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Evaluation of the immunoprotective power of a multiple antigenic peptide against Aah II toxin of *Androctonus australis hector* scorpion

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ABSTRACT

Scorpion envenoming (SE) is a public health problem in developing countries. In Algeria, the population exposed to the risk of SE was estimated at 86.45% in 2019. Thus, the development of a vaccine to protect the exposed population against scorpion toxins would be a major advance in the fight against this disease.

This work aimed to evaluate the immunoprotective effect of a Multiple Antigenic Peptide against the Aah II toxin of *Androctonus australis hector* scorpion, the most dangerous scorpion species in Algeria. The immunogen MAP1Aah2 was designed and tested accordingly. This molecule contains a B epitope, derived from Aah II toxin, linked by a spacer to a universal T epitope, derived from the tetanus toxin.

The results showed that MAP1Aah2 was non-toxic despite the fact that its sequence was derived from Aah II toxin. The immunoenzymatic assay revealed that the 3 immunization regimens tested generated specific anti-MAP1Aah2 antibodies and cross-reacted with the toxin. Mice immunized with this immunogen were partially protected against mortality caused by challenge doses of 2 and 3 LD_{50} of the toxin. The survival rate and developed symptoms varied depending on the adjuvant and the challenge dose used. In the *in vitro* neutralization test, the immune sera of mice having received the immunogen with incomplete Freund's adjuvant neutralized a challenge dose of 2 LD50.

Hence, the concept of using peptide dendrimers, based on linear epitopes of scorpion toxins, as immunogens against the parent toxin was established. However, the protective properties of the tested immunogen require further optimizations.

Introduction

Scorpion envenomation (SE) is a serious public health problem in different regions of the world, particularly in developing countries. The annual number of scorpion stings exceeds 1.2 million cases globally, resulting in over 3,250 deaths [1]. In Algeria, due to its health and financial impact, SE has been considered a public health problem since the 1980 s [2,3]. Indeed, an average of 47,773 stings and 47 deaths were recorded annually between 2009 and 2020 [4]. In 2019, the population at risk of SE was estimated at 86.45 % [5].

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Regardless of the envenomation severity, the cornerstone of the treatment of stung individuals is serotherapy with scorpion antivenom [6]. This treatment was first produced in 1909 by Todd [7] by immunizing horses with *Buthus quinquestriatus* venom. Over a century after its first production, no scientific breakthrough has practically replaced the use of scorpion antivenom. Besides, the production and availability of antivenoms in countries where populations are at risk of SE is problematic. African and South American authors argued that the reliable availability of antivenoms in these regions is limited [8,9]. A WHO workshop report stated that the production and quality control of these life-saving products in developing countries are dependent on available funding [10]. Venom scarcity, the lengthy and costly purification process, and the low toxin proportion in venom are among the major difficulties encountered in antivenom production.

An alternative therapeutic approach to serotherapy would be a breakthrough in SE management and, more generally, in envenomation treatment. The development of a vaccine against the toxins of one or several scorpions, intended for the population at risk of SE, is a particularly interesting option. This vaccine could also be used to produce a potent antivenom in animals or humans to treat non-vaccinated patients. Furthermore, the high demand for venom would be significantly reduced with such a vaccine. Research has been conducted to explore innovative immunization approaches to protect animals against scorpion toxins with encouraging results for some studies [11–15]. Still, no vaccine candidate has been tested in humans to date.

Multiple Antigenic Peptides (MAPs), are rational and controlled systems of immunogenic multimeric peptides [16–18]. They have several advantages as potential vaccines which are mainly due to the immunogenic signal amplification arising from the multiple copies of the immunogenic sequence [19]. This makes it possible to circumvent the ineffectiveness of linear peptides in generating an immune response. Besides, unlike peptide-protein conjugates, MAPs consist almost of pure antigens and are thus focused on a targeted immunological response with no risk of an allergic reaction against a carrier protein [16]. Their relatively low production cost by solid phase synthesis also justifies the interest shown in them [20].

The aim of this work was to assess the immunoprotective capacity of a MAP against Aah II toxin of *Androctonus australis hector* (Aah) scorpion. Aah II is the most potent toxin of Aah venom, which is considered the most dangerous scorpion species in Algeria [21–24]. The MAP in this study was tested for its potential toxicity and immunogenicity through *in vivo* and *in vitro* assays.

Materials and methods

Animals

Female BALB/c mice weighing 20 ± 2 g were used. These mice were provided by Pasteur Institute of Algeria (IPA) and had *ad libitum* access to food and water.

