EFFECTS OF ANTITHROMBIN III AND ANTIVENOM ON PROCOAGULANT ACTIVITY OF RUSSELL'S VIPER VENOM IN A WHOLE BLOOD MODEL

Ralf Clemens[1], Reinhard Lorenz[2], Sasithon Pukrittayakamee[1], Benjanee Punpoowang[1], Sirivan Vanijanonta[1] and Pricha Charoenlarp[1]

[1]Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [2]Medizinische Klinik and Poliklinik TU Munchen, Klinikum Rechts der Isar, Munchen, FRG

Abstract. The procoagulant activities of Russell's viper venom were assessed in an *in vitro* whole blood model. Sequetial samplings showed that the generation of fibrinopeptide A (FPA), a marker of thrombin activity, and platelet factor 4 (PF4), a marker of platelet activity, exhibited bi-phasic kinetics with an initial slow phase followed by a rapid phase of secretion. In the presence of Russell's viper venom, the generation of both FPA and PF4 was accelerated with the bi-phasic kinetics of PF4 being maintained while that of FPA completely disappeared. Administration of either antivenom (1,600 ng) or 10 IU antithrombin III (AT-III) had no antagonistic effect against the venom but combination of both resulted in a significant prolongation of both FPA and PF4 release ($p < 0.05$). High dose AT-III (20 IU) resulted in normalization of both FPA and PF4 kinetics and serial levels of both parameters were lower than those treated with the combined regimen, although these were not statistically significant. Unlike the untreated venom activated whole blood, there was no clot formation following treatment with either the combined regimen or high dose AT-III. The results of this study suggested that the effect of Russell's viper venom on the clotting cascade is more potent and direct than that on platelet activity. There were complementary effects between antivenom and AT-III in controlling of both FPA and PF4 release induced by the venom. Furthermore, in this *in vitro* experiment, AT-III alone when administered in a sufficient dose, abolished the procoagulant effects of Russell's viper venom.

INTRODUCTION

More than 200 years ago, Fontana observed that viper venom promptly clotted whole blood and rendered the circulating blood of envenomed animals incoagulable (Fontana, 1787). Systematic studies during the last few decades on the biochemistry and biological action of different venoms have revealed that bites by certain species of hematotoxic snake particularly the vipers and rattlesnakes can produce hypofibrinogenemia with or without accompanying thrombocytopenia (Myint-Lwin *et al,* 1985; Warrell *et al,* 1976; Barrantes *et al,* 1985; Stocker, 1978; Reid *et al,* 1963; Kamiguti and Cardoso, 1989; Hasiba *et al,* 1975). These effects depend on differences in the mechanisms of action of snake venoms on the clotting cascade and on platelet function (Stocker, 1978; Mitrakul, 1979).

Russell's viper (Viper russelli) is an important hematotoxic snake and is the major cause of snake bite morbidity and mortality in most Southeast Asian countries (Looareesuwan *et al,* 1988; Viravan *et al,* 1992; Mahasandana *et al,* 1980; Myint-Lwin *et al,* 1985; Jeyarajah, 1984; De Silva and Ranasinghe, 1983). The principle coagulant action of Russell's viper venom is activation of factors X and V (Pukrittayakamee *et al,* 1983, 1987). This leads finally to fibrin formation and consumption of clotting factors. Typical clinical manifestations of systemic envenoming after Russell's viper bites are incoagulable blood and, to a lesser extent, thrombocytopenia, spontaneous systemic bleeding and acute renal failure (Than-Than *et al,* 1988, 1989; Looareesuwan *et al,* 1988; Mahasandana *et al,* 1980; Myint-Lwin *et al,* 1985).

The specific action of Russell's viper venom on factor X and, subsequently, prothrombin suggests that the venom enzymes may be inhibited by plasma protease inhibitors such as antithrombin III (AT-III). AT-III inhibits several clotting factors by complex formation, especially with thrombin and factor X and, to a lesser extent, factors IX and XI, plasmin and prekallikrein (Rosenberg and Damus, 1973).

