Original Article

Cellular Immunity in Mice Vaccinated with Recombinant Phospholipase D Toxoid of *Hemiscorpius lepturus* Scorpion

Narges Safari-Foroushani[1], Mohammad Hossein Modarressi[2], Kamran Pooshang Bagheri[3], Mahdi Behdani[3], *Delavar Shahbazzadeh[3]

[1]Islamic Azad University, Science and Research Branch, Tehran, Iran
[2]Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[3]Venom and Bio therapeutics Molecules Lab, Medical Biotechnology Department, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

***Corresponding author:** Dr Delavar Shahbazzadeh, Email: shahbazzadeh@yahoo.com

(Received 15 June 2021; accepted 22 Sep 2021)

Abstract

Background: *Hemiscorpius lepturus* is one of the most dangerous scorpions in Iran and the world. Numerous studies have been conducted on phospholipases, especially phospholipase D, in this scorpion's venom, and the results have shown this protein to be the main cause of death. Therefore, one of the most effective ways of preventing fatalities is to produce a toxoid vaccine from the deadly toxin of the venom. The present study was conducted to assess the non-toxicity of this toxoid and the safety of the vaccine candidate in BALB/c mice.

Methods: The production of interferon-gamma and interleukin-4 cytokines in the spleen cells of the mice was measured using ELISpot assay 28 days following immunization with rPLD toxoid.

Results: The unpaired t-test results showed a significant increase in the concentration of IFN-γ cytokine in the vaccinated mice (P= 0.001), indicating that the immune system is directed toward the Th1 pattern, while no significant difference was observed in the levels of IL-4 (P= 0.16) despite an increase in this cytokine. The in-vivo tests showed that the mice immunized with interval doses of 80µg of toxoid were completely protected against 10 × the LD_{100} of the venom. Moreover, the toxoid had no dermonecrotic effects and caused no necrotic and inflammatory complications in the rabbit skin.

Conclusion: As a vaccine, the toxoid has the potential to increase the Th1 cytokine response and, subsequently, increase acquired cellular immunity. Thus, this toxoid appears to be able to provide an effective vaccine against the venom of *Hemiscorpius lepturus*.

Keywords: *Hemiscorpius lepturus*; Toxoid; Interferon-gamma (IFN-γ); Interleukin 4 (IL-4); Cellular immunity

Introduction

Scorpion sting is one of the main public health problems in the world (1). The six dangerous scorpion species in Iran include *Hemiscorpius lepturus* (*Scorpionidae*), *Androctonus crassicauda*, *Hottentotta saulcyi*, *Hottentotta schach*, *Odontobuthus doriae*, and *Mesobuthus eupeus* (Buthidae) and among them, *H. lepturus* is considered the most dangerous (2). *Hemiscorpius lepturus*, or Gadim scorpion (its local name), is blamed for 50% of all scorpion stings nationwide, while it is responsible for 89% of the reported deaths (3). In addition to adults, this scorpion's sting is highly deadly in sensitive groups, particularly children, and causes 60 times more deaths than other scorpion species in Iran (3, 4). Stinging by this scorpion causes clinical signs and symptoms not previously described for other scorpion species (5). Unlike other scorpions, whose venom causes neurotoxic signs and symptoms, this scorpion's venom produces cytotoxic signs and symptoms with systemic effects such as dermonecrosis, inflammation, arterial leak, and renal failure (6, 7). These signs and symptoms are attributed to the venom's enzymatic compounds (5, 8). Recently, a 32-kDa

http://jad.tums.ac.ir
Published Online: March 31, 2022

protein, called phospholipase D, has been identified in *H. lepturus* scorpion's venom (9). It is a dermonecrotic toxin that causes signs and symptoms fully similar to those of the complete venom of *H. lepturus* and is the most important molecule in the scorpion venom (10, 11). These signs and symptoms are fully similar to the main clinical signs and symptoms of the brown *Loxosceles intermedia* sting, which is responsible for many signs and symptoms and deaths in humans (9, 12). The treatment currently used for Gadim venom in Iran is the equine polyvalent antiserum against the six venomous scorpions in the country but there is still no standard protocol for the management of people afflicted with the venom of this scorpion (2). Moreover, there are reports of anaphylactic shock and death in the people stung by this scorpion, especially young children (7). The use of antiserum is particularly effective against pathogens that can activate the immune system in the unstimulated state (13). Based on a previous study, we assumed that toxoid (the first non-toxic form of phospholipase D toxin produced using formalin) would be able to induce active immunity in BALB/c mice and, consequently, could be used as a vaccine (10). This hypothesis has previously been confirmed by using toxoids as vaccines for the prevention of diphtheria (14, 15, 16), tetanus (15, 17), and botulism (18, 19). Accordingly, the present study was conducted to test the non-toxicity of the toxoid and assess the efficiency of the immunogenicity of the produced toxoid for its use as a vaccine.

This study assesses the vaccine by investigating inflammatory and anti-inflammatory cytokines in the immunized mice. The authors believe that if it is demonstrated that pro inflammatory cytokines are significantly increased in the present study, the results were confirmed by repeated tests, it can then be concluded that the present vaccine increases cellular immunity in the mice and can also cause acquired immunity of the cellular type.