Immunogen

Since octavalent MAPs did not show a higher potency compared to tetravalent ones [16], an asymmetric tetravalent MAP was used as the immunogen. The peptide sequence composing the MAP branches was determined based on several criteria. The 4 branches are made of the same peptide sequence which is partly derived from Aah II toxin. Each branch displayed two tandem epitopes separated by a spacer to reduce the antigenicity of any potential neo-epitope [25,26]. One epitope is a T epitope whose role is to increase the MAP immunogenicity [27,28]. The N-terminal epitope (distal to the PAM core) should have B epitope properties, which is associated with a stronger immune response [27–29]. The sequence of the immunogen was established as follows:

▪ Determination of epitope B

Two linear epitopes of Aah II toxin are known for their ability to induce neutralizing antibodies upon conjugation to a carrier protein – the major antigenic determinant *aa50-59* (sequence KLPDHVRTKG) [30] and the B epitope *aa1-8* (sequence VKDGYIVD) [31]. The determinant *aa50-59* has also T epitope properties. Due to its dual role in terms of antigenicity (major determinant) and toxicity (sodium channel binding site), the *aa50-59* epitope was used in the immunogen design [30]. This peptide was associated with the spacer by its C-terminal residue; the N-terminal residue remaining free.

▪ Determination of the T-helper epitope

The sequence of the T epitope which was included in the MAP, in order to enhance the immunogenicity of the B epitope, was defined *in silico* using the Immune Epitope Database (IEDB) prediction resource [32]. This tool was used to determine a 15 amino acids T epitope, presentable by mouse MHC II molecules, using the Consensus approach, recommended by the IEDB. This tool covers the H2-IE^{d} and H2-IA^{d} alleles found in the BALB/c strain mice used in this study [33]. IEDB generates a percentile rank by comparing the score of the considered sequence with the scores of five million random 15-mers selected from the SwissProt database. The IEDB recommends selecting peptides with a percentile rank below 10 % [34,35]. The analysis of Aah II sequence using this tool revealed 3 sequences with a percentile rank lower than 10 %. These are the overlapping sequences aa45-59, aa46-60 and aa44-58 (percentiles 7.5, 7.65 and 8.6 respectively for the H2-IE^{d} allele) (Table S1 in the supplementary information). Given the inclusion of most amino acids of these sequences in B epitope, the use of a universal T epitope from tetanus toxin was preferred. In silico analysis of tetanus toxin revealed fifty-five sequences with a percentile rank below the threshold recommended by the IEDB. The T epitope selected for this study is the sequence with the lowest percentile among these (sequence *aa86-100:* FTVSFWLRVPKVSAS with a percentile of 0.47 for the H2-IE^{d} allele) (Table S2 in the supplementary information).

▪ Determination of the spacer

The sequence of the spacer was defined based on the results of a previous study which successfully allowed the development of an immunogen based on a MAP composed of B and T epitopes in tandem [25].

Fig. 1 is a representation of the designed MAP1Aah2 immunogen. It was synthesized by Proteogenix (France).

Toxin and venom

Pure (>95 %) Aah II toxin of synthetic origin was obtained from Smartox Biotechnology (France). Aah scorpion venom, extracted in 2014 from M'Sila region (Algeria) scorpions, was provided by PIA.

Single dose toxicity assessment of the immunogen

This assay aimed to assess the potential toxicity conferred by Aah II toxin sequence included in the immunogen structure. A group of 6 mice received an intraperitoneal injection of the immunogen at a dose equivalent to 1000 times the toxin LD50 mass. The control group received the vehicle (sodium chloride 0.9 %) [11–13]. The animals monitoring included: [1] mortality determination 20 h after injection [11–13;2] semi-quantitative physiological and behavioural evaluation according to Irwin test, before administration and at 30 min, 2 h, 6 h, and 14 days after administration [36;3] daily observations for 14 days to detect any clinical abnormalities [25]; and [4] daily animal weighing. This monitoring allows the detection of SE signs detectable in mice (Table 1) [37–39]. Weight averages and Irwin test parameters were statistically analysed [40]. Subsequently, in the absence of mortality or signs of toxicity resembling those of SE, the immunogen would be

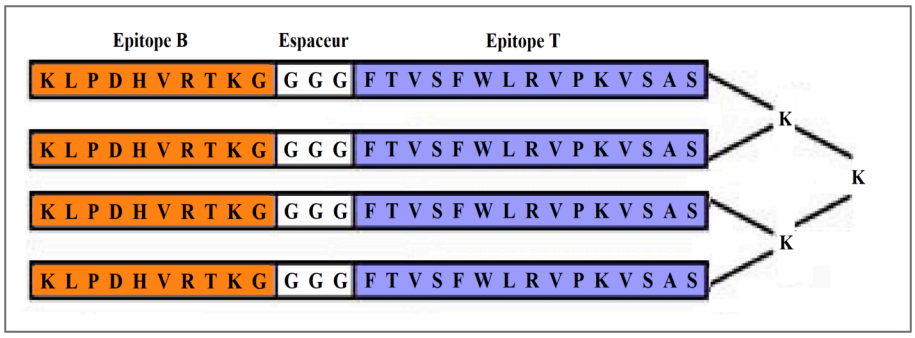

Fig. 1. Representation of the multiple antigenic peptide MAP1Aah2 used as an immunogen against Aah II toxin of Androctonus australis hector. The immunogen is a tetravalent diepitopic MAP composed of a B epitope, derived from Aah II toxin, linked by a spacer to a universal T epitope, derived from the tetanus toxin. The distal B-epitope consists of aa50-59 epitope of Aah II toxin, which has a dual role in terms of antigenicity and toxicity. The proximal T-epitope, which aims to increase immunogenicity, was defined in silico using the IEDB prediction resource.