We assessed in an *in vitro* native whole blood model (Lorenz *et al,* 1988a; 1988b) the procoagulant effects of Russell's viper venom through sequential determination of fibrinopeptide A (FPA), a marker of thrombin activity, and platelet factor 4 (PF4), a marker of platelet activity. Subsequently, we analyzed the effects of AT-III concentrate, specific antivenom or the combination of both on the activation of the hemostatic system by Russell's viper venom.

MATERIALS AND METHODS

Crude whole lyophilized Russell's viper venom (*Daboia russelli siamensis*) was kindly provided by DA Warrell, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford, UK.

The antivenom used was unrefined, equine freezedried monospecific Russell's viper antivenin serum, obtained from the Thai Red Cross Society (TRC), Bangkok.

The antithrombin III (AT-III) concentrate used was the pasteurized, freeze dried commercially available product manufactured by Behringwerke, Marburg, FRG.

A solution used as anticoagulant and as a platelet inhibitor (containing 20 U/ml heparin, 5% EDTA and 50 mmol/l chloroquine) was provided by Dr Pelzer, Behringwerke, Marburg, Germany.

In vitro model of hemostasis in native whole blood

Determinations of FPA generation and PF4 release were performed on whole blood taken from six healthy volunteers who had given informed consent. The procedure of blood sampling was according to a well standardized technique (Lorenz *et al,* 1988b). A 24 gauge butterfly needle was inserted in the antecubital vein and 30 ml blood was drawn into a plastic syringe. To prevent interference with the generation of FPA and PF4 release, minimal suction pressure was used and no anticoagulant was added to the syringe. An aliquot of 1.5 ml was taken immediately, then the blood was incubated at 37°C and further aliquots were taken every minute for 9 minutes. The generation of FPA and PF4 in each blood aliquot was terminated at each time point by adding 1.5 ml of the anticoagulant and platelet inhibitor solution. The mixtures were centrifuged at 4°C for 15 minutes at 2,000g and for a further 15 minutes at 8,000g. The platelet poor plasma supernatant was collected and stored at -20°C for subsequent determination of FPA and PF4 levels.

The coagulant effect of Russell's viper venom on native whole blood was determined by adding 100 ng venom (dissolved in 1 ml Ringer's lactate) to 30 ml of whole blood. For neutralization experiments, 1 ml of Ringer's lactate containing either AT-III (10 IU or 20 IU) or antivenom (1,600 ng) or both (10 IU AT-III and 1,600 ng antivenom) was immediately added to 30 ml of the venom activated whole blood. Both AT-III (10 IU and 20 IU) and antivenom (1,600 ng) were also tested to determine their direct effect on the native whole blood. From each set of experiments, aliquots of blood were sequentially sampled for determination of FPA and PF4 levels as in the native whole blood experiment. For each plasma sample, 6 measurements for FPA and PF4 levels were carried out and the results were expressed as median values.

Determination of FPA and PF4 levels

Commercially available radioimmunoassays were used for the determination of FPA (Mallinckrodt Inc, St Louis, Mo) and PF4 (Abbott Diagnostics Division, Wiesbaden). The test systems were standardized and controlled by means of standard plasma samples as described previously (Lorenz *et al,* 1988b).

Statistics

The differences of FPA and PF4 levels at each time point between the untreated and treated whole blood models were assessed by the Wilcoxon rank sum test.

RESULTS

The coagulant effect of Russell's viper venom

The generation of FPA and PF4 release in native whole blood, exhibited parallel kinetics with an initial slow phase followed by a fast phase of secretion (Fig 1). There was no clot formation during the 9 minute incubation period. In the presence of Russell's viper venom, FP4 generation showed an immediate sharp rise omitting the