Materials and Methods

Expression and purification of recombinant phospholipase D

The phospholipase D (PLD) gene of the Iranian venomous scorpion *H. lepturus* was expressed and the product purified based on a previous design (10, 11). Briefly, pET-22b plasmid was used in gene expression. This gene has the gene bank accession number: KY287766. *E. coli* BL21 bacterium was transformed using the PLD gene. The transformed bacterium was cultured in LB medium containing ampicillin at 37 °C until optical absorption at 600nm wavelength reached 0.6. The induction was carried out using 0.1mM isopropyl beta-d-1-thiogalactosidase (IPTG) (Thermo Fisher Scientific Co. Waltham, MA, USA). The cells were kept at 30 °C for 3.5 hours, then centrifuged at 10000×g for 10 minutes at 4 °C and suspended in the lysis buffer. To purify the recombinant protein, bacterial cells were first dissolved in 1mL of the lysis buffer per 0.1g of the precipitate and placed on ice for 30 minutes. Then, the host cell membrane was disintegrated by sonication (Hielscher co. Teltow, Germany) over 15 minutes at 30% amplitude with a cycle frequency of 30. Next, the lysed cells were centrifuged, and the supernatant containing the soluble protein was purified by affinity chromatography with Ni-NTA agarose column (Qiagen co. Hilden, Germany). The recombinant protein was dialyzed using PBS and analyzed by SDS-PAGE (20).

Toxoid production from recombinant phospholipase D

To produce the toxoid, detoxification, recombinant PLD was first diluted in sterile PBS and then added to a 10kD dialysis sac (10). The protein was exposed to 0.25 methanol formalin for 10 days at room temperature. The samples were gently dialyzed with PBS buffer at 4 °C, over 24 hours (the buffer was changed twice during this procedure) and filtered through a 0.22μm membrane to remove excess residue.

http://jad.tums.ac.ir
Published Online: March 31, 2022

The toxoid concentration was determined using the BCA kit following the manufacturer's instructions (i NtRoN Biotechnology Co. Seoul, Korea). The residual formalin test was performed to ensure the absence of formalin in the toxoid samples (20).

Animals

The present study had a code of ethics from Pasteur Institute of Iran for using animals in research (No: PII.REC.1394.38). Two 3±1 months-old New Zealand white rabbits weighing 2kg each, and one hundred- twenty 6±1 months-old female BALB/c mice weighing 20–25g each were procured from the Pasteur Institute of Iran. Before the experiments, the animals were kept in standard laboratory conditions at a temperature of 22–25 °C with 12 hourly alternating light/dark cycles and relative humidity of 40–60% with free access to food and water for one week, after which time, the mice were randomly divided into 2 groups (60 mice per group).

Toxoid toxicity test

The toxoid toxicity was assessed using dermonecrotic activity so that 3µg of the toxoid was brought to a volume of 100µl of sterile PBS and injected subcutaneously into a rabbit with the hair on its back shaven. The presence of inflammation and necrosis was assessed after 4, 8, 16, 24, 48, 72, and 96 hours, and the injection site diameter was measured with a caliper. PBS and rPLD1 toxin were used as negative and positive controls.

Toxoid immunogenicity in mice

One hundred-twenty female BALB/c mice were assigned into two sixty mice groups. The first group was chosen for vaccinating with toxoid- Freund's adjuvant and the second group for toxoid- Alum adjuvant as described in detail below.

Immunization by toxoid-Alum adjuvant

In this series of experiments each of sixty other mice received 80µg of toxoid. This amount of toxoid was mixed with 100µl of sterile PBS and then mixed with 100µl of Alum adjuvant (Thermo Fisher Scientific Co. Waltham, MA, USA.) followed by stirring until a homogenous solution was obtained. 200µl of this solution was subcutaneously injected to each mouse on days 1, 14, 28, 42, 56 and 70. Here also, 6 mice that only received sterile PBS-alum under the same conditions were considered as negative controls.

Immunization by toxoid-Freund's adjuvant

Based on previous studies (Safari et al. 2018), mice were immunized in 10 groups with two-weeks intervals on days 15, 29, 43, 57 and 71. The first injection was administered using complete Freund's adjuvant and the following injections using incomplete Freund's adjuvant. 80µg of toxoid was homogenized with 100µl of Freund's adjuvant at 4 °C for 4 hours, so that the antigen could completely mix with the adjuvant, diluted in PBS to 200µl, and injected subcutaneously. The control group was injected with 100µl of PBS and 100µl of Freund's adjuvant. The animals were examined in terms of health and weight in all stages of the experiment. Here also, 6 mice that only received sterile PBS-Freund under the same conditions were considered as negative controls.

Challenging tests in immunized mice

The mice immunized by toxoid were challenged one week after the last injection. In the case of the mice which have been immunized by toxoid- Freund's adjuvant, the first group of them was divided into five groups of six mice. Each mouse in group 1 received 10 (37µg), group 2; 20 (74µg), group 3; 50 (185µg), group 4; 100 (370µg) and group 5; 200 (740µg) LD 100 of the rPLD. Also, the other 30 mice from these mice were assigned to five groups of six mice and received different doses of crude venom, as group 1 received 1 (100µg), group 2= 2 (200µg), group 3= 5 (500µg), group 4= 7 (700 µg) and the group 5= 10 (1000µg) LD 100 of the crude venom. The other 60 mice that were immunized by toxoid-alum adjuvant received

http://jad.tums.ac.ir
Published Online: March 31, 2022

the same doses of rPLD and crude venom respectively as mentioned above. All the injections were done intraperitoneal, and mice were monitored for 2 weeks. Those that survived were regarded as the survivors and non-immunized mice were regarded as the negative controls.