Table 1
Daily observations conducted and signs particularly sought during the single dose toxicity assessment of MAP1Aah2 (1–4).

Observations conducted to seek signs of clinical alterations	
Appearance	- Body condition
	- Coat and skin condition
	- Secretions
	- Ophthalmic examination
	- Oral examination
Behaviour	- Social interactions
	- Stereotypes
	- Posture and mobility
Body fonctions	- Breathing
	- Body temperature
	- Auditory reflex
	- Visual reflex
	- Response to pain
	- Grip strength
Environment	- Faeces appearance
	- Bedding appearance
Others	- Other symptoms/signs observed
Symptoms of scorpion envenoming sought	
Central nervous system	- Agitation
	- Hyperirritability
	- Stereotypes*
	- Aggressivity*
	- Sedation, loss of consciousness
	- Hypo-locomotion*, akinesia/bradykinesia, ataxia
	- Convulsions, myoclonus
	- Paralysis
Autonomous nervous system	- Ptosis
	- Piloerection*
	- Lacrimation*, hypersalivation
Respiratory system	- Polypnea
Digestive system	- Diarrhoea
Others	- Deaths

* Symptoms observed during the LD50 determination test of Aah II toxin.

considered non-toxic [12,13]. Otherwise, its LD50 would have been determined [41].

Immunization protocols

- ***Immunization for the evaluation of the immuno-protective capacity against Aah II toxin***

Two groups of mice were immunized with 0.6 mg of immunogen. The immunizing preparations of group MAP1Alu-0.6 were adjuvanted with aluminium hydroxide, while those of group MAP1IFA-0.6 received incomplete Freund's adjuvant (IFA) Imject™. These preparations were administered intraperitoneally (half of the product) and subcutaneously (remaining half). Each group of mice was boosted 7 and 17 days later using the same procedure. A negative control group receiving the vehicle (sodium chloride 0.9 %) was established [11,12].

For each test group, two sets of mice were created: Set (I) of 12 mice intended to provide immune sera for immunoenzymatic tests and the neutralizing capacity evaluation of these sera. These sera were obtained 45 and 75 days after the first immunogen injection and were stored at −20 °C. A satellite group was formed to replace potential dead mice after blood sampling. Set (II), composed of 24 mice [42], was intended for *in vivo* protection assay against a challenge with the toxin (Tables 2 and 3) [11,12].

- ***Immunization for the evaluation of the immuno-protective capacity against Aah venom***

Two groups of mice were immunized with the immunogen at a dose of 0.6 mg and 0.9 mg per mouse for group MAP1IFA-0.6 and group MAP1IFA-0.9, respectively. The immunizing preparations were adjuvanted with IFA. The route of administration and the immunization schedule were the same as those described in section 2.5 (Tables 2 and 4) [11,12].

ELISA assays

The immune sera obtained on days 45 and 75 from Set (I) mice from three immunized groups (groups MAP1Alu-0.6, MAP1IFA-0.6 and MAP1IFA-0.9) were tested using ELISA technique to titrate specific anti-MAP antibodies and test their cross-reactivity with the toxin.

The specific antibodies titre was determined by individually analysing the reactivity of the immune sera from the 12 mice with the immunogen. The average titre of these antibodies per group was defined by calculating the geometric mean of the individual titres. The cross-reactivity of the immune sera with the toxin was determined by analysing a pool of sera from the 12 mice obtained at day 45, as well as a pool of group MAP1IFA-0.9 sera obtained at day 75.

The protocol used was adapted from Zenouaki's protocol [11] after its validation for specific antibody titration [43,44]. One hundred microliters of antigen (MAP1Aah2 at 20 μg/ml or Aah II at 10 μg/ml) in 0.1 M sodium bicarbonate buffer (pH 9.6) were adsorbed onto NUNC Maxisorp 96-well microplates overnight at + 4 °C. After washing the plates three times with PBS/Tween (0.05 %), 200 μl of 1 % BSA in PBS buffer were added and incubated for 1 h at 37 °C. After washing the plates three times, dilutions of the serum to be tested in PBS/Tween were added to wells 1 to 10. The dilutions ranged from 1/100 to 1/51200 for specific antibody titration, and from 1/20 to 1/10240 for cross-reactivity assessment (using 2-fold dilutions). Column 11 contained the negative control consisting of serum from non-immunized mice at

Table 2
Immunization protocols for the evaluation of the immuno-protective capacity against Aah II toxin and against Aah venom (16,107,286).