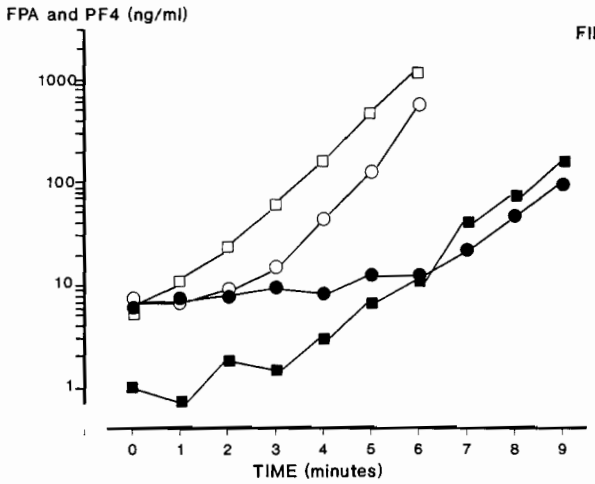

Fig 1–Concentrations of fibrinopeptide A (FPA) (□ ■) and platelet factor 4 (PF4) (○ ●) of native whole blood in the presence (open symbols) and absence (closed symbols) of Russells's viper venom. Concentrations are given as median values of six tests.

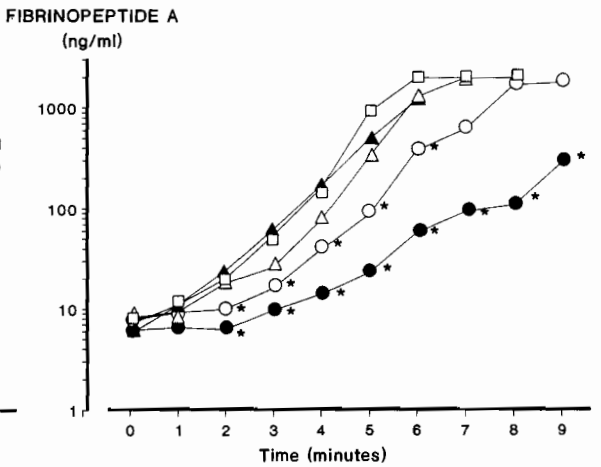

Fig 2–Activation of FPA generation induced by snake venom in untreated group (▲); treated with 10 IU antithrombin-III (Δ); or antivenom (□); or both (○) or with 20 IU AT-III (●). Each value is the median of six experiments. *$p < 0.05$ *vs* untreated whole blood activated with venom.

slow phase and the serial FPA levels were all significantly higher than those in native whole blood (Fig 1). The PF4 levels during the first 3 minutes were similar to that in the native whole blood but then became significantly higher until the end of the experiments. All Russell's viper venom activated whole blood clotted and the mean (SD) time for clot formation was 6.7 (2) minutes.

Neutralization of coagulant effects of Russell's viper venom

Administration of either antivenom or low dose AT-III (10 IU) had no effect on the coagulant activity of Russell's viper venom. The serial changes of FPA and PF4 levels in the treated whole blood models were similar to those in the venom activated whole blood (Fig 2, 3). When low dose AT-III was combined with antivenom, there was a statistically significant prolongation of both FPA secretion and PF4 release compared to those of the untreated venom activated whole blood (Fig 2, 3). High dose AT-III (20 IU) resulted in normalization of FPA kinetics and almost abolished the venom effects on PF4 release (Fig 2, 3). The serial levels of FPA and PF4 in the presence of 20 IU AT-III at most time points were lower

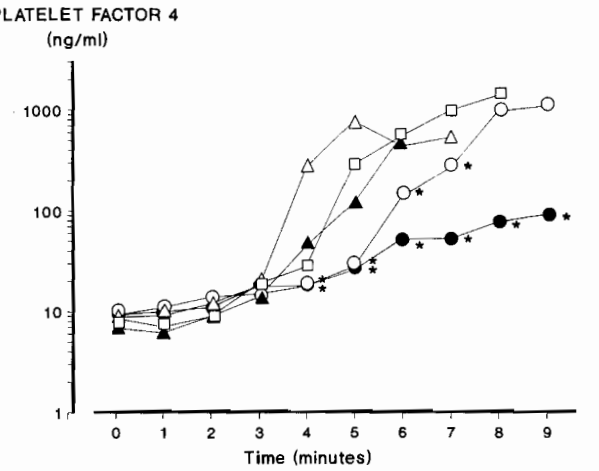

Fig 3–Activation of PF4 generation induced by snake venom in untreated group (▲); treated with 10 IU antithrombin-III (Δ); or antivenom (□); or both (○) or with 20 IU AT-III (●). Each value is the median of six experiments. *$p < 0.05$ *vs* untreated whole blood activated with venom.

than those in the presence of low dose AT-III combined with antivenom but this was not statistically significant ($p > 0.05$). Unlike the untreated venom activated whole blood, there was no clot formation following treatment with either the combined regimen or high dose AT-III.

