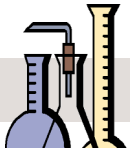

Xanthine Oxidase and Tumor Necrosis Factor Alpha: Possible Mediators of Remote Tissue Injury after Viper Envenomation

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Key words: viper, envenomation, xanthine oxidase, tumor necrosis factor, systemic effects, mediation

Abstract

Background: Tumor necrosis factor is associated with various local and systemic inflammatory sequelae following snakebite. Xanthine oxidase is a principal mediator of remote tissue injury (e.g., lungs, heart, liver).

Objective: To investigate in a snakebite-like animal model the as yet unexplored role of TNF and XO in mediating organ damage following snakebite.

Methods: Sprague-Dawley rats were injected intramuscularly with a non-lethal 500 μg/kg dose of *Vipera aspis* venom (n=10) or saline (n=10). Blood pressure and heart rate were continuously monitored, TNF-α was measured in the blood, and total XO + xanthine dehydrogenase activity was assessed in various tissues. Lung histology and permeability indices were analyzed.

Results: Venom injection caused a significant ($P<0.05$) reduction in both heart rate and invasive arterial pressure. The blood circulating TNF levels were significantly higher in the intoxicated group ($P<0.05$ vs. saline group), with changes seen at 30 minutes from intoxication in both groups. Total XO + XDH activity in the kidney, lung and liver of the venom-injected group was significantly ($P<0.05$) higher than in the saline group, while the activity in the heart was similar.

Conclusions: The mediation of remote organ and hemodynamic changes following intramuscular injection of a non-lethal dose of *Vipera aspis* venom can be attributed partly to TNF and partly to XO. More research is needed to better understand the role of either compound and the time frame of their activity before specific antagonists can be introduced for snakebite management.

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TNF = tumor necrosis factor
XO = xanthine oxidase
XDH = xanthine dehydrogenase

Snakebites are a public health problem throughout the world [1]. The incidence of death from snakebites is low in most countries because of the quick availability of medical care even in rural areas. In the United States, the mortality rate from snakebite is less than 1% for victims who received antivenin [2], compared to 0.5% in France [3]. Worldwide, some 30,000–40,000 persons die every year as a result of venomous snakebite. We suspect that these numbers may very well be underestimations in the developing countries.

Snake venoms are a complex mixture of enzymes, low molecular weight polypeptides, glycoproteins, and metal ions. Clinical manifestations following low grade envenomation result in local and limited edema, necrosis and marked pain [4], but there can often be systemic effects such as nausea and vomiting, generalized edema, bleeding disorders, cardiovascular instability, renal and respiratory failure, and central nervous system depression that may later be fatal [5].

TNF-α, a potent pro-inflammatory compound, is synthesized as a 26 kDa precursor protein that is later cleaved to its mature and active 17 kD form. The matrix metalloproteinases have been implicated in the syntheses of TNF-α [6,7]. Viper venoms are indeed rich in zinc metalloproteinases, which very closely resemble metalloproteinases [8] and were shown to cleave recombinant pro-TNF-α to form the biologically active mature TNF-α [9]. This observation has raised the suggestion that TNF actually plays an important role in the generation of both local and systemic sequelae of snakebites.

Xanthine oxidase and its reduced form, xanthine dehydrogenase, have long been considered principal mediators of remote tissue injury (e.g., lungs and heart) [10–12]. Isolated perfused lungs exposed to XO-rich effluent from livers that had been damaged earlier were found to suffer from acute lung injury and increased alveolar capillary leak [11]. In other studies, hemorrhagic shock in a rat model [13] was associated with an increase in serum XO activity. The possible role of XO-generated oxidants as mediators of

damage following snakebites has never before been explored.

We designed this study to test the hypothesis that TNF and/or XO play a damaging role in systemically mediating damage to remote organs following experimental viper envenomation.