		Evaluation of the immuno-protective capacity against Aah II toxin assay			Evaluation of the immuno-protective capacity against Aah venom assay		
		Group MAP1Alu-0.6	Group MAP1IFA-0.6	Control group	Group MAP1IFA-0.6	Group MAP1IFA-0.9	Control group
Injected preparations composition (for one mouse)	**MAP1Aah2**	0.6 mg	0.6 mg	/	0.6 mg	0.9 mg	/
	Aluminium hydroxyde	120 µg	/	/	/	/	/
	Incomplete Freund Adjuvant	/	0.25 ml	/	0.25 ml	0.25 ml	0.25 ml
	Sodium chloride in water (0.9 %)	0.5 ml	0.25 ml	0.5 ml	0.25 ml	0.25 ml	0.25 ml
Route of administration	**Intraperitoneally**	Half the volume			Half the volume		
	Subcutaneously	Half the volume			Half the volume		
Chronology of the immunization protocol	**Immunogen/vehicle injection**	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0
		Day 7	Day 7	Day 7	Day 7	Day 7	Day 7
	(Set I and II)	Day 17	Day 17	Day 17	Day 17	Day 17	Day 17
	Blood sampling (Set I)	Day 45 [a, b]	Day 45 [a, b]	/	/	Day 45 [a, b]	/
		Day 75 [a]	Day 75 [a]			Day 75 [a]	
	***In vivo* protection assay (Set II)**	Day 45	Day 45	Day 45	Day 45	Day 45	Day 45

[a]Samples used in immunoenzymatic tests.
[b]Samples used in the neutralizing capacity assessment.

Table 3
Number and group assignment of mice as part of the immunization protocol for the evaluation of the immuno-protective capacity against Aah II toxin.

Assignment of mice	Number of mice				
	Group MAP1Alu-0.6	Group MAP1IFA-0.6		Control group	
	Set I	Set II	Set I	Set II	
Blood sampling at day 45 [a, b] and day 75 [a]	12		12		
***In vivo* protection assay at day 45**		24		24	24
Satellite group [c]	6		6		
Total per set	18	24	18	24	
Total per group	42	42	24		
Total	**108**				

[a]Samples used in immunoenzymatic tests.
[b]Samples used in the neutralizing capacity assessment.
[c]Group intended for the replacement of potential dead mice following blood collection.

Table 4
Number and group assignment of mice as part of the immunization protocol for the evaluation of the immuno-protective capacity against Aah venom.

Assignment of mice	Number of mice			
	Group MAP1IFA-0.6	Group MAP1IFA-0.9		Control group
		Set I	Set II	
Blood sampling at day 45 [a] and day 75 [a]		12		
***In vivo* protection assay at day 45**	24		24	24
Satellite group [b]		4		
Total per group	24	40		24
Total for 2 test groups and a control group	**88**			

[a] Samples used in immunoenzymatic tests.
[b]Group intended for the replacement of potential dead mice following blood collection.

the lowest dilution used. Column 12 contained the diluent and served as blank. The samples were incubated at 37 °C for 90 min. After washing the plates three times, 100 µl of diluted mouse anti-immunoglobulin conjugated to peroxidase were added and incubated at 37 °C for 90 min. After washing the plates four times, 100 µl of a 0.5 mg/ml OPD solution in a stable hydrogen peroxide buffer were added and incubated in the dark at room temperature for 20 min. The reaction was stopped by adding 50 µl of 2 N sulfuric acid. The absorbance was measured at 490 nm with a microplate reader. The test was performed in duplicate wells [43,44]. The specific antibodies titre corresponds to the highest dilution factor that gives an absorbance greater than the cut-off (mean OD of negative control × 1.22). The cross-reactivity estimated in titre corresponds to the highest dilution factor that gives an absorbance greater than the cut-off (mean OD of negative control + 3SD) [43,44].

Evaluation of the immuno-protective capacity against Aah II toxin

- ***In vivo protection assay against the toxin***

This test assessed the protection of immunized mice against a challenge dose of Aah II. First, weekly weight monitoring and daily examinations of Set (II) mice and control mice were conducted during the immunization period and for 4 weeks thereafter. This aimed to detect any signs of clinical abnormalities that could affect the study results. Four weeks after the completion of immunizations, mice in each test group received an intraperitoneal challenge dose of Aah II: 12 mice received 2 LD50 of toxin, and another 12 mice received 3 LD50. Twelve non-immunized mice received 2 LD50 of the toxin and served as negative controls. The symptoms presented by the mice and mortality time were noted. The signs particularly sought were those of SE noticeable in mice (Table 1). Mortality was determined 20 h after toxin administration.

In vivo protection against the toxin, expressed as the percentage of mouse survival, was determined for each challenge dose. Groups with 100 % survival were considered completely protected, while those with 0 % survival were considered unprotected. Groups with survival percentages higher than 0 % and lower than 100 % were considered partially protected [12]. The average time of death and the symptoms presented by the mice were statistically analysed [45].

