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Hematological alterations and splenic T lymphocyte polarization at the crest of snake venom induced acute kidney injury in adult male mice

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ABSTRACT

Snake venom induced acute kidney injury (SAKI) is of great clinical relevance in tropical countries. Involvement of T cell, a key mediator of AKI and its remission, is least explored in SAKI. In the present study the *in vivo* hematological alterations and associated splenic T cell polarization is probed in order to investigate the immune response at the crest of Russell's viper venom (RVV) induced AKI in experimental murine model. Based on a dose and time kinetic study intra muscular injection dose of 20 μg RVV/100 gm body weight of mice and incubation period of 60 h was selected for induction of SAKI. Renal involvement in SAKI group was confirmed from oliguria, significantly elevated urinary microprotein ($p < 0.001$), decreased urinary creatinine ($p = 0.003$) and creatinine clearance ($p < 0.001$) compared to control. Hematological analyses revealed a significant neutrophilic leukocytosis ($p < 0.001$) associated with a reduced lymphocyte percentage ($p < 0.001$) favoring a state of acute inflammation in SAKI group. Immunophenotyping study of splenocytes showed a significant decrease in $CD4^+/CD8^+$ ratio ($p < 0.001$) with a significant increase in regulatory ($CD25^+FoxP3^+$) helper and cytotoxic subset of T cell ($p < 0.001$). Significant increase in IL-10+ regulatory helper and cytotoxic T cell ($p < 0.001$) further confirmed the internal milieu favoring immunosuppression. Apart from these the $CD25^-FoxP3^+$ reservoir regulatory T cells were also found to be significantly elevated in SAKI group compared to that of control ($p < 0.001$). Taken together, the results of the present study clearly indicated a state of acute inflammation and splenic T cell polarization towards regulatory subset at the crest of SAKI.

1. Introduction

Snake envenomation is a significant public health issue in tropical countries especially in rural parts, due to associated morbidity and mortality (Bagcchi, 2015; Warrell, 2010). Snake envenomation is often associated with a wide array of pathological manifestation, and direct toxicity of the venom components are thought to be the principle culprit. Among these, acute kidney injury (AKI) secondary to snake envenomation (especially vasculotoxic) is of great clinical relevance (Kohli and Sakhuja, 2003; Mohamed et al., 2015). Pathophysiological mechanism of snake venom induced AKI (SAKI) includes, direct toxicity resulting in necrosis of the cellular component/s, thrombotic microangiopathy in glomerular capillaries, resulting in ischemic condition, and inflammation and activation of immune response (Barsoum et al., 1999).

In recent times the involvement of human intrinsic component in snake envenomation related pathologies has been suggested (Leon et al., 2011). These indirect toxic effects are primarily brought about by the circulatory species, which are also the key immune as well as inflammatory mediators and regulators. Snake envenomation is characterized on the one side by neutrophilic leukocytosis associated with lymphocytopenia, macrophage activation and thrombocytopenia (Ahmed et al., 2008; Silva et al., 2012) and by inflammatory response, cellular infiltration of different organ and release of pro-inflammatory cytokines and chemokines on the other (Leon et al., 2011; Moreira et al., 2016; Sunitha et al., 2015; Voronov et al., 1999). Despite these facts there are very few reports on the involvement of lymphocytes, neutrophil, and

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macrophage in snake venom pathologies and secondary complications.

Among the circulatory components, lymphocyte characterization & its involvement in snake-bite induce primary and secondary complication is often neglected. Studies on venom-lymphocyte interaction generally focus on lymphocyte activation, proliferation, genotoxicity & therapeutic potential of crude and/or purified fraction of venom (Marcussi et al., 2013; Vyas et al., 2013) normally in very low dose. Lymphocytes in snake envenomation associated pathologies are often treated as silent bystander or poor victim despite their immune regulatory potentials. A recent study by Waikhom et al. (2011) shows that SAKI patients suffering from Herpes labialis have lowered peripheral $CD4^+/CD8^+$ T-cell ratio suggesting a probable link between secondary complications (rather tertiary) of snake envenomation and lymphocyte sub population. Another study by Kou et al. (2014) shows that oral supplementation of denatured Naja venom has no significant effect on $CD4^+$ and $CD8^+$ T-cell profile but have suppressive effect in dinitrofluorsence induced delayed typed hypersensitivity. Similar observations were also reported by Favoretto et al. (2011) who shows that crude and purified fraction of *Crotalus durissus terrificus* venom inhibits human serum albumin induced humoral and cellular responses.