Materials and Methods

Experimental protocol

Adult male Sprague-Dawley rats (n=20) weighing 350–400 g were used in the study. The animals were treated in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals at the Tel Aviv University, and the Medical Center Committee for Animal Research approved the study. The rats were randomized into venom-administered (n=10) and saline-injected (n=10) groups and anesthetized with intraperitoneal ketamine 125 mg/kg. Following an intramuscular injection of a non-lethal dose of *Vipera aspis* venom (500 μg/kg in 0.5 ml; Unite des Venins, Institut Pasteur, Paris, France) or a similar volume of 0.9% sodium chloride solution, the animals were kept for observation for 4 hours during which additional intraperitoneal injections of ketamine 60 mg/kg were given if necessary. Heart rate and invasive blood pressure were continuously recorded. At the end of the experiments the rats were sacrificed by a lethal dose of thiopental.

Biochemical measurements

Blood samples (2 ml) were obtained via the arterial line to determine TNF levels before drug injection (baseline), at 2 hours, and at the end of the experiment. The results of a pilot study had shown that no changes in TNF blood levels would occur within the first 90 minutes of envenomation. Blood volume was always replaced by 3:1 volumes of 0.9% sodium chloride solution. All samples were immediately centrifuged (15,000 rpm) for 3 minutes at 4°C and the serum was frozen at -70°C until analyzed. At the end of the experiment, just before the animal was sacrificed, blood samples for I^{125} assessment were taken and 20 ml of saline was injected into the right ventricle in order to wash out the blood from the pulmonary circulation. A specimen of the lung tissue was then removed for a blood-to-tissue radioactivity count ratio.

TNF assay

TNF activity was measured using a commercially available ELISA kit (Cytoscreen^{TM} rat kit TNF-α, Immunoassay kit, Biosource, Camarillo, CA, USA). Values in blood were expressed as pg/ml.

XO assay

XO activity was analyzed as previously described [14]. Activity was quantified by spectrophotometrically monitoring the formation of uric acid from xanthine at 292 nm. XDH was similarly determined separately after nicotinamide dehydrogenase was added to the reaction. One unit of activity was defined as 1 μmol/min of uric acid formed at 37°C, pH 7.5. Activity was expressed in mU/g wet weight of the various tissues.

Histological assessment of organ injury

The lungs of the sacrificed animals were inflated with 10% formaldehyde to a pressure of 25 cm H_2O. Following standard fixation, microthin sections were taken from the inferior aspect of each lung and stained with hematoxylin and eosin for light microscopic analysis. Neutrophil sequestration indicating injury to the lung was achieved by quantifying the alveolar septal wall polymorphonuclear cells; entrapment was expressed as the mean number of PMN per 10 high power fields in an equivalent number of alveoli. The pulmonary pathologist who analyzed the microscopic sections was blinded to the protocol.

Lung permeability

I^{125} albumin (0.1 μCi) with an activity of approximately 1,000,000 CPM in 1 ml of saline was injected at the beginning of the experiment through the femoral vein. The resultant circulating I^{125} activity was recorded in both the 4 hour blood sample (end of experiment) and the lung specimen (after being washed and weighed). Lung permeability was expressed as the ratio between radioactivity in the lung tissue and that in the serum (permeability index = $CPMg_{Lung}/CPMg_{serum}$).

Statistics

All results are reported as mean ± SEM. Statistical significance was tested by one-way analysis of variance (ANOVA) and significant results were reanalyzed using an unpaired Student's *t*-test. Differences were considered significant at $P<0.05$.

Results

Hemodynamic data

Fifteen minutes after the injection of the venom, there was a gradual decrease in mean heart rate of the treated group (data not shown), which became statistically ($P<0.05$) different from the saline group heart rate 15 minutes later [Figure 1]. The mean arterial blood pressure [Figure 2] initially (at 15 minutes) and briefly increased to non-significant values, after which (at 30 minutes) it decreased significantly ($P<0.05$) compared to the saline group. Both cardiovascular parameters remained lower than in the saline group for the rest of the experiment.