- ***Neutralizing capacity evaluation of immune sera against the toxin***

This test assessed the ability of immune sera of immunized mice to protect naive mice against a toxin challenge dose. The immune sera obtained 45 days after the immunizations start from Set (I) mice were thawed and pooled on an equal volume basis. For each group, two sets of a mixture of 0.2 ml of immune serum were incubated with 2 LD50 or 3 LD50 of toxin for 90 min at 37 °C. The resulting samples were administered intraperitoneally to four groups of 6 mice, with each group receiving a mixture of immune serum from one group of mice combined to a specified amount of toxin (2 LD50 or 3 LD50). Mortality was determined 20 h after administration.

The neutralizing capacity, expressed as the percentage of mouse survival, was determined for each group against each challenge dose. Sera from non-immunized mice, serving as negative controls, were pooled and evaluated for their neutralizing capacity as well. This effect was categorized as described for *in vivo* protection assay. Additionally, the minimal neutralizing titre was calculated for sera with 100 % survival (neutralizing sera) [12].

Evaluation of the immuno-protective capacity against Aah scorpion venom

This test assessed the protection of immunized mice against a challenge dose of Aah venom. The *in vivo* protection assessment methodology was the same as described in section 2.7 [11,12].

Statistical analysis

Data statistical analysis was carried out using IBM® SPSS® Statistics software (version 29) as follows [40]:

- **Continuous variables analysis:** Student *t*-test was used to compare the means of two batches for normally distributed variables. Mann-Whitney *U* test was used to compare the means of batches for non-normally distributed variables. The one-way ANOVA test was used to compare the means of several batches for normally distributed variables.
- **Ordinal variables analysis:** Mann-Whitney test was used to compare the means of variables/parameters evaluated according to a semi-quantitative ordinal scale (Likert-type) considering the small size of the sample (n = 6) [46].
- **Nominal variables analysis:** Chi-square test was used to study variables associations or independence.

Results

Single dose toxicity of the immunogen

After the injection of 13.83 mg of MAP1Aah2 (equivalent to 1000 LD50 of toxin using corrected mass), no mortality or signs of toxicity resembling those caused by the toxin, were observed during the monitoring period. The daily monitoring of the body weight revealed no significant difference between the test and control groups over the 14 days following the immunogen administration (illustrated in Figure S1 in the supplementary information).

ELISA assays

All three immunization schedules generated specific anti-MAP1Aah2 antibodies. The highest antibody titres were obtained with group MAP1IFA-0.9 mice, with titres of 7610.9 at D45 and 2135.7 at D75. Group MAP1IFA-0.6 mice sera had titres of 1223.6 at D45 and 377.6 at D75. The lowest titres were obtained with sera from group MAP1Alu-0.6, with values of 156.1 at D45 and 123.9 at D75 (Fig. 2). It should be noted that one mouse in group MAP1IFA-0.9 satellite died approximately 2 min after the third immunization.

Furthermore, all four pools of immune sera also reacted with Aah II toxin. The highest titre was observed with the serum pool from group MAP1IFA-0.9 obtained at D45 (titre of 320). The group MAP1IFA-0.6 serum obtained at D45 and the group MAP1IFA-0.9 serum obtained at D75 had a titre of 80. The lowest titre was obtained with the serum from group MAP1Alu-0.6 obtained at D45 (titre of 20) (Fig. 3).

In vivo protection against Aah II toxin

During the immunization phase and subsequent monitoring, some clinical anomalies were observed in group MAP1IFA-0.6 mice, but without a degradation of the general condition. These anomalies included muscle cramps (100 % occurrence), reversible abscesses (79 %), and moderate hyperthermia (58 %). One mouse developed an