Despite the well-known immune-regulatory potentials of snake venom (Stone et al., 2013; Zoccal et al., 2011) and lymphocytes, specially T lymphocyte (Jang and Rabb, 2015), and their relation with AKI (Wang and Tao, 2015) scarce reports are available about the polarization of T-cell, the major immune regulator cells, into different subsets in SAKI. In the present study we endeavoured to establish a Russell's viper venom (RVV) induced murine AKI model and to evaluate associated renal and hematological changes and the splenic T-lymphocytes polarization.

2. Material and methods

2.1. Materials

2.1.1. Chemicals and reagents

Urine analysis kit (Orinasys Strip lot no.URS4080206), Creatinine kit (Merck Specialities Pvt. Ltd., India; B. No.:G52401713), Micro-Protein kit (Coral Clinical Systems, India; Lot No.:MCP1143A). Rat anti-mice antibodies: CD4- FITC (catalogue no. 553729), CD8-APC-CY7 (catalogue no.557654), CD25- V450 (catalogue no.55719), Fox-P3-Alexa Flour 647 (catalogue no.560402), IL10-PE (catalogue no.554467), Fixation/Permeabilization Solution Kit (BD Cytofix/Cytoperm solution catalogue no.554714) were purchased from BD Bio-Science.

2.1.2. Venom

Crude Viper Venom (RVV) was a gift from Dr. Debanik Mukherjea, Field Biologist (Herpetology), Biodiversity Parks Programme, Centre for Environmental Management of Degraded Ecosystems (CEMDE), School of Environmental Studies, University of Delhi. New Delhi -110007. The venom was dissolved in sterile 0.85% physiological saline before use and stored at 4 °C.

2.1.3. Animals

Male Swiss albino mice (weight: 18–20 g) were collected from CNCI, Kolkata. They were housed under regulated ambience (RT: 22±4°c, relative humidity: 60± 5%) and received balanced diet and water *ad libitum*. Animal ethical committee, Department of Physiology, University of Calcutta endorse the animal experiments in accordance with the guideline of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India (IAEC-III/proposal/RM-02/2012 dated 24.04.2012).

2.2. Lethality assay & LD_{50} determination and selection of dose

The intra-muscular (i.m.) medial lethal dose (LD_{50}) of RVV in a dose range of 10–100 μg RVV/100 gm mice body weight was assessed by i.m. injection in the right hind limb and followed over next 24 h. Five animals were used for each venom dose. The LD_{50} was determined by the Spearman-Karber method and calculated by Probit method (Bross, 1950).A sub-lethal dose of 20 μg RVV/100 gm mice was selected for induction of SAKI in experimental group.

2.3. Selection of AKI induction time period

To ensure renal involvement in mice after i.m. injection of sub-lethal dose of RVV, time kinetic study were performed and it was observed that at 48–72 h after the venom injection kidney injury was prominent among the experimental animals.

2.4. SAKI experimental model

Animals were randomly classified into two groups- sham control (Group I, n = 12) and venom treated group (Group II, n = 12). The animals of group II were given i.m. injection of 50 μl of saline containing RVV (dose: 20μg/100 gm) and sham control received equal amount of saline via same route. The animals were housed for 60 h under identical condition with free access to food and water. Urine was collected at the end of 60 h and animals were then sacrificed after proper anaesthesia and blood and tissues were collected.

2.5. Sample collection

2.5.1. Urine collection

For urine collections both group of mice were pooled in small batch of three mice/batch. Each mice received 1.2 ml of saline intra peritoneal (i.p.) at 46 h after the venom injection. Mice were then kept in urine cage to urinate till completion of the incubation period, i.e. for 14 h.

2.5.2. Blood collection

Both groups of animals were bled by retro orbital plexus and the blood samples were collected in a heparinised vial.

2.6. Assessment of renal markers

The volume of excreted urine after 14 h incubation was measured for each batch of animals. Uristrip analysis was performed and results were evaluated according to manufacturer's guideline. The urinary and plasma creatinine were assessed by the alkaline picrate method and micro protein was assessed by pyrogallol red method according to manufacturer's guideline.

2.7. Renal histology

Kidney was excised aseptically from the cervically dislocated animal and cut into small pieces transversely and subsequently fixed in 10% formol buffered saline (pH-7.4) for 24 h. After fixation, the pieces of kidney were then dehydrated using graded ethanol series (50%–100%) and embedded in paraffin (m.p.-56-58 °C). 6 μm thick section were cut and stained with hematoxylin and eosin and examined under light microscope (10x, 40x and 100x objective).