Isolation of splenocytes from mouse spleen

The vaccinated mice were put down by spinalization four weeks after the last injection. Then, they were submerged in 70% alcohol for a few seconds. The spleen was removed from each mouse by opening the peritoneum in fully sterile conditions under a microbiological hood and was completely cleaned of excess matter and adipose tissue. The spleen was quickly transferred to a small sterile petri dish with 5 ml of RPMI culture medium containing 0.5% FBS and penicillin and streptomycin antibiotics. The spleen cells were suspended using several injections of RPMI culture medium. The resulting suspension was centrifuged at 600×g and 4 °C for 10 minutes and Tris-NH4Cl was then added to it. The cell mixture was centrifuged after inverting it several times. The cell deposit was suspended in the RPMI culture medium containing antibiotics. Cells were counted using a hemocytometer slide, and the percentage of the surviving cells was determined. The resulting cell supernatant was kept at -20 °C until analysis. Counting was carried out using the following formula: Number of cells counted in 4 squares in the corners of the NeoBar slide × 2 (dilution coefficient) × 10^4 (volume coefficient)

Measurement of IL-4 and IFN-γ cytokines

To place the mouse anti-IL-4 and anti-IFN-γ monoclonal antibodies, first, 100µl of the antibody in question at a concentration of 5µg/ml in sterile PBS buffer was added to each well in a 96-well plate and kept at 4 °C overnight for 16 hours. The wells were rinsed three times with PBS and kept with 200µl of complete RPMI culture medium at laboratory temperature for 2 hours. The spleen cells isolated from each mouse were diluted, and 2× 10^5 cells in a volume of 100µl were added to wells in triplicates. A volume of 50µl of the toxoid at a concentration of 10µg/ml was added to each well to stimulate cells. Only sterile PBS was added to the control wells. Plates were kept at 37 °C for 48 hours. The culture medium was drawn from each well, and wells were rinsed once with cold sterile distilled water and three times with PBS buffer containing 0.05% Tween 20; then, 100µl of the mouse biotin-avidin detection MBA in PBS buffer with 1% BSA at a concentration of 2µl was added to the wells. Plates were kept at laboratory temperature for 2 hours, and then unbound antibodies were removed and each well was rinsed with PBS buffer containing 0.05% tween 20. Diluted streptavidin-HRP was added to each well at a 1:1000 ratio in PBS containing 1% BSA and then kept at 37 ºC for 1 hour. The wells were rinsed three to five times with PBS buffer, and 100µl of the final substrate solution BCIB-BCIP/NBT Blue One Component AP Membrane Substrate (Surmodics IVD, Inc., USA) was added. One hour after incubation at 37 °C, the wells were rinsed with sterile distilled water, and plates were air-dried. The spots that were developed were counted automatically through Enzyme-Linked Immuno-Spot (ELISpot) assay. IL-4 and IFN-γ cytokines were measured using ELISpot IFN-γ (ab 64029) and IL-4 (ab 64033) kits purchased from ABCAM Company, USA. Each test was repeated three times,and the results were assessed as mean ± SD.

Statistical analysis

The statistical analysis was performed using IBM SPSS 19.0 (New York, USA). The results were expressed as the mean ± SD and were compared using Student's t-test. Differences were considered statistically significant at values of $P \leq 0.05$.

http://jad.tums.ac.ir
Published Online: March 31, 2022

Results

Detection of dermonecrotic activity of phospholipase D toxoid

Given that the main indicator of the effect of *H. lepturus* scorpion venom is phospholipase D with dermonecrotic properties, this test was used to assess the toxic properties of the produced toxoid, and the results were assessed after conducting the dermonecrotic test. The results showed that in 96 hours, the toxoid injection had no dermonecrotic effect in rabbits and no wounding or inflammation signs were observed on rabbit skin (Fig. 1A). Therefore, detoxification of phospholipase D to produce toxoid was successful, while 72 hours after phospholipase D injection (positive control), the necrosis area reached 0.72±0.3cm^3 (Fig. 1B), and PBS was considered as the negative control (Fig. 1C).

Immune response of cytokines IL-4 and IFN-γ to toxoid vaccine

Following immunization of the mice with toxoid -Freund/Alum adjuvants, the supernatant obtained from their spleen was used to evaluate the anti-inflammatory IL-4 cytokine and the inflammatory IFN-γ cytokine. No fatalities occurred in the course of the study. The mean immunity indices of IFN-γ and IL-4 cytokines were measured in the study and control groups. The mean concentrations of IFN-γ cytokine in the vaccinated group with toxoid -Freund/Alum adjuvants increased compared to the control. As a result, IFN-γ cytokine in the vaccinated group with toxoid - Alum adjuvant, extremely close results were observed with toxoid- Freund adjuvant. The mean concentration of IL-4 cytokine in the vaccinated group with toxoid -Freund/Alum adjuvants slightly increased compared to the control. There was a significant difference between all vaccinated groups in terms of the concentration of the inflammatory IFN-γ cytokine ($P\leq 0.05$) but no significant difference was observed in the concentration of anti-inflammatory IL-4 cytokine between these groups ($P\geq 0.05$) (Table 1). The concentration of inflammatory and anti-inflammatory cytokines was determined in vaccinated and control groups using the ELISpot technique, and the results were compared. The results showed an increase of 400–500 units or 400–500% in the inflammatory IFN-γ level in the study group compared to the control, and this increase was significant (P= 0.001). The level of the anti-inflammatory IL-4 cytokine increased by 1.1–1.33 units or 1.1–1.3% in the study groups compared to the control group. Despite this slight increase, the mean level of IL-4 showed no significant difference between the study and control groups (P= 0.16). The comparison of the levels of inflammatory IFN-γ and anti-inflammatory IL-4 cytokine with each other in the study groups showed an increase of 400–432 units or 400–432% in the IFN-γ level compared to the IL-4 level, and the difference between them was significant (P= 0.001) (Fig. 2). Thus, an increase in IFN-γ activity leads to a decrease in IL-4 activity, and they have a regulatory effect on each other. Comparisons were based on the unpaired t-test. $P\leq 0.05$ indicates a significant difference in all cases. Only IFN-γ concentration from Freund/Alum -immunized mice showed statistically significant results (P= 0.001) (Fig. 2). Thus, an increase in IFN-γ activity leads to a decrease in IL-4 activity, and they have a regulatory effect on each other.

