Toxicology Reports 5 (2018) 318–323

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Montivipera bornmuelleri venom has immunomodulatory effects mainly up-regulating pro-inflammatory cytokines in the spleens of mice

Tania Yacoub[a], Mohamad Rima[b], Riyad Sadek[c], Walid Hleihel[d], Ziad Fajloun[e,f], Marc Karam[a,*]

[a] Department of Biology, University of Balamand, Kourah, Lebanon
[b] Sorbonne Universités, Department of Neuroscience, Institute of Biology Paris-Seine, CNRS UMR 8246, INSERM U1130, F-75005 Paris, France
[c] Department of Biology, American University of Beirut, Lebanon
[d] Faculty of Sciences, USEK, Kaslik, Jounieh, Lebanon
[e] Department of Biology, Faculty of Sciences III, Lebanese University, Lebanon
[f] Laboratory of Applied Biotechnology, Azm Center for Research in Biotechnology and its Applications, Doctoral School of Sciences and Technology, Tripoli, Lebanon

ARTICLE INFO

Keywords:
Montivipera bornmuelleri
Venom
Th1
Th2
Th17
Treg
Cytokines

ABSTRACT

Beside their toxicity, snake venom components possess several pharmacological effects and have been used to design many drugs. Recently, the cytotoxic, antibacterial, vasorelaxant, pro- and anti-coagulant as well as inflammatory activities of *Montivipera bornmuelleri* venom have been described *in vitro*. However, the *in vivo* effects of this Lebanese snake venom on the immune system has not been established yet. Here, we investigate the immunomodulatory effects of *M. bornmuelleri* venom on the murine splenic levels of TNF-α, IFN-γ, IL-4, IL-10, IL-1ß and IL-17 at 6 and 24 h post treatment. Different doses of the venom (1 mg/kg, 2 mg/kg, 4 mg/kg and 6 mg/kg) were injected intraperitoneally in BALB/c mice. Using the logit method, LD_{50} of *M. bornmuelleri* was proved to be 1.92 mg/kg in our experimental conditions. This study also shows that 1 mg/kg and 2 mg/kg of *M. bornmuelleri* venom are able to modulate the levels of cytokines in the spleen of mice, as assessed by ELISA. In fact, this snake's venom up-regulates TNF-α, IFN-γ, IL-1ß and IL-17 with a trend in decreasing IL-4 and IL-10. Therefore, by favoring Th1 and Th17 over Th2 and Treg responses, *M. bornmuelleri* venom might have important clinical implication especially in the field of cancer immunotherapy.

1. Introduction

Snake venom studies trace back to Aristotle (384–322 BCE); however, they were not experimentally used until the 18th century (by Felice Fontana) [1]. Snake venoms contain a mixture of mostly proteins and peptides that constitute 90–95% of the venom's dry weight including enzymes, non-enzymatic peptides and toxins. Although being dangerous, snakes have always been associated with healing; the true and authentic symbol of medicine is the Rod of Asclepius, illustrating a staff with a snake coiled around it. In fact, venom components form a pool of pharmaceutical products involved in many medical practices and drug discovery. For example, Captopril is the first drug based on a peptide from the pit viper *Bothrops jararaca* and is used to treat hypertension and some forms of congestive heart failure [2,3]. In addition, snakes venom are capable of modulating the immune system especially at the level of cytokines production. A member of the *Elapidae* family exhibited anti-arthritic and anti-inflammatory activities in arthritic rats mainly through reducing serum levels of the pro-inflammatory cytokines IL-1β, IL-17 and TNF-α [4]. However, most immunomodulatory studies focused on the venom of the *Viperidae* family especially the subfamily of *Crotalinae* with its both genera *Bothrops* and *Crotalus*. Intraperitoneal injection of *Bothrops atrox* and *Bothrops erythromelas* venoms in mice resulted in an inflammatory reaction characterized by the up-regulation of many serum and splenic cytokines such TNF-α, IL-6, IL-10, IL-12p70 as well as IFN-γ [5,6]. Conversely, *Crotalus* envenomations failed to generate a significant inflammatory reaction at the site of bite and induced an anti-inflammatory status in a mouse experimental model of colitis [7]. The distinct effects of Bothrops and Crotalus venoms on the inflammatory response might be attributed to the inability of *Crotallus durissus terrificus* to induce the expression of COX-2 and therefore the synthesis of the prostanoids PGD_2 and PGE_2 [8].

Montivipera bornmuelleri is a venomous snake belonging to the family of *Viperidae* and endemic to high-altitude Lebanese mountains [9]. A proteomic analysis of the venom of *M. bornmuelleri* showed that it contains 65 protein compounds corresponding to enzymatic protein families such as serine proteases, phospholipases A2 and metalloproteases III [10]. The crude venom of *M. bornmuelleri* possesses

* Corresponding author.
E-mail addresses: mohamad.rima@upmc.fr (M. Rima), marckaram1@gmail.com (M. Karam).

https://doi.org/10.1016/j.toxrep.2018.02.011
Received 14 October 2017; Received in revised form 27 February 2018; Accepted 27 February 2018
Available online 01 March 2018

antimicrobial activities against Gram positive and Gram negative bacteria, with the most significant effect on *Staphylococcus aureus* and *Morganella morganii,* as well as against the fungus *Candida albicans* [10]. *M. bornmuelleri* venom also affects the hemostatic system since it has been shown to have pro- and anti-coagulant activities on human plasma at different concentrations [11]. Vipers venom are known to reduce blood pressure; this is also true for *M. bornmuelleri* venom. In fact, the venom displays vasorelaxant effects by acting synergistically on different pathways. It can possibly act on endothelial cells inducing the release of the vasoactive mediator NO, reducing Ca^{2+} influx through voltage dependent calcium channels and inhibiting angiotensin I induced vaso-constriction [12]. Furthermore, it was suggested that *M. bornmuelleri* venom lacks direct lytic factors since it induces indirect hemolysis in the presence of PLA2, which is able to hydrolyze lecithin [11]. To further characterize the role of PLA2, it was purified and its biological activity was tested showing that it exhibits strong anti-bacterial, hemolytic, anti-coagulant and pro-inflammatory activities [13,14].

