RUSSELL'S VIPER VENOM FRACTIONS AND NEPHROTOXICITY

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Abstract. Apparently healthy Wistar rats of body weight 250-300 g were chosen for the experiments. A group of 6 rats were assigned for each fraction. The dose of Russell's viper venom (RVV) fraction used for *in vivo* experiments was 0.75 µg/g body weight. Of each batch of 6 rats 3 were sacrificed on the third day and the remaining 3 on the fifth day after the administration of test venom fractions. Daily urine output with proteinuria and serum creatinine were determined on the day they were sacrificed. Kidneys from the rats were also examined under light microscopy after hematoxylin and eosin staining. In the *in vitro* experiment, kidney slices (1 mm thickness) from normal rat was incubated with RVV fractions of 5 mg/ml concentration, The predominant renal lesions observed in both sets of animal experiments were tubular degeneration and necrosis. The changes were mostly confined to proximal tubules. Glomerular changes were mild. Similar tubulotoxic effects were produced by whole RVV as well as single fractions. Therefore, it is possible that RVV contains a common nephrotoxic (protein) component which is present in all fractions of the venom. The renal damage caused by RVV seemed to be due to both systemic effects (mainly DIC and renal ischemia) and direct tubulotoxic effects of the venom.

INTRODUCTION

In Russell's viper (*Daboia russelii siamensis*) bite, acute renal failure (ARF) is known to be one of the serious, common complications and it is not prevented by monospecific anti-snake venom (ASV) therapy even when given within 4 hours after bite (Myint Lwin *et al*, 1985).

Renal lesions reported include glomerulitis (Sant and Purandare, 1972); glomerulonephritis (Seedat *et al*, 1974): arteritis (Sitprija *et al*, 1974); interstitial nephritis (Sant and Purandare, 1972); tubular necrosis (Sitprija *et al*, 1973, 1974); cortical necrosis (Oram *et al*, 1963); renal infarct (Raab and Kaiser, 1966). In most reports, renal failure was attributed to tubular necrosis and cortical necrosis.

It was shown in animal experiments as well as in victims of RV bite that tubular ischemia resulted from the obstruction of glomerular capillaries by coagulated material, giving rise to tubular necrosis and subsequent renal failure (Chugh *et al*, 1975; Aung Khin, 1978; Than Than *et al*, 1989).

In spite of these studies, the pathogenesis is not clearly understood and particular nephrotoxic protein(s) responsible for renal lesions have not been identified yet.

The purpose of the study was to isolate fractions from Russell's viper venom (whole or crude) and study their nephrotoxic effect *in vivo* and/or direct nephrotoxicity *in vitro*. It was considered that such a study might identify the particular protein with nephrotoxic action and might clarify the mechanism involved in Russell's viper envenomation.

MATERIALS AND METHODS

Dried crystals of *V. russelii* venom were obtained from the Biological Laboratories of the Union of Myanmar, 1988. Sephadex C-25 and molecular weight marker proteins (Sigma Chemical Co, St Louis MO, USA) and SDS Page marker III (Fluk AG, Buchs, Switzerland) were used.

V. russelii venom was fractionated according to the method described by Viswanath, *et al* (1987). Briefly, 500 mg venom in 2 ml of 0.02M phosphate buffer (pH 7.0) was loaded on CM-Sephadex C-25 column (1.2 × 120 cm) and was eluted stepwise using phos-phate buffers of molarities (a) 0.02 M, pH 7.0; (b) 0.03 M, pH 7.0; (c) 0.04 M, pH 7.0; (d) 0.05 M, pH 7.0; (e) 0.075 M, pH 7.0; (f) 0.09 M, pH 7.3; (g) 0.1 M, pH 7.5; (h) 0.15 M, pH 8.0; (i) 0.2 M, pH 8.0; (j) 0.3 M, pH 8.0; (k) 0.4 M, pH 8.0.