Vaccination Trials in the Mice

The results indicated that the mice vaccinated with Toxoid-Freund/Alum adjuvants challenged either by rPLD or crude venom of *H. lepturus*, survived throughout the study. The results obviously indicated that all mice vaccinated with the toxoid were completely protected against a wide range of rPLD or crude venom concentrations, using either of these two adjuvants (Table 2). Non-immunized negative control mice in all groups died after injecting one LD_{100} of rPLD or one LD_{100} of crude venom. From these results, it can be deduced that the amount of vaccine (toxoid= 80

http://jad.tums.ac.ir
Published Online: March 31, 2022

μg/mouse) which promoted the efficacy of the immune response was certainly adequate to protect the mice against rPLD or crude venom of *H. lepturus*. As a result, toxoid vaccine developed an effective cellular immunity against the scorpion venom of *H. lepturus*.

Table 1. *Hemiscorpius lepturus* toxoid concentration of inflammatory IFN-γ and anti-inflammatory IL-4 in the mice vaccinated with toxoid -Freund /Alum adjuvants and control groups

Variable	Case group		Control group		P*
	Mean	SD	Mean	SD	
IFN-γ (a)	263.33	6.77	42.66	2.51	0.001
IFN-γ (b)	231.51	4.13	40.24	3.14	
IL-4 (a)	53.55	5.26	38.17	2.31	0.16
IL-4 (b)	50.21	3.42	35.82	4.14	

(a): IFN-γ/ IL-4 in the mice vaccinated with toxoid- Freund adjuvant. The mice that only received Freund-Adjuvant + PBS (control group)
(b): IFN-γ/ IL-4 in the mice vaccinated with toxoid- Alum adjuvant. The mice that only received Alum-adjuvant + PBS (control group). *Comparison based on unpaired t-test. P≤ 0.05 indicates a significant difference in all cases. The IFN-γ variable is statistically significant

Table 2. In vivo vaccination trials in immunized mice

Number of mice	rPLD Challenge ($LD_{100}s$)[a]	Survival Rate (%)	Number of mice	Crude Venom Challenge ($LD_{100}s$)(b)	Survival Rate
6	10	100	6	1	100
6	20	100	6	2	100
6	50	100	6	5	100
6	100	100	6	7	100
6	200	100	6	10	100
Neg. Cont.(C) 6	1	0	**Neg. Cont.**(C) 6	1	0

(**a**): One LD_{100} of rPLD is equal to 3.7μg. (**b**): One LD_{100} of *Hemiscorpius lepturus* scorpion crude venom is equal to 100μg (**c**): The mice that only received Adjuvant + PBS (negative controls)

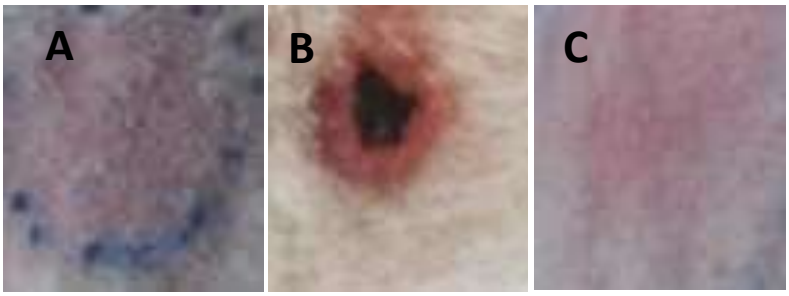

Fig. 1. *Hemiscorpius lepturus* toxoid Intracutaneous injection of 3μg of PLD toxoid into rabbit's shaved skin. No dermonecrotic activity was observed in 96 hours after injection (A). Injection of 3μg of PLD as the positive control showed a specific dermonecrotic activity, and the necrosis site reached 0.72cm^3 after 72 hours (B). PBS was used as the negative control (C). Each experiment was conducted with two repeats