Despite the extensive studies of the Viperidae venom's biological activities, the immunomodulatory effect of the *M. bornmuelleri* venom has not been previously tested *in vivo*. Therefore, we investigate here the effect of the intraperitoneal injection of several *M. bornmuelleri* venom doses on the levels of various cytokines (TNF-α, IFN-γ, IL-4, IL-10, IL-1ß and IL-17) in spleen tissue of BALB/c mice, which will help to further evaluate the potential use of the venom in immunotherapy.

2. Materials and methods

2.1. Chemicals

Bovine Serum Albumin (BSA), NP-40, Phosphate Buffer Saline (PBS), Sodium Chloride (NaCl), Sodium Dodecyl Sulftae (SDS) and Sodium Deoxycholate were obtained from Sigma-Aldrich chemie, Steinheim, Germany. Tris-Hydrochloride (Tris-HCl) and tween-20 were obtained from Bio Basic Inc., Ontario, Canada.

2.2. Venom

Venom was supplied by Dr. Riad Sadek (American university of Beirut) in its lypholised form and stored at −20 °C. Venom was dissolved in PBS prior to the experiment and filtered through 0.2 µm sterile syringe filters.

2.3. Mice handling

Eight to ten weeks old female BALB/c mice, procured from the University of Balamand animal house, were fed a standard diet and kept at 25 °C in 12 h day/night cycle. They were handled according to the Guide for Care and Use of Laboratory Animals of the Faculty of Sciences.

Mice were placed in groups and injected with different doses of venom: 1 mg/kg, 2 mg/kg, 4 mg/kg and 6 mg/kg. Control animals were injected with either PBS (negative control) or fed a normal diet (naïve). Mice were sacrificed by neck dislocation at time of death or at 6 and 24 h following the injections. Spleens were removed, weighed and kept in eppendorf tubes at −80 °C. All experimental procedures were carried out with ethics committee approval from the University of Balamand and with strict adherence to the ethical guidelines for the study of experimental pain in conscious animals [15].

2.4. Determination of the median lethal dose

According to the world health organization (WHO), the median lethal dose (LD_{50}) is the amount of venom causing the death of 50% of treated animals. Most of the mice injected with 4 mg/kg and 6 mg/kg of venom died by 24 h, with death occurring mainly between 2 and 4 h post injection. These two doses were thus considered as toxic and not of relative significance to the rest of the results. Six out of 10 of the mice injected with 2 mg/kg of venom were dead by 24 h, whereas, mice injected with 1 mg/kg of venom remained all viable except for 1. Plotting the natural logarithm of the proportion of mice dead for each injection versus their corresponding logarithmic concentration generates a linear curve used to determine the toxicity of a certain compound on living organisms.

2.5. Tissue preparation

Frozen samples were homogenized in 1.5 ml RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS pH = 7.6) supplemented with Protease inhibitors at 4 °C. The homogenate was incubated on ice for 30 min and then centrifuged at 10 000*g* for 30 min at 4 °C. Following centrifugation, the supernatants were transferred to labeled eppendorfs and stored at −80 °C for cytokine measurement.

2.6. Cytokine measurement

Quantitative measurement of the levels of cytokines was performed using Mini Enzyme-Linked Immunosorbant Assay (ELISA) Development Kits (Peprotech). 96-well plates were set up according to the manufacturer's instructions and read using an ELISA plate reader at 405 nm with 650 nm as the correction wavelength. Concentrations of the cytokines TNF-α, IFN-γ, IL-4, IL-10, IL-1ß and IL-17 were estimated using standard curves established with the appropriate recombinant cytokines. The results were expressed as pg/ml and then pg/g of tissue.

2.7. Statistical analysis

Differences among groups were analyzed using GraphPad Prism 6.00 software (GraphPad Software Inc., San Diego USA) by one-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test. Results were expressed as means ± SEM (n = 4–3 animals/group). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Determination of LD50 of Montivipera bornmuelleri venom

Mice were injected once with the following venom concentrations: 1 mg/kg, 2 mg/kg and 4 mg/kg and the LD50 was estimated. Our results show that the intraperitoneal LD_{50} of *M. bornmuelleri* venom is 1.92 mg/kg is in our experimental conditions (Fig. 1). This finding therefore explains the observed lethality of the 2 mg/kg dose on almost half of the injected mice.

3.2. The effect of M. bornmuelleri venom on the levels of IFN-γ in the spleen of mice

Intraperitoneal injections of mice with 1 mg/kg of venom failed to induce IFN-γ up-regulation 6 h post-treatment (Fig. 2A). However, 24 h following the treatment an increase in IFN-γ levels, though insignificant, was observed (Fig. 2B). Furthermore, intraperitoneal injections of mice with 2 mg/kg was able to induce a significant increase in IFN-γ levels as compared to the control ($p < 0.01$).

3.3. The effect of M. bornmuelleri venom on the levels of TNF-α in the spleen of mice

The dose of 1 mg/kg of *M. bornmuelleri* venom was able to significantly induce the production of TNF-α as compared to the control groups ($p < 0.01$) at 6 h (Fig. 3A) but not 24 h post-treatment (Fig. 3B). However, injecting 2 mg/kg of venom caused a significant