Fractionation was carried out at room temperature (25°C). The flow rate was adjusted to 40 ml/hour and 4 ml fractions were collected by Frac 100

automatic fraction collector (LKB, Bromma, Sweden). Protein elution was monitored at 280 nm. Individual fractions of the peaks was pooled, dialysed, lyophilized and stored at -18°C. The enzyme elution from individual peaks as well as the pooled column fractions were assayed for phospholipase and coagulase enzymatic activities according to the methods described by Marinetti (1965) and by Dimitrov *et al* (1968), respectively. Protein concentration was measured by a Coomassie dye binding procedure (Biorad).

The molecular weight was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (1969). The mobilities of proteins were plotted against the molecular weights of some standard proteins.

In vivo animal experiment

Apparently healthy Wistar rats (28) of body weight 250-300 g were fed on normal diet (rat chow) and fluid through out the experimental period. Intake and output of fluid were recorded daily from one day prior to the administration of the test venom fractions. The dose used was 0.75 µg/g body weight, dissolved in 0.9% normal saline, maximum volume adjusted to 5 ml, administered subcutaneously into the left thigh.

A group of 6 rats were assigned for each fraction. Three were sacrified on days 2 and 5 after injection. Blood samples were collected for determination of serum creatinine on the day they were sacrified as well taking as both kidneys for histological examination. Values for urine output (ml) and albumin excretion per 24 hours prior to the administration of venom (*ie* from 6 animals) were taken as controls. Serum creatinine control values were also determined on 24 apparently healthy rats. Kidney tissues taken from these healthy rats was also examined under light microscopy.

Incubation of Russell's viper venom and its fractions with kidney slices *in vitro*

These experiments were performed according to the method described by Soe Soe *et al* (1993). Slices of kidney tissue (1 mm in thickness), containing both cortex and medulla, were obtained immediately after sacrifice, rinse thoroughly in normal saline and then incubated in the following solutions:

(i) 5 mg/ml of RVV, reconstituted in normal saline.

(ii) 5 mg/ml of RVV fraction 1(F1), 2(F2), 4(F4), 7(F7), 9(F9) and 10(F10), reconstituted in normal saline.

(iii) One ml of normal saline.

Test tubes containing the above slices were incubated in a water bath (37°C) for 2 hours and histological examination of the kidney was made after hematoxylin and eosin staining.

(iv) Slices were also put into 10% buffered formalin without incubation and processed for histology (iii and iv served as controls).

RESULTS

Altogether ten pooled fraction (1-10) were obtained. The total amount of protein recovered was 457.5 mg (*ie* 91.3%). The protein present in each pool, enzyme activities (phospholipase, coagulase and anticoagulase) are shown in Table 1.

Venom fractions 1, 2, 3, 4 had coagulase (procoagulant) enzyme activity, while fractions 5, 6, 7, 8, 9 and 10 had anticoagulase enzymatic activity. The latter 6 fractions showed higher phospholipase enzyme activity.

SDS-PAGE electrophoresis of pooled column fractions showed high MW proteins in fractions 1-5. In contrast, the remaining fractions (*ie* 6-10) exhibiting phospholipase activity and poor procoagulant activity contained low MW proteins.

Urine output was reduced in all of the rats receiving RVV fractions and urine albumin excretion was also noted. High serum creatinine values were observed in all envenomated rats after the administration of venom fractions (Table 2).

Renal histology

Renal histology at the day of sacrificed (*ie* at day 2 and day 5 after the administration of RV venom fractions) was described as showing glomerular, tubular and interstitial changes. Vascular congestion and hemorrhages in the interstitium was a

Table 1

Coagulase and phospholipase activities of pooled column fractions of Russell's viper (amount of Russell's viper venom used was 500 mg).

Pooled column fractions	Total protein per pool (mg)	% protein recovery	Coagulase activity	Phospholipase activity (Units)
1	384.75	76.95%	procoagulant	6.074
2	12.6	2.32%	,,	37.62
3	4.86	0.92%	,,	25.98
4	6.52	1.3%	,,	19.98
5	4.82	0.92%	soft clot only	85.69
6	6.75	1.35%	non clot	78.76
7	5.62	1.1%	,,	136.37
8	7.56	1.5%	,,	223.04
9	16.38	3.2%	,,	77.67
10	7.65	1.5%	,,	79.16

Table 2

Mean serum creatinine values in experimental animals at Day 2 (48 hours), Day 5 (120 hours) after the administration of RVV and its fractions.