http://jad.tums.ac.ir
Published Online: March 31, 2022

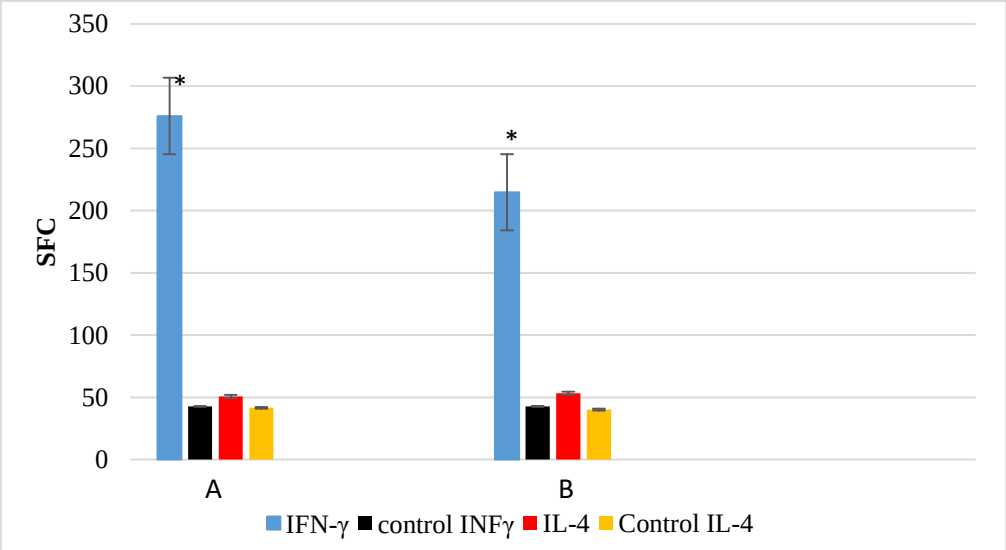

Fig. 2. *Hemiscorpius lepturus* concentration of inflammatory IFN-γ from mice vaccinated by toxoid -Freund/Alum adjuvants and anti-inflammatory IL-4 from mice vaccinated by toxoid -Freund/Alum adjuvants and control groups by ELISpot method. (A) assay was performed on Freund-immunized mice. The mice that only received Freund-Adjuvant + PBS (negative control). (B) assay was performed on Alum-immunized mice. The mice that only received Alum-adjuvant + PBS (negative control). The mean ± S.E.M is shown in each column

Discussion

Given the high percentage of the annual mortality due to *H. lepturus* sting in the warm regions of Iran, especially in the southern areas (Khuzestan Province) (3), the main solution is prevention instead of treatment. Prevention occurs with vaccine production, and currently, the only effective treatment against *H. lepturus* sting is the use of the polyvalent antiserum obtained from horses immunized against the whole venoms of the six venomous scorpions of Iran (2). The main disadvantages of the common antisera on the market include their lack of specific activity against particular scorpion venoms and the complications they cause, especially in susceptible groups of the population (21). Various methods are used in the preparation of vaccines, and among them, formalin use is the most common (22) and basic (23) method. Toxoid vaccines obtained by using formalin on the venom of Cobra (24) and Habu (25) snakes are currently used in southern Asian countries, especially in India. When toxoid is used as a vaccinating agent, an augmented immune response ensues that forms an immunological memory against the toxoid immunogenic epitopes (26). Based on previous reports, we used formalin for the first time to detoxify the most venomous component of *H. lepturus* venom (phospholipase D protein) to produce a toxoid vaccine (10). Detoxification of this protein and its conversion into toxoid can provide a suitable candidate for vaccinating individuals against *H. lepturus* venom if the three-dimensional structure of the protein is preserved. The use of formalin modifies histidine residues and produces uncommon imidazole derivatives in the protein (27). The resulting toxoid can neutralize the deadly effect of the phospholipase D toxin and the complete *H. lepturus* venom (10). After developing this new molecule, in the present study, we assessed the toxicity of this toxoid and the active immunity type of the vaccine. Dermonecrotic activity assessment is the most important confirmatory test for toxoids developed from toxins. It can be inferred from the results that the produced toxoid does

http://jad.tums.ac.ir
Published Online: March 31, 2022

not have any enzymatic activity even to the slightest degree, while all toxins in the whole venom, such as phospholipase D toxin, and the whole venom contain this activity (9, 28). In agreement with our results, it has previously been reported that recombinant PLD antisera inhibit the necrotic activity of the whole venom of Gadim scorpion (11). Other findings confirm that some antibodies against dermonecrotic toxins can fully neutralize the dermonecrotic activity of the scorpion's whole venom. For instance, in the study conducted in Brazil, anti-dermonecrotic antibodies were produced against the brown *Loxosceles intermedia* spider venom, which were fully capable of neutralizing the spider's venom (29). Thus, the antisera obtained by using the toxoid produced in the previous study can be regarded as an effective agent in preventing necrotic wounds caused by *H. lepturus* venom (10). Studies have shown that inflammatory effects are observed following the scorpion sting (30).

The toxicity of the toxoid and the effectiveness of the vaccine were assessed through dermonecrotic test and T-helper (Th) cell immune response, respectively. To transmit a message and affect other cells, the immune system cells must secrete the products that mediate the transmission of this message (26). In response to microbes and other antigens, cells of the innate and specific immunity system secrete protein molecules, generally known as cytokines, that produce different effects (26, 31). Two subgroups of Th immune cells, Th1 and Th2, secrete different types of cytokines (26). Among the cytokines secreted by Th2 cells, the main interest is in interleukin-4 (IL-4), which has the key role in the synthesis of IgE (32). The IL-4 producing cells stimulate and differentiate lymphocytes and switch isotypes from IgM to IgE production in B lymphocytes and thus have a major role in inducing and maintaining allergies, inflammation, and necrosis (33). IL-4 stimulation can affect mast cell responses through IgE signaling (34). Studies have shown that the increase in serum IL-4 levels is directly associated with allergic symptoms and inflammation (33, 34). IL-4 has a central role in type 1 allergic reactions and can have a role as a mediator in the T-cells differentiation (35). On the other hand, as the most important cytokine synthesized by Th1 cells, IFN-γ has a role in cell immunity and inhibits Th2-related immune response (36, 37). Studies have shown that IFN-γ has a highly important role in Th2 lymphocyte dependent allergic reactions (37, 38). IFN-γ can cause the release of pro inflammatory cytokines during allergic reactions (39). Th1 and Th2 cytokines' role in the immune response and their mutual balance have decisive effects on disease incidence and complications (26, 37). Differentiation of T-helper lymphocytes can be induced through the effect of various cytokines and antigens (40). IFN-γ cytokine can cause differentiation of Th0 toward Th1 (39). Activation of the Th1 immune response has pro inflammatory properties and activates B cells and macrophages and induces IgG toward IgG2β, IgG2α in mice, resulting in the activation of the cellular acquired immune system (37, 39-40). IL-4 cytokine causes differentiation of Th0 toward Th2 (39). Activation of the Th2 immune response, which has anti-inflammatory properties, results in the growth and differentiation of lymphocytes, production of immunoglobulin E, basophils, mastocytes, inactivation of macrophages, recruitment of eosinophils, and induction of IgG toward IgG1 in mice and causes inhibition of the immune system (26, 37).