TNF activity

TNF blood levels in the venom-injected rats started to rise within 15 minutes after the inoculation of the toxin, becoming significantly ($P<0.05$) higher than at baseline 15 minutes later. TNF levels reached a peak value at the 2 hour

PMN = polymorphonuclear cells

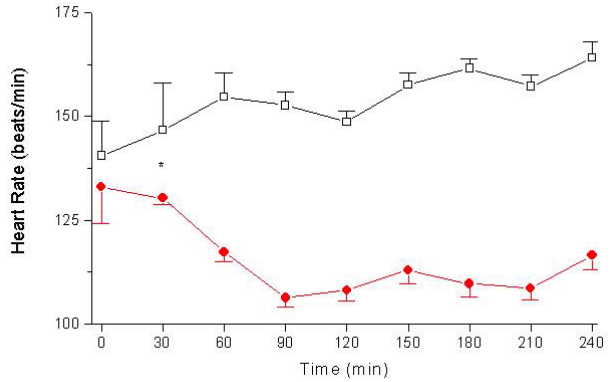

Figure 1. Mean heart rate. Data are expressed as means ± SEM. Open squares = saline group, solid circles = venom group. *P<0.05 vs. saline group.

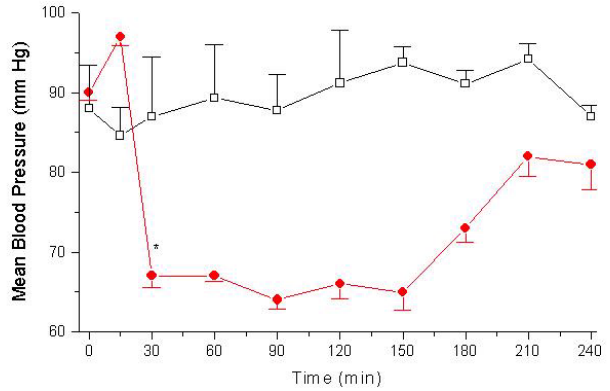

Figure 2. Mean arterial blood pressure. Data are shown as means ± SEM. Open square = saline group, solid circle = venom group. *P<0.05 vs. saline group.

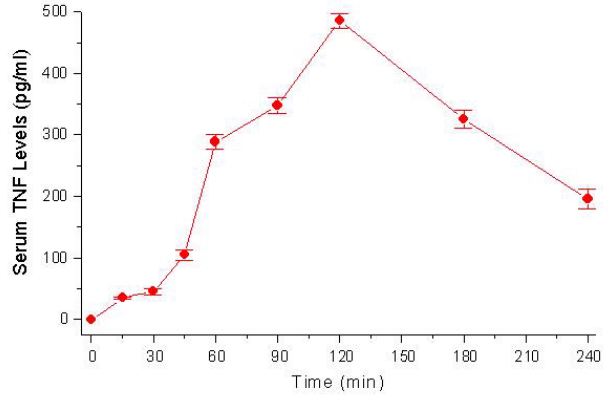

Figure 3. Serum TNF levels. Data are shown as means ± SEM. Open square = saline group, solid circle = venom group. *P<0.05 vs. saline group.

experimental time point, whereas no increase whatsoever in the TNF blood value was detected in the saline group [Figure 3].

XO activity

No significant differences in the total XO (XO + XDH) activity were detected in the heart tissues of animals in either group. However, activity was significantly (P<0.05) higher in the kidney, lungs and liver tissues of the venom group compared to those in the saline group [Table 1].

Lung permeability and histology

There was no histological evidence of lung injury in the venom group. The lung permeability index was not statistically higher compared to the saline group, and the number of PMN sequestrated within the lung septa were similar in both groups (data not shown).