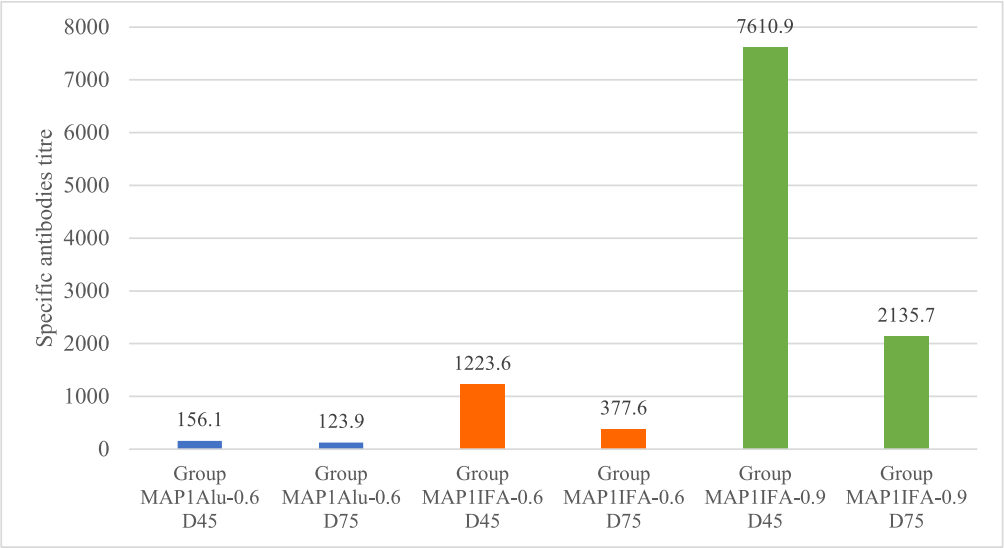

Fig. 2. Anti-MAP1Aah2 specific antibodies titer of immune sera generated in mice. Three groups of mice were immunized with MAP1Aah2. For group MAP1Alu-0.6, 0.6 mg of the immunogen was adjuvanted with aluminium hydroxide, while for group MAP1IFA-0.6, the same dose of immunogen was associated with incomplete Freund's adjuvant (IFA). Group MAP1IFA-0.9 received 0.9 mg of the immunogen associated with IFA. These preparations were injected intraperitoneally (half of the product) and subcutaneously (remaining half). The mice were boosted 7 and 17 days later using the same procedure. The immune sera obtained 45 and 75 days after the first immunisation, from 12 mice of each immunized group, were tested using ELISA technique to titrate specific anti-MAP antibodies.

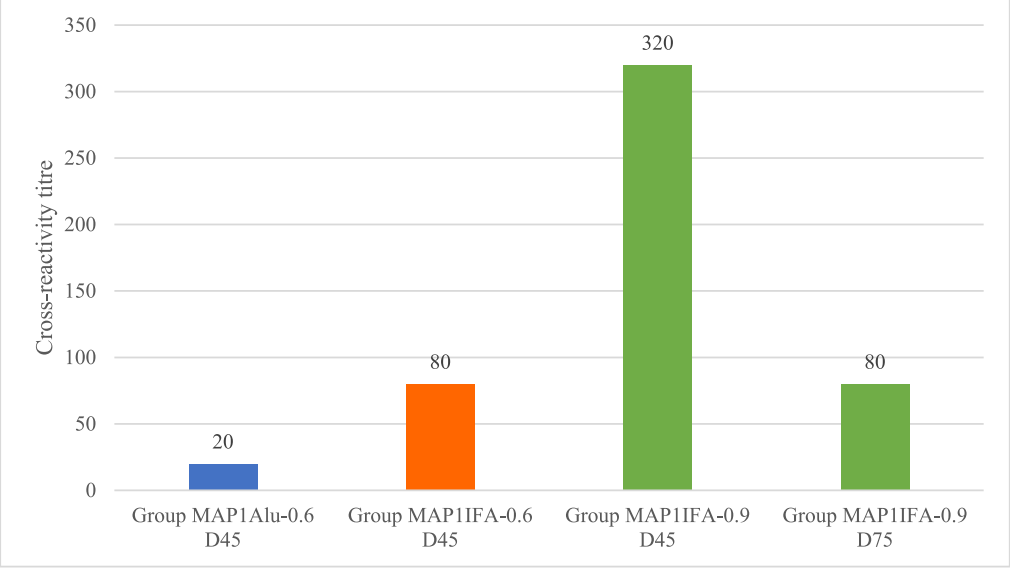

Fig. 3. Cross-reactivity of immune sera, generated using the immunogen MAP1Aah2, with Aah II toxin. Three groups of mice were immunized with MAP1Aah2. For group MAP1Alu-0.6, 0.6 mg of the immunogen was adjuvanted with aluminium hydroxide, while for group MAP1IFA-0.6, the same dose of immunogen was associated with incomplete Freund's adjuvant (IFA). Group MAP1IFA-0.9 received 0.9 mg of the immunogen associated with IFA. These preparations were injected intraperitoneally (half of the product) and subcutaneously (remaining half). The mice were boosted 7 and 17 days later using the same procedure. The immune sera obtained 45 days after the first immunisation from 12 mice of each immunized group and the sera obtained after 75 days from 12 mice of group MAP1IFA-0.9 were tested using ELISA technique to determine the cross-reactivity of anti-MAP antibodies with Aah II toxin.

ulcerated abscess. These signs were not observed in group MAP1Alu-0.6 and the control group. A continuous increase in average weights was noted for the two test groups as well as the control group, with no significant differences between these groups (Figure S2 in the supplementary information).

The *in vivo* protection assessment showed that while both immunized groups were partially protected against toxin-induced mortality, the percentage of mice survival and the symptoms developed varied depending on the adjuvant and challenge dose. Indeed, group MAP1IFA-0.6 exhibited survival rates of 41.6 % and 25 % against 2 LD50 and 3 LD50, respectively, while Group MAP1Alu-0.6 had mice survivals of 25 % and 8.3 % against 2 LD50 and 3 LD50, respectively. All control mice died to 2 LD50 (Table 5).