In the present study, to assess the inflammatory effects of the vaccine and type of immunity conferred by it, concentrations of inflammatory IFN-γ and anti-inflammatory IL-4 cytokines in the supernatant of the spleen of the mice immunized with toxoid was determined and their relationship with cellular immunity was investigated. Given the sensitivity of this experiment, the immune response was measured by ELISpot assay to evaluate the toxoid as a vaccine candidate. This test is 20 to 200 times more sensitive than the ELISA test and

http://jad.tums.ac.ir
Published Online: March 31, 2022

its results are almost 100% accurate (41). In addition to the increase in humoral immunity that was assessed using the ELISA test in our previous article (10), the present study provides evidence for an increase of 400–500 % in the concentration of the inflammatory cytokine in the immunized mice compared to control groups. This increase is significant and has noticeably increased the cellular immunity. However, the anti-inflammatory cytokine increased by 1.1–1.33% compared to the control group, and this increase was not significant. To evaluate the potency of this toxoid protein as a vaccine candidate and its effectiveness in neutralizing the lethality of crude venom of *H. lepturus* scorpion, the following experiments were performed. The groups of mice vaccinated by toxoid using Freund or alum adjuvants, were challenged by whole venom of *H. lepturus*. Their survival rate was documented for two weeks' post-injection and over longer time periods. This survival strongly affirms that all mice were completely protected against wide range of different lethal dosages of crude venom (10 LD100, equal to 50mg/Kg), thus illustrating a successful and 100% immunization in the mice. The results obtained confirm that the developed vaccine, in addition to inducing humoral immunity, can increase the Th1 cytokine response within lymphocytes and consequently increase cellular immunity. In agreement with the present study results, a study conducted by Borchani et al. showed that a type of PLD in *H. lepturus* called hemonecrolysin can increase the secretion of pro-inflammatory factors TNF-α and IL-6 on human monocytes by 3.5 times compared to the control. This PLD can also double the level of the anti-inflammatory factor IL-10 compared to the control (42). Furthermore, in agreement with the present study results, it has been reported that the recombinant phospholipase D obtained from the brown *Loxosceles recluse* is also able to increase pro-inflammatory cytokines TNF-α, IL-1β, IL-8 on human fibroblasts (43). These results show that the vaccine produced from toxoid has the same effect on T-cell as the whole venom, recombinant phospholipase D from the scorpion venom, or recombinant phospholipase D of the brown spider and can activate inflammatory processes and that it can be used as a booster of the immunological memory against scorpion/spider stings. In a study conducted by Homayoon et al. (44), it was shown that *Pasteurella multocida* vaccine, inactivated by formalin, can significantly increase pro-inflammatory cytokines, and significantly increase cellular immunity in the immunized BALB/c mice compared to the control group. In agreement with the results obtained, the vaccines produced from *Leishmania major* also significantly increased the ability to produce IFN-γ and resulted in the Th1 response in mice, while the production of IL-4 and the Th2 cytokine response decreased significantly (45). Overall, lack of clinical assessments was among the limitations of the present study and is proposed to be addressed in future studies. Moreover, according to the present study results, this vaccine, as a protective agent in people exposed to *H. lepturus* scorpion sting, can increase Th1 cytokines and thus increase the acquired immunity of the cellular kind.

Conclusion

The present study results showed for the first time that toxoid, as a vaccine, can increase the production of certain components of Th1 and Th2 cytokine response in the serum of mice immunized with rPLD toxoid, and this increase is significant in terms of the level of IFN-γ cytokine. Furthermore, it was shown that the supportive immunity produced in the mice was associated with the activity of Th1 cytokines.

Acknowledgements

This article is a part the Ph.D. thesis of Narges Safari-Foroshani approved by the Pasteur Institute of Iran, Tehran, Iran. This re-

http://jad.tums.ac.ir
Published Online: March 31, 2022

search did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical considerations

Male BALB/c mice (18–20g) were purchased from Pasteur Institute of Iran. The animals were allowed to adapt for a week in standard conditions with a dark/light cycle of 12h. The room temperature was 22±1 °C and the relative humidity adjusted at 50±5%. The animals were received a standard pellet diet and fresh tap water. All experiments were approved by Ethical Committee of the Pasteur Institute of Iran (code number IR.PII.REC.1394. 38).

Conflict of interest statement

Authors declare that there is no conflict of interest.