Table 1. Total xanthine oxidase activity (mU/g wet weight)

Organ	Saline	Venom	*P
Liver	209±49	264±40	0.047
Lung	288±41	457±16	0.013
Kidney	96±44	159±45	0.036
Heart	67±16	72±24	NS

Data are expressed as means ± SEM.
* Student's t test

Discussion

The intramuscular injection of snake venom in the intact rat caused a systemic inflammatory process. This was expressed by an abnormal and brief increase followed by a decrease in mean blood pressure associated with the contrasting bradycardia in the venom rats. The abnormally high blood TNF levels and the increased XO activity in the tissue support our contention that these compounds may have taken part in a systemic spread of pro-inflammatory activity that followed the injection of snake venom. The abnormally high XO activity in various organs might indicate a process that could ultimately lead to conditions similar to clinical multiple organ failure.

The clinical features that follow different snakebites are well described. In a study on 102 cases of viper bites, local edema was the most prominent finding in 97 patients [5]. The authors documented a correlation between the levels of venom antigens in the urine or blood and the severity of the clinical signs following envenomation [5]. The authors of a recent report also observed a correlation between the amount of Russell's viper venom that can be detected in the serum (measured by ELISA) and the patient's clinical signs [15]. In each report, while a bite to the finger or toe was characteristically followed by mild systemic effects due to poor absorption, severe local skin blistering, infection and necrosis did occur. The inflammatory response generated in a model of a mouse subjected to a subcutaneous injection of *Bothrops asper* venom to the footpad was characterized by a

rapid increase in serum interleukin-6 concentration that was observed 3–6 hours after the envenomation [16]. In contrast, serum TNF-α and IL-1 were not detectable at any time during their 4 hour study period [16]. Similarly, we were able to demonstrate a significant inflammatory process within 2 hours after the injection of *Vipera aspis* venom in a murine model, as expressed by the increase in serum TNF levels. Our results are in agreement with the finding that viper venom is rich in zinc metalloproteinases [8] and with the suggestion that these proteins can cleave pro-TNF-α into its mature active form [9]. Our findings of an increase in TNF blood levels, albeit a delayed one, differ from those of Lomonte et al. [16] and are apparently due to the different protocols of venom intoxication: subcutaneous injection into the footpad was suggested to induce local generation of TNF that results in only regional necrosis and without systemic consequences, as was also reported in humans [5,15]. In contrast, the intramuscular administration of venom mimics a snakebite that releases its venom systemically. This route of envenomation and the systemic absorption of the toxin in our experiment led to the generation of TNF associated with systemic changes, i.e., loss of hemodynamic stability, all occurring almost at the same time. The relative bradycardia initially associated with hypotension that was documented only in the viper group may have resulted from an acute and severe pro-inflammatory process, thus lending further support to our contention of a systemic mediation following the intoxication. Bradycardia after venom immunotherapy administration in one reported case [17] was suggested to have resulted from a direct negative chronotropic effect of the venom on the rat's heart.