Days after venom injection	RVV	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	Control mean (range)
Day 2 (48 hrs)	1.5	1.6	1.6	2.3	1.8	1.1	0.9	1.5	1.1	2.1	1.0	0.91
Day 5 (120 hrs)	1.2	1.4	1.9	2.1	1.8	1.1	1.3	1.2	1.4	1.6	1.5	(0.3-1.8)

Table 3(a)

Histological changes on renal tubules after the administration of RVV and its fractions to experimental animals (Day 2).

Tubular and interstitial changes	RVV	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Degeneration	+++	+++	+++	+++	++	++	++	++	++	++	++
Necrosis	+++	+++	++	+++	+	+	+	+	+	+	+
Casts	++	+	+	+	+	-	-	+	+	+	+
Regeneration	-	-	-	-	-	-	-	-	-	-	-
Fibrosis	-	-	-	-	-	-	-	-	-	-	-
Vascular congestion and hemorrhage	+++	++	++	++	++	+	+	+	+	+	+

(-) nil, (+) mild, (++) moderate, (+++) severe

Table 3(b)

Histological changes on renal tubules after the administration of RVV and its fractions to experimental animals (Day 5).

Tubular and interstitial changes	RVV	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Degeneration	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
Necrosis	+++	+++	+	+++	+++	++	+++	+++	++	++	+++
Casts	++	++	++	++	++	++	++	++	++	++	++
Regeneration	+	+	+	+	-	-	-	-	-	+	+
Fibrosis	-	-	-	-	-	-	-	-	-	-	-
Vascular congestion and hemorrhage	++	++	+	++	+	+	+	+	+	+	+

(-) nil, (+) mild, (++) moderate, (+++) severe

Table 4(a)

Histological changes on renal glomeruli after the administration of RVV and its fractions to experimental animals (Day 2).

Glomerular changes	RVV	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Capillary ballooning	-	+	+	+	+	-	-	-	-	+	++
Glomerular necrosis	-	+	+	-	-	-	-	-	-	+	+
Hypercellularity	+	-	+	-	+	+	+	++	++	++	++
Fluid/Adhesion	-	+	+	+	+	-	+	++	+	-	++
GMB thickening	-	-	-	-	-	-	-	-	-	-	-
Endothelial cell swelling	-	-	-	-	-	-	-	-	-	-	-

(-) nil, (+) mild, (++) moderate, (+++) severe

Table 4(b)

Histological changes on renal glomeruli after the administration of RVV and its fractions to experimental animals (Day 5).

Renal glomerular changes	RVV	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Capillary ballooning	++	+	++	++	++	+	-	-	-	+	++
Necrosis	+	+	+	++	++	+	-	++	++	++	+
Hypercellularity	+	+	++	++	++	+	++	++	++	++	+
Fluid/Adhesion	+	+	+	++	-	++	++	++	+	+	-
GMB thickening	+	-	-	-	-	-	-	-	+	+	-
Endothelial cell swelling	+	-	-	-	-	-	-	-	+	+	-

(-) nil, (+) mild, (++) moderate, (+++) severe

Table 5

Renal histological changes after incubation with RVV and its fractions, *in vitro*.

Glomerular changes	F1	F2	F4	F7	F9	F10	NS	RVV
Capillary ballooning	-	-	-	-	-	-	-	++
Necrosis	-	-	-	-	-	-	-	+
Hypercellularity	-	-	-	-	-	-	-	++
Fluid/adhesion	-	-	-	-	-	-	-	+
GMB thickening	-	-	-	-	-	-	-	-
Endothelial swelling	-	-	-	-	-	-	-	-
Tubular and interstitial changes								
Degeneration	+++	+	+	+	++	++	-	+++
Necrosis	+++	+	+	+	++	+	-	+++
Casts in tubules	+++	+	+	+	++	+	-	+++
Regeneration	++	-	-	-	-	-	-	++
Fibrosis	-	-	-	-	-	-	-	-
Cellular infiltration	+	-	-	-	-	-	-	+
Vascular congestion and hemorrhage	+	+	+	-	-	-	-	+++