References

1. Jalali A, Rahimi F (2014) Epidemiological review of scorpion envenomation in Iran. Iran J Pharm Res. 13(3): 743–756.
2. Latifi M, Tabatabai M (1979) Immunological studies on Iranian scorpion venom and antiserum. Toxicon. 17(6): 617–621.
3. Shahbazzadeh D, Srairi-Abid N, Feng W, Ram N, Borchani L, Ronjat M, Akbari A, Pessah IN, De Waard M, El Ayeb M (2007) Hemicalcin, a new toxin from the Iranian scorpion *Hemiscorpius lepturus* which is active on ryanodine-sensitive Ca2+ channels. Biochem J. 404(1): 89–96.
4. Seyedian R, Jalali A, Babaee MH, Pipelzadeh MH, Rezaee SA (2012) A biodistribution study of *Hemiscorpius lepturus* scorpion venom and available polyclonal antivenom in rats. J Venom Anim Toxins Incl Trop Dis. 18(1): 375–383.
5. Pipelzadeh MH, Dezfulian AR, Jalali MT, Mansouri AK (2006) In vitro and in vivo studies on some toxic effects of the venom from *Hemiscorpious lepturus* scorpion. Toxicon. 48(1): 93–103.
6. Jalali A, Pipelzadeh MH, Sayedian R, Rowan EG (2010) A review of epidemiological clinical and in vitro physiological studies of envenomation by the scorpion *Hemiscorpius lepturus* (Hemiscorpiidae) in Iran. Toxicon. 55(2): 173–179.
7. Pipelzadeh MH, Jalali A, Taraz M, Pourabbas R, Zaremirakabadi A (2007) An epidemiological and a clinical study on scorpionism by the Iranian scorpion *Hemiscorpius lepturus.* Toxicon. 50(7): 984–992.
8. Jalali A, Pipelzadeh MH, Taraz M, Khodadadi A, Makvandi M, Rowan EG (2011) Serum TNF- a levels reflect the clinical severity of envenomation following a *Hemiscorpius lepturus* sting. Eur Cytokine Netw. 22(1): 5–10.
9. Borchani L, Sassi A, Shahbazzadeh D, Strub JM, Tounsi H, Boubaker MS, Akbari A, Van Dorsselaer A, El Ayeb M (2011) Heminecrolysin the first hemolytic dermonecrotic Toxin purified Scorpion venom. Toxicon. 58(1): 130–139.
10. Safari Foroushani N, Modarressi MH, Behdani M, Shahbazzadeh D, Torabi E, Pooshang Bagheri K (2018) Developing recombinant phospholipase D1 (rPLD1) toxoid from Iranian *Hemiscorpius lepturus* scorpion and its protective effects in BALB/c mice. Toxicon. 152: 30–36.
11. Torabi E, Shahbazzadeh D, Behdani M, Hosseininejad M, Moazzami R, Sabatier JM, Khalaj V, Bagheri KP (2017) Characteristics and lethality of a novel recombinant dermonecrotic venom phospholipase D from *Hemiscorpius lepturus.* Toxins (Basel). 9(3): 102.
12. Tambourgi DV, Magnoli, FC, Van den Berg CW, Morgan BP, De A raujo PS, Alves EW, Da Silva WD (1998) Sphingomy-

elinases in the venom of the spider *Loxosceles intermedia* are responsible for both dermonecrosis and complement-dependent hemolysis. Biochem Biophys Res Commun. 251(1): 366–373.
13. Mortimer N, Nathan T (2015) Parasitoid wasp venom SERCA regulates drosophila calcium levels and inhibits cellular immunity. Proc Natl Acad Sci U S A. 110(23): 9427–9432.
14. Metz BW, Jiskoot WE, Hennink DJA, Crommelin, Kersten GFA (2003) Physicochemical and immunochemical techniques predict the quality of diphtheria toxoid vaccines. Vaccine. 22(1): 156–167.
15. Farizo KM (2007) *Diphtheria* and *Tetanus* toxoids absorbed FDA. Iran Academy Press. 8(2): 33–34.
16. Erlich P (1897) Die werthbemessung des diphtheria heil-serum und deren theoretische grundlagen. Klin Jahrb Jena. 6: 299–326.
17. Moloney PG (1926) The preparation and testing of *Diphtheria* toxoid (Anatoxin-Ramon). Am J Public Health (NY). 16 (12): 1208–1210.
18. Lonard AS (2009) Botulism and vaccines for its prevention. Vaccine. 27 Suppl 4: D33–39.
19. Weinberg M, Goy P (1924) Recherches sur la toxin botulinque. Compt Rend Soc Biol. 90: 269–271.
20. El-Maghraby AS, Abd El-hakim MA (2020) Sensitivity of different methods used in determination of formalin residues in inactivated veterinary vaccines. Glob Vet. 14(4): 546–552.
21. Teakston RDG, Warrell DA, Griffiths E (2003) Report of a WHO 574 workshop on the standardization and control of antivenoms. Toxicon. 41(5): 541–557.
22. Ramon G (1924) Sur la toxin et sur l' anatoxine diphteriques. Ann. Inst. Pasteur. 38: 1–10.
23. Glenny AT, Hopkins, BE (1923) Diphtheria toxoid as an immunizing agent. Br Exp Pathol J. 4(5): 283–288.
24. Someya S, Murata R, Sawai Y, Kondo H, Ishii A (1972) Active immunization of man with toxoid of *trimeresurus flavoviridis* (Habu) venom. Jpn J Med Sci Biol. 25(1): 47–51.
25. Kondo S, Sadahiro S, Yamauchi K, Kondo H, Murata R (1971) Preparation and standardization of toxoid from the venom of *Trimeresurus flavoviridis*. Jpn J Med Sci Biol. 24(5): 281–294.
26. Piersma Sytse J, Leenaars Marlies PPAM, Guzylack-Piriou Laurence, Summerfield Artur, Hendriksen Coenraad FM, McCullough Ken C (2006) An in vitro immune response model to determine tetanus toxoid antigen (vaccine) specific immunogenicity: Selection of sensitive assay criteria. Vaccine. 24(2): 3076–3083.
27. Bizzini B, Raynaud M (1974) Detoxification of protein toxins by formol: Supposed mechanisms and new developments. Biochimie. 56(2): 297–303.
28. Borchani L, Sassi A, Yehlefa R, Safra E, Ayeb M (2011) Heminecrolysin a potential immunogen for monospecific antivenom production against *Hemiscorpius lepturus* scorpion. Toxicon. 58(8): 681–688.
29. Alvarenga LM, Martins MS, Moura JF, Kalapothakis E, Oliveira JC, Mangili OC, Granier C, Chávez-Olórtegui C (2003) Production of monoclonal antibodies capable of neutralizing dermonecrotic activity of Loxoceles intermedia spider venom and their use in a specific immunometric assay. Toxicon. 42(7): 725–731.
30. Dragulev B, Bao Y, Ramos-Cerrillo B, Vazquez H, Olvera A, Stock R, Algaron A, Fox JW (2007) Upregulation of IL-6, IL-8, CXCL1, and CXCL2 dominates gene expression in human fibroblast cells exposed to *Loxosceles reclusa* sphingomyelinase D: insights into spider venom