XO is a ubiquitous enzyme existing in most normal functioning tissues, mainly in the XDH form. However, once the tissue is exposed to a metabolic stress, such as inflammation, hypoxia or ischemia, the enzyme converts to the oxidized form. In the presence of adequate amounts of substrate and oxygen, XO enables the generation of cytotoxic oxidants such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) [18]. The entire cascade of XO-generated oxidative events was proven to be damaging in animals [19, 20] and in humans [21]. The elevated total XO activity in the liver, the lung and the kidney documented in the current study is suggestive of stress oxidant mediation of multiple organ dysfunction that followed envenomation. The absence of histological damage in the lungs in our study is probably due to the short time that elapsed between the injection of the venom and the animal execution. These findings, therefore, do not exclude this cascade of events. Since the toxin would pass through the liver soon after its absorption, attempts to detoxify it might involve the recruitment of powerful endogenous anti-oxidative activity. That is, due to the large amount of XO that is withheld within the hepatic cells, part of that oxidative activity could leak out into the circulation [19]. The capability of rat organs to bind circulating XO, especially if their endothelial area is damaged (e.g., in the lung) [11], could explain both the abundant deposition of XO in these organs and the injury in three remote organs, independently of the factor that initiated this cascade of oxidative events. Indeed, it was observed that XO binds to glycosaminoglycans of vascular endothelial cells [13], fibroblasts, and components of various basement membranes [22]. Because of this XO capability, the concentration of XO on the cell surfaces might become a mechanism that would supply the circulation with oxidants and do so uninterruptedly. This concept of binding and concentrating XO within tissues and later propagating the damage further is supported by our previous observation that total XO activity in lung tissue increased by 14-fold following lung reperfusion with the effluent of a post-ischemia liver that contained high XO activity [11]. The fact that the current study used an intact animal rather than isolated organs is not relevant, since a similar mechanism of XO-derived propagated damage was demonstrated in an intact animal model of ischemia-reperfusion as well [23].

Finally, there is the possibility of a combined toxic interrelationship between TNF-α and XO that could lead to the systemic hemodynamic instability and organ damage that we witnessed. This eventuality is currently under investigation.

In conclusion, intramuscular injection of *Vipera aspis* venom in the rat results in systemic circulatory deterioration and the effects of multi-organ damage. TNF and XO were involved in damaging processes that could be attributed to the mediation of each one. Clarification of their combined effect may pave the way for new and enhanced therapeutic modalities for patients with snakebites.

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IL = interleukin

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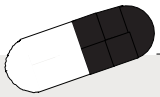
Capsule



HIV and scrub typhus

Typically, when HIV-infected people become infected with other pathogens, the level of the AIDS virus in their blood skyrockets. But in the 5 August issue of *The Lancet*, researchers report that AIDS patients infected with the bacillus that causes scrub typhus – a mite-borne disease that produces fever but usually isn't fatal – have precisely the opposite reaction: their HIV levels plummet. The idea for the study began 2 years ago, when George Watt, a tropical disease specialist at the United States Armed Forces Research Institute of Medical Sciences in Bangkok, became intrigued by one HIV-infected patient who developed scrub typhus. Caused by *Orientia tsutsugamushi* (formerly *Rickettsia*), scrub typhus is transmitted by chiggers that fall off rodents and then live in the scrub bush.

As the researchers report in *The Lancet*, their massive, year-long screening program in Thailand uncovered 10 people who clearly had both infections and no others. First, the Thai team compared viral loads in these patients to a control group of five HIV-infected people who did not have scrub typhus but did have either malaria or leptospirosis. Over the 28 day study, the investigators found that the patients with scrub typhus had significantly lower HIV viral loads than those with the other diseases. In two of these people, in fact, the levels fell so low that the most sensitive tests could not detect HIV. In another curious twist, the scrub typhus patients happened to have more damaged immune systems, with an average of only 117 CD4 white blood cells, as opposed to an average of 255 CD4s in the control group. (By definition, an HIV-infected person has AIDS when CD4 counts drop below 200.)

More evidence that scrub typhus somehow suppresses HIV came from a second experiment, a comparison of viral variants in the same 10 scrub typhus patients and another control group whose CD4 counts were more closely matched. As HIV disease progresses, several researchers have shown that the virus typically evolves to a form more adept at destroying immune system cells. Specifically, once these nastier HIV variants infect cells, they can readily fuse with other cells to form "syncytia." None of the 10 patients with scrub typhus had a syncytia-inducing HIV variant, whereas five of the seven controls did.

Watt and his co-workers attempted to tease out how *0. tsutsugamushi* might thwart HIV. Preliminary data in both mouse and test tube experiments with human sera point to antibodies against scrub, which for some unknown reason seem also to bind HIV. These new insights might provide clues for developing both AIDS treatments and vaccines.

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