NS - normal saline

prominent histological change, observed as early as 24 hours after the administration of different venom fractions and persisting till the 7th day of envenomation. Tubular changes (*ie* degeneration and necrosis) was the pathonomonic feature noted both at day 2 and day 5. Severe changes were observed with fractions exhibiting strong coagulase enzymatic activity, especially fraction No. 3 where the mean serum creatinine was also found to be the highest. Glomerular changes compared to tubular changes were milder. But hypercellularity, capillary ballooning and mild to moderate degree of necrosis were observed, particularly at day 5 (Tables 3a, b; 4a, b).

In vitro renal histological changes are listed in Table 5.

DISCUSSION

There was reduction in urine output, a varying degree of proteinuria and elevation of serum creatinine value throughout the envenomated state. This indicated that renal damage occurred in animals tested with both whole RVV and single fractions.

The renal histopathological features noted following envenomation with either whole RVV or fractions were in conformity with those reported by other investigators who also studied RVV in experimental animals (Aung Khin, 1978; Myint Myint Than, 1978). They were also similar to biopsy and autopsy findings reported by Maung Maung Aye (1976); Chugh *et al* (1975); Date and Shastry (1982); Aung Khin (1978) and Soe Soe *et al* (1993).

From experiments in rats, it was demonstrated that both whole RVV and its fractions produced renal lesions. The main morphological features observed were tubular degeneration and necrosis. Proximal tubules were most affected. Both ischemia and nephrotoxic effects were seen and were as toxic as whole venom.

The tubular lesions induced by whole venom had the same histological appearances as those produced by specific fractions, and the changes *in vivo* were comparable to those observed *in vitro*. However, different effects of whole RVV and its fractions on glomeruli were observed *in vivo* and *in vitro*. Glomeruli were minimally affected by whole venom, as compared to its fractions when investigated *in vivo*. In contrast, they were more severely damaged by whole RVV *in vitro* with no significant damage observed with fractions. The explanation for the difference could be that RVV consists of different proteins which exert a synergistic effect

on renal tissue when tested *in vitro*. Hence, injurious effects were observed on both glomeruli and tubules. In contrast, the nature of the proteins contained in single fractions might show more selective toxicity *eg* for renal tubules rather than glomeruli.

RVV is a complex mixture of proteins and enzymes; 30% of the total protein has phospholipase A2 enzyme activity. Phospholipase leucine aminopeptidase activity was significantly increased after rats were given *Agkistrodon piscivorus* venom. This increase enzymatic activity suggested that the kidney was damaged by the venom. These observation may partly explain the direct action of RVV and its fractions on renal tubular cells, *ie* through phospholipase A2 activity.

Morphological changes in various organs (*ie* heart, liver, lungs, kidney and muscle) have been produced by black mamba venom (which also contains phospholipase A2) given to experimental rats (Zaki and co-workers, 1970).

It has been well established and reported by many workers that RVV contains procoagulants and it is their activity that produces disseminated intravascular coagulation with fibrin deposits in both animal experiments and human autopsy findings.

Both sets of experiments in this study demonstrated that the nephrotoxic effect did not seem to be related specifically to coagulase or phospholipase A2 activity. It appeared that coagulase and phospholipase A2 have their own mechanisms for producing renal injury because the experimental tubular lesions produced were fairly uniform. All fractions appeared to be nephrotoxic indicating that the effects were non-specific or that the venom contained a common nephrotoxic component that was present in all polypeptide fractions.

Multiple factors are responsible for the deterioration in renal function and lesions resulting from RV bite. Although disseminated intravascular coagulation has been reported to be the major factor in the pathogenesis of renal lesions, direct nephrotoxicity should not be ignored. There is a strong possibility that a common component with nephrotoxic properties is present in RVV as well as its fractions.

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