Neither AT-III (10 IU or 20 IU) nor antivenom alone or in combination had any effects on coagulation in the native whole blood model. Both the generation of FPA and PF4 in whole blood supplemented with any of these reagents were similar to those in the native whole blood model (data not shown)

DISCUSSION

In this *in vitro* whole blood model, the generation of FPA was measured as a marker of thrombin activity (Kaplan *et al,* 1981; Nossel *et al,* 1974) and the plasma concentrations of PF4, secreted from activated platelets, were determined as a measure of platelet activity (Doyle *et al,* 1980; Kaplan and Owen, 1981). An advantage of this whole blood model is that the kinetics of both FPA and PF4 can be assessed through sequential determinations (Rybak *et al,* 1981; Shuman and Levine, 1980). Russell's viper venom, accelerated and increased both the generation of PFA and PF4 release but the bi-phasic kinetics of PF4 was maintained while that of FPA completly disappeared. The effects of the venom on the clotting cascade, being more direct and potent than that on platelet activity, may reflect its ability to activate factor X and subsequently prothrombin leading to thrombin-induced platelet stimulation.

In human victims of Russell's viper bite, the venom injected is usually insufficient to cause massive fatal intravascular coagulation within a short time (Than-Than *et al,* 1989; Looareesuwan *et al,* 1988; Myint-Lwin *et al,* 1985). However, thrombin generation induced by activation of the clotting cascade, if untreated, leads to the consumption of fibrinogen and other coagulant factors as well as of the main inhibitor AT-III. Finally the syndrome of disseminated intravascular coagulation may develop. This is characterized by two distinct coagulation abnormalities: spontaneous systemic hemorrhage due to defibrination and deposition of microthrombi in the microvasculature due to depletion of plasma inhibitors (Marder, 1980). Acute renal failure, the most common fatal complication after systemic envenomation of Russell's viper bite (Matthai and Date, 1981; Myint-Lwin *et al,* 1985; Than-Than *et al,* 1988; Looareesuwan *et al,* 1988) is probably caused by deposition of microthrombi in the kidney which compounds the direct nephrotoxicity (Ratcliffe and Pukrittayakamee, 1985; Ratcliffe *et al,* 1989). Clinical data indicate that antivenom, when given within hours of the bite, may completely neutralize the circulating venom toxins and may restore the clotting factor levels to normal (Myint-Lwin *et al,* 1985; Jeyarajah, 1984). However, despite restoring the coagulation system, renal failure may still develop if irreversible tubular damage or extensive fibrin obstruction of the renal vessels has already occurred.

The role of heparin in treatment of venom associated coagulopathy has been controversial (Warrell *et al,* 1976; Weiss *et al,* 1973). Heparin works primarily as a catalyst of AT-III/thrombin complex formation (Rosenberg and Damus, 1973; Mammen *et al,* 1985). Thus, in situations with decreased AT-III levels, as in Russell's viper venom envenomation, heparin treatment is ineffective because of the substrate insufficiency.

The specific activation of Russell's viper venom on factor X as well as the clinical evidence of disseminated intravascular coagulation, suggests that AT-III, as the main coagulation inhibitor is consumed after systemic envenomation. Thus the effects of this venom on hemostasis may be minimized by AT-III supplementation. In our whole blood model, AT-III substitution at high dose but not at low dose abolished both the FPA generation as well as the PF4 release induced by the venom. The dose of antivenom used in this study (venom: antivenom ratio = 1:16), was unable to inhibit the venom coagulopathic effects. It is possible that a larger dose of antivenom would have an inhibitory effect on the activation of coagulation. However, the same dose of antivenom in combination with low dose AT-III results in a slower kinetics of both FPA and PF4 compared to either administered alone. This synergistic effect is probably due to direct neutralization of venom by the antivenom, while AT-III complements the activity of antivenom by inhibiting the already activated procoagulant components. A similar complementary effect of antivenom and AT-III has been observed in rats envenomed with Malayan pit viper venom (Pukrittayakamee *et al,* 1990).