The mean time of death after receiving a 2 LD50 challenge dose was the shortest in the control group with a 41.6 min average. The mean time of death was 62.2 min for group MAP1Alu-0.6 and 117.1 min for group MAP1IFA-0.6. Furthermore, this time after a challenge dose of 3 LD50 was 40.9 min for group MAP1Alu-0.6 and 71.1 min for group MAP1IFA-0.6 (Fig. 4). There was a significant difference in the death time between group MAP1IFA-0.6 and the control group after receiving 2 LD50 ($p = 0.025$) but no significant difference was found for group MAP1Alu-0.6.

Clinically, there was a significant decrease in the percentage of mice with akinesia and dead mice in group MAP1IFA-0.6 compared to the control group [0–1] hour after challenge. Similarly, there was a significant decrease in the percentage of mice experiencing loss of consciousness, akinesia, piloerection, polypnea, and mortality in group MAP1IFA-0.6 compared to the control group [1–3] hours after challenge. No significant decrease was found in the percentage of dead mice or those developing these symptoms in group MAP1Alu-0.6 compared to the control group.

In vivo protection against Aah venom

Both immunized mice groups were partially protected against the mortality caused by 2 LD50 of venom. Group MAP1IFA-0.9 exhibited a survival rate of 25 %, while mice survival was 16.6 % in group MAP1IFA-0.6 following this challenge dose. Partial protection against 3 LD50 was obtained in group MAP1IFA-0.9 with a survival rate of 8.3 %. Furthermore, no mice from group MAP1IFA-0.6 were protected against 3 LD50. All control mice died at these venom doses (Table 5).

Neutralizing effect of immune sera against Aah II toxin

A total protection was observed against 2 LD50 of the toxin when mixed with immune sera from group MAP1IFA-0.6 mice (100 % survival). The pool of these sera was considered neutralizing, with a minimal neutralizing titre estimated at 10 LD50/ml. However, partial protection was observed against 2 LD50 with sera from group MAP1Alu-0.6 mice (33.3 %). Partial protection was also obtained against 3 LD50 of the toxin with sera from group MAP1Alu-0.6 (16.6 %) and group MAP1IFA-0.6 mice (50 %). The immune sera from the latter groups were considered non-neutralizing. The control mice did not survive the challenge dose (Table 5).

Table 5
Results of the *in vivo* protection assays and neutralizing capacity assessment conferred by the immunogen MAP1Aah2 against the Aah II scorpion toxin and Aah venom.

Toxin/venom challenge dose	*In vivo* protection against Aah II toxin (survival rate %)		*In vivo* protection against Aah venom (survival rate %)		Immune serum neutralizing capacity against Aah II toxin (survival rate %)	
	Group MAP1Alu-0.6	Group MAP1IFA-0.6	Group MAP1IFA-0.6	Group MAP1IFA-0.9	Group MAP1Alu-0.6	Group MAP1IFA-0.6
2 LD50	25 %	41.6 %	16.6 %	25 %	33.3 %	100 %
3 LD50	8.3 %	25 %	0 %	8.3 %	16.6 %	50 %