http://jad.tums.ac.ir
Published Online: March 31, 2022

dermonecrosis. J Invest Dermatol. 127(5): 1264–1266.
31. Khosravi A, Motevalibashi M, Babaahmadi E, Davodian A, Jafarzadeh L, Manian Azodi M (2012) The effect of car smoke on the level of TNF-α, INF-γ and IL-2 in rat serum. Res Med. 36(5): 99–131.
32. Kashiwada M, Deborah M, McKeag L, Murray K, Schröder AJ, Canfield SM, Geri Traver G, Rothman PB, Coffman RL (2009) IL-4-induced transcription factor NFIL3/E4BP4 controls IgE class switching. Proc Natl Acad Sci USA. 107(2): 821–826.
33. Saba MA, Akbari H, Banihashemian SM, Jazayeri H, Talaei SA, Banihashemian SH, Ghaforian S (2013) Relationship between serum levels of IL-4 and IgE with disease severity in allergic asthma. Feyz J Kashan Univ Med Sci. 17(4): 366–372.
34. Haas H, Falcone F, Holland M, Schramm G, Haisch K, Gibbs B (1999) Early interleukin–4: its role in the switch towards a th2 response and IgE mediated allergy. Int Arch Allergy Immunol. 119(2): 86–94.
35. Fietta P, Delsante G (2009) The effector T helper cell triade. Riv Biol. 102(1): 61–74.
36. Holgate S, Polosa R (2008) Treatment strategies for allergy and asthma. Nat Rev Immunol. 8(3): 218–229.
37. Paul WE, Zhu J (2010) How are TH2-type immune responses initiated and amplified? Nat Rev Immunol. 10(4): 225–235.
38. Teixeira L, Fonseca B, Barboza B, Viola J (2005) The role of interferon-γ on immune and allergic responses. Mem Inst Oswaldo Cruz. 100 Suppl 1: 137–144.
39. Torre D, Speranza F, Giola M, Matteelli A, Tambini R, Biondi G (2002) Role of Th1 and Th2 cytokines in immune response to uncomplicated *Plasmodium Falciparum Malaria.* Clin Diagn Lab Immunol. 9(2): 348–351.
40. Rosloniec EF, Latham K, Guedez YB (2002) Paradoxical roles of INF-γ in models of Th1-mediated auto immunity. Arthritis Res. 4(6): 333–336.
41. Boulet S, Ndongala M, Peretz Y, Boisvert M.P, Boulassel MR, Tremblay C, Routy JP, Sekaly RP, Bernard NF (2007) A Dual Color ELISPOT Method for the Simultaneous Detection of IL-2 and IFN-γ HIV-Specific Immune Responses J Immunol Methods. 320(1): 18–29.
42. Borchani L, Sassi A, Gharsa HB, Safra I, shahbazzadeh D, Lasfar ZB, El-Ayeb M (2013) The pathological effects of Heminecrolysin, a dermonecrotic toxin from *Hemiscorpius lepturus* scorpion venom are mediated through its lysophospholipase D activity. Toxicon. 68: 30–39.
43. Adib-Conquy M, Cavaillon JM (2009) Compensatory anti-inflammatory response syndrome. Thromb. Haemost. 101(1): 36–47.
44. Homayoon M, Tahamtan Y, Kargar M (2020) The comparison detection of cytokines (IL-6 and IL-12) from spleen cells and serums in BALB/c mice after immunization with killed *P. multocida* vaccines co formulated with bacterial DNAs as adjuant. Arch Clin Microbiol. 11(1): 1105–1109.
45. Roberts MT, B-Stober C, Mckezie A, Blackwell JM (2005) Interleukin-4 (IL-4) and IL-10 collude in Vaccine failure for novel exacerbatory antigens in murine *leshmania* major infection. Infect Immun. 73(11): 7620–7628.

http://jad.tums.ac.ir
Published Online: March 31, 2022