In this native whole blood model, high dose AT-III concentrate alone and, to a lesser extent, AT-III concentrate in combination with monovalent antivenom proved to be effective in controlling FPA generation and PF4 release after Russell's viper venom administration. Further laboratory and clinical experiences are warranted to verify the

potential role of AT-III in the treatment of hematotoxic envenoming.

ACKNOWLEDGEMENTS

This study was part of the Wellcome-Mahidol University-Oxford Tropical Medicine Research Program supported by the Wellcome Trust of Great Britain. We are grateful to Dr Nicholas White for his encouragement of the study.

REFERENCES

Barantes A, Solis V, Bolanos R. Alteracion de los mecanismos de la coagulacion en el envenenamiento pro bothrops asper (terciopelo). *Toxicon* 1985; 23 : 399-407.

De Silva A, Ranasinghe L. Epidemiology of snake-bite in Sri Lanka: a review. *Ceylon Med J* 1983; 28 : 144-54.

Doyle DJ, Chesterman CN, Cade JE, *et al.* Plasma concentration of platelet-specific proteins correlated with platelet survival. *Blood* 1980; 55 : 82-4.

Fontana F. Treatise on the Venom of the Viper. London: J Murray 1787.

Hasiba U, Rosenbach LM, Rockwell D, Lawis JH. DIC-like syndrome after envenomation by the snake, Crotalus horridus horridus. *N Engl J Med* 1975; 292 : 505-7.

Jeyarajah R. Russell's viper bite in Sri Lanka, a study of 22 cases. *Am J Trop Med Hyg* 1984; 33 : 506-10.

Kamiguti AS, Cardoso JLC. Haemostatic changes caused by the venoms of South American snakes. *Toxicon* 1989; 27 : 955-63.

Kaplan KL, Drillings M, Lesznik G. Fibrinopeptide A cleavage and platelet release in whole blood *in vitro*. Effects of stimuli, inhibitors and agitation. *J Clin Invest* 1981; 67 : 1561-8.

Kaplan KL, Owen J. Plasma levels of B-thromboglobulin and platelet factor 4 as indices of platelet activation *in vivo*. *Blood* 1981; 57 : 199-202.

Looareesuwan S, Viravan C, Warrell DA. Factors contributing to fatal snake bite in the rural tropics: analysis of 46 cases in Thailand. *Trans R Soc Trop Med Hyg* 1988; 82 : 930-4.

Lorenz R, Gebhart S, Wirth U, Classen M, Heptner W. Methodische Untersuchungen zur Bildung von Fibrinipeptide A und zur H Sektion von Plattchenfaktor 4 in gerinnendem Vollblut. *Med Klin* 1988a; 83 : 81-7.

Lorenz R, Gebhart S, Heptner W, Classen M. *In vitro* studies of secretion of platelet factor 4 and generation of fibriopeptide A in native whole blood. *Throm Res* 1988b; 50 : 231-5.

Mahasandana S, Rungruxsirivorn Y, Chantarangkul V. Clinical manifestations of bleeding following Russell's viper and green pit viper bites adults. *Southeast Asian J Trop Med Public Health* 1980; 11 : 285-93.

Mammen EF, Miyakawa T, Phillips TF. Human antithrombin concentrates and experimental disseminated intravascular coagulation. *Semin Thromb Hemost* 1985; 11 : 373-83.

Marder VJ. Microvascular thrombosis. In : Lichtmann MA, ed. The Science and Practice of Clinical Medicine. New York: Grune and Stratton, 1980: pp 230-4.

