. Philadelphia, WB Saunders, 1971

16. Atichartakarn V, Marder VJ, Kirby EP, Budzynski AZ: Effects of enzymatic degradation on the subunit composition and biologic properties of human factor VIII. Blood 51:281, 1978

17. Ratnoff OD, Menzie C: A new method for the determination of fibrinogen in small samples of plasma. J Lab Clin Med 37:316, 1951

18. Dintenfass L, Kammer S: Re-evaluation of heat precipitation method for plasma fibrinogen estimation: Effect of abnormal proteins and plasma viscosity. J Clin Pathol 29:130, 1976

19. Castelan DJ, Hirsch J, Martin M: Latex-bound antifibrinogen test for plasma fibrinogen assay. J Clin Pathol 21:638, 1968

20. Mancini GA, Carbonara O, Heremans J: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235, 1965

21. Budzynski AZ, Marder VJ, Parker ME, Shames P, Brizuela BS, Olexa SA: Antigenic markers of fragment DD, a unique plasmic derivative of human crosslinked fibrin. Blood 54:794, 1979

22. Bick RL, Kovacs I, Fekete LF: A new two-stage functional assay for antithrombin III (heparin cofactor): Clinical and laboratory evaluation. Thromb Res 8:745, 1976

23. Friberger P, Knös M, Gustavsson S, Aurell L, Claeson G: Methods for determination of plasmin, antiplasmin and plasminogen by means of substrate S-2251. Haemostasis 7:138, 1978

24. Laurell CB: Antigen antibody crossed-electrophoresis. Anal Biochem 10:358, 1965

25. Neville DM Jr: Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J Biol Chem 246:6328, 1971

26. Olexa SA, Budzynski AZ, Marder VJ: Modification of high molecular weight plasmic degradation products of human crosslinked fibrin. Biochim Biophys Acta 576:39, 1979

27. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and applications. Proc Natl Acad Sci USA 76:4350, 1979

28. Avrameas S, Guilbert G: A method for quantitative determination of cellular immunoglobulins by enzyme-labeled antibodies. Eur J Immunol 1:394, 1971

29. Brecher G, Schneiderman M, Cronkite EP: The reproducibility and constancy of the platelet count. Am J Clin Pathol 23:15, 1953

30. Niewiarowski S, Budzynski AZ, Morinelli T, Brudzynski TM, Stewart GJ: Interaction of fibrinogen with human platelets altered by proteolytic enzymes. J Biol Chem 256:917, 1981

31. Holmsen H, Setkowsky-Dangelmaier CA: Adenine nucleotide metabolism of blood platelets. X. Formaldehyde stops centrifugation-induced secretion after A23187-stimulation and causes breakdown of metabolic ATP. Biochim Biophys Acta 497:46, 1977

32. Gimbrone MA, Jr Shefton EJ, Cruise SA: Isolation and primary culture of endothelial cells from human umbilical vessels. Tissue Cult Assoc Manual 4:813, 1978

33. Ives HE, Schultz GS, Galardy RE, Jamieson JD: Preparation of functional smooth muscle cells from the rabbit aorta. J Exp Med 148:1400, 1978

34. Bergmeyer HU, Bernt E: Lactic dehydrogenase, in Bergmeyer HU (ed): Methods of Enzymatic Analysis (ed 2). New York, Academic, 1974, pp 574

35. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248, 1976

36. Moroz L, Gilmore NJ: A rapid and sensitive ^{125}I-fibrin solid-phase fibrinolytic assay for plasmin. Blood 46:543, 1975

37. Knight LC, Budzynski AZ, Olexa SA: Radiolabeling of human fibrinogen using the Iodogen technique. Thromb Haemostas 46:593, 1981

38. Pandya BV, Rubin RN, Olexa SA, Budzynski AZ: Unique degradation of human fibrinogen by proteases from western diamondback rattlesnake (*Crotalus atrox*) venom. Toxicon 21:515, 1983

39. Marder VJ, Budzynski AZ: Data for defining fibrinogen and its plasmic degradation products. Thromb Diath Haemorrh 33:199, 1975

40. Bdolah A: The venom glands of snakes and venom secretion. Handb Exp Pharmacol 52:41, 1979

41. Seegers WH, Ouyang C: Snake venoms and blood coagulation. Handb Exp Pharmacol 52:684, 1979

42. Pandya BV, Budzynski AZ, Rubin RN, Olexa SA: Anticoagulant proteases from western diamondback rattlesnake venom. Fed Proc 40:1790, 1981

43. Olexa SA, Budzynski AZ: Evidence for four different polymerization sites involved in human fibrin formation. Proc Natl Acad Sci USA 77:1374, 1980

44. Olexa SA, Budzynski AZ: Localization of a fibrin polymerization site. J Biol Chem 256:3544, 1981

45. Bagley EA, Lonberg-Holm K, Pandya BV, Budzynski AZ: 2D gel electrophoretic analysis of plasma proteins from a patient bitten by a rattlesnake. Electrophoresis 4:238, 1983

46. Kirby EP, Niewiarowski S, Stocker K, Kettner C, Shaw E, Brudzynski TM: Thrombocytin: A serine protease from *Bothrops atrox* venom. I. Purification and characterization of the enzyme. Biochemistry 18:3564, 1979

47. Niewiarowski S, Kirby EP, Brudzynski TM, Stocker K: Thrombocytin: A serine protease from *Bothrops atrox* venom. II. Interaction with platelets and plasma clotting factors. Biochemistry 18:3570, 1979

48. Schmaier AH, Claypool W, Colman RW: Crotalocytin: Recognition and purification of a timber rattlesnake platelet aggregating protein. Blood 56:1013, 1980

49. Schmaier AH, Colman RW: Crotalocytin: Characterization of the timber rattlesnake platelet activating protein. Blood 56:1020, 1980

50. Niewiarowski S, Budzynski AZ, Morinelli T, Brudzynski TM, Stewart GJ: Interaction of fibrinogen with human platelets altered by proteolytic enzymes. J Biol Chem 256:917, 1981

51. LaGrange R, Russell F: Blood platelet studies in man and rabbits following crotalus envenomation. Proc West Pharmacol Soc 13:99, 1970

52. Bond TP, Guest MM: Hemostatic imbalance resulting from western diamondback rattlesnake venom. Fed Proc 34:290, 1975

53. McKay DG, Moroz C, DeVries A, Csavossy I, Cruse V: The action of hemorrhagin and phospholipase derived from *Vipera palestinae* venom on the microcirculation. Lab Invest 22:387, 1970

54. Sandbank U, Jerushalmy Z, Ben-David E, DeVries A: Effect of *Echis colaratus* venom on brain vessels. Toxicon 12:267, 1974

55. Ownby CL, Bjarnason J, Tu AT: Hemorrhagic toxins from rattlesnake (*Crotalus atrox*) venom. Am J Pathol 93:201, 1978

56. Crabtree GR, Kant JA: Molecular cloning of cDNA for the α, β and γ chains of rat fibrinogen. J Biol Chem 256:9718, 1981

57. Wik KO, Tangen O, McKenzie FN: Blood clotting activity of reptilase and bovine thrombin in vitro: A comparative study on seven different species. Br J Haematol 23:37, 1972

58. Grace TG, Omer GE: The management of upper extremity pit viper wounds. J Hand Surg 5:168, 1980

59. Niewiarowski S, Senyi AF, Gillies P: Plasmin induced platelet aggregation and platelet release reaction. Effects on hemostasis. J Clin Invest 52:1647, 1973