Matthai TP, Date A. Acute renal failure in children following snake bite. *Ann Trop Paediatr* 1981; 1 : 73-6.

Mitrakul C. Effect of five Thai snake venoms on coagulation fibrinolysis and platelet aggregation. *Southeast Asian J Trop Med Public Health* 1979; 10 : 266-75.

Myint-Lwin, Phillips RE, Tun-Pe, *et al.* Bites by Russel's viper (Vipera russelli siamensis) in Burma: haemostatic, vascular and renal disturbances and response to treatment. *Lancet* 1985; ii : 1259-64.

Nossel HL, Yudelman J, Canfield RE, *et al.* Measurement of fibrinopeptide A in human blood. *J Clin Invest* 1974; 54 : 43-53.

Pukrittayakamee S, Esnouf MP, McMichael AJ. Purification and inactivation of the factor X activator of Russell's viper venom with monoclonal antibodies. *Molec Biol Med* 1983; 1 : 123-35.

Pukrittayakamee S, Ratcliffe PJ, McMichael AJ, Warrell DA, Bunnag D. A competitive radioimmunoassay using a monoclonal antibody to detect the factor X activator of Russell's viper venom. *Toxicon* 1987; 25 : 721-9.

Pukrittayakamee S, Desakorn V, Clemens R, *et al.* Synergy of antithrombin III concentrate and antivenom in preventing coagulopathy in a rat model of Malayan pit viper envenoming. *Trans R Soc Trop Med Hyg* 1990; 84 : 880-4.

Ratcliffe PJ, Pukrittayakamee S. Direct nephotoxicity of Russell's viper venom demonstrated in the isolated perfused rat kidney. *Toxicon* 1985; 23 : 605.

Ratcliffe PJ, Pukrittayakamee S, Ledingham JGG, Warrell DA. Direct nephrotoxicity of Russell's viper venom demonstrated in the isolated perfused rat kidney. *Am J Trop Med Hyg* 1989; 40 : 311-9.

Reid A, Chan KE, Thean PC. Prolonged coagulation defect (defibrination syndrome) in Malayan viper bite. *Lancet* 1963; i : 621.

Rosenberg RD, Damus PS. The purification and mechanism of action of human antithrombin heparin cofactor. *J Biol Chem* 1973; 248 : 6490-505.

Rybak ME, Lau HK, Tomkins B, Rosenberg RD, Handin RI. Relationship between platelet secretion and prothrombin cleavage in native whole blood. *J Clin Invest* 1981; 68 : 405-12.

Shuman MA, Levine SP. Relationship between secretion of platelet factor 4 and thrombin generation during *in vitro* blood clotting. *J Clin Invest* 1980; 65 : 307-13.

Stocker K. Defibrinogenation with thrombin-like snake venom enzymes in fibrinolytics and anti-fibrinonolytics. In: Markwardt F, ed. Handbook of Experimental Pharmacology. Berlin: Springer-Verlag 1978; 46 : pp 451-84.

Than-Than, Hutton RA, Myint-Lwin, *et al.* Haemostatic disturbances in patients bitten by Russell's viper (Viper russelli siamensis) in Burma. *Br J Haematol* 1988; 69 : 513-20.

Than-Than, Francis N, Tin-Nu-Swe, *et al.* Contribution of focal haemorrhage and microvascular fibrin deposition to fatal envenoming by Russell's viper (Vipera russelli siamensis) in Burma. *Acta Trop* 1989; 46 : 23-38.

Viravan C, Looareesuwan S, Kosakarn W, *et al.* A national hospital-based survey of snakes responsible for bites in Thailand. *Trans R Soc Trop Med Hyg* 1992; 86 : 100-6.

Warrell DA, Pope HM, Prentice CRM. Disseminated intravascular coagulation caused by the carpet viper (Echis carinatus). Trial of heparin. *Br J Haematol* 1976; 33 : 335-42.

Weiss HJ, Phillips LL, Hopewell WS. Heparin therapy in a patient bitten by a saw-scaled viper (Echis carinatus), a snake whose venom activates prothrombin. *Am J Med* 1973; 54 : 653.