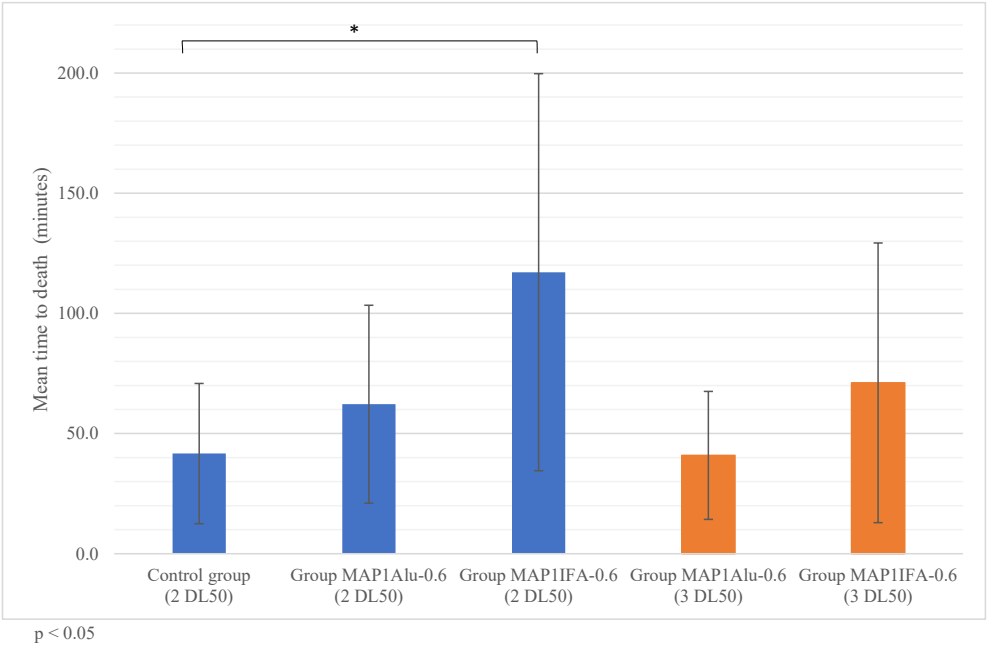

Fig. 4. Mean time to death of mice after receiving a challenge dose of 2 LD50 or 3 LD50 of Aah II toxin. Two groups of mice were immunized with 0.6 mg of MAP1Aah2. For group MAP1Alu-0.6, the immunogen was adjuvanted with aluminium hydroxide, while for group MAP1IFA-0.6, it was associated with incomplete Freund's adjuvant. These preparations were injected intraperitoneally (half of the product) and subcutaneously (remaining half). The mice were boosted 7 and 17 days later using the same procedure. A negative control group receiving the vehicle was established. Four weeks after the completion of immunizations, mice in each test group received an intraperitoneal challenge dose of Aah II (12 mice received 2 LD50 and 12 others received 3 LD50). The control group received 2 LD50 of toxin. The time to death was noted and statistically analysed.

Discussion

The development of MAPs derived from Aah II toxin aimed to create a non-toxic immunogen that generates protective antibodies against this toxin. Considering the absence of mortality or Aah II toxic signs following the injection of MAP1Aah2 dose equivalent to 1000 LD50 of Aah II, the immunogen was considered non-toxic [11–13,41]. This can be explained by the fact that linear sequences carried by the immunogen arms, with a sequence identity of 23.4 % with the toxin [47], are incapable of causing steric hindrance to VSD4 domain of sodium channels [48].

The presence of clinical anomalies in group MAP1IFA-0.6 during the immunization period and their absence in group MAP1Alu-0.6 suggest an effect caused by IFA. This hypothesis was confirmed after the administration of IFA without the immunogen to a group of 6 mice with similar characteristics. In the literature, immunization with IFA is generally described as painful. However, cramps, which are usually associated with painful phenomena in rodents, were not specifically mentioned [49,50]. Moreover, local reactions such as sterile abscesses have been described in humans during administration of influenza vaccine associated with IFA [51]. This observation is consistent with the abscesses presented at the injection site in this study. Nevertheless, body weight monitoring supports a good overall health status. The choice of IFA as an adjuvant for this study was motivated by the fact that several studies investigating immunization procedures in animals against scorpion venoms or toxins, which successfully produced vaccines or passive protection, utilized IFA as an adjuvant [14,15,31,52–54]. Aluminium hydroxide was also successfully used for this purpose by some authors [12,13,55], which justifies the use of this adjuvant. Despite the known local adverse effects caused by IFA, it has been shown that this adjuvant induce higher antibodies titres than those obtained with aluminium-based adjuvants [56], which was also confirmed in an internal study where the immune sera from rabbits immunized with *Aah* venom adjuvanted with IFA gave a higher neutralizing capacity than the sera of rabbits immunized with *Aah* venom with aluminium hydroxide (13.33 and 7.92 LD50/ml/20 gr mouse, respectively) [57]. Considering the current stage of this research, which is a Proof-of-Concept study, it was preferred to maintain IFA rather than testing novel alternative adjuvants. Nevertheless, since the concept has been proved, the limits associated with the use of IFA in humans [58] and the modest titres obtained with aluminium hydroxide require the exploration of other, better tolerated, adjuvants such as MF-59, Montanide® adjuvants (ISA-51 or ISA-720) or liposomes as part of the forthcoming immunogen optimization studies.

ELISA assays showed that all three immunization schedules generated specific antibodies. A dose–response correlation was observed, and it was shown that IFA elicited a better immune response than aluminium hydroxide, which is consistent with the literature [56]. While some studies described a concordance between the reactivity of immune sera with Aah2 and passive protection [11,31], in the study conducted by Ait-Amara, the reactivity with the toxin was not associated with its neutralization [59]. Although there was a decrease in specific antibody titres between D45 and D75, these antibodies remained detectable for all three groups at D75. However, the protective capacity of the remaining antibodies was not tested. This indicates the necessity of periodic booster doses to maintain sufficiently high antibody levels for optimal protection against Aah II toxin. Nevertheless, in a study conducted by Zenouaki and colleagues, where *Swiss* mice were immunized with 3 injections of the compound $(Abu)_8$AahII, a non-toxic synthetic analogue, 3 out of 6 mice survived after receiving 6 LD50 doses of Aah II one month after the beginning of immunization period. Two months into the immunization, *in vivo* protection was even more effective, with all six mice surviving after receiving the same dose, without requiring booster injections of the immunogen. The authors argued that the decrease in circulating antibody levels and the continued *in vivo* protection suggest the presence of additional protective mechanisms beyond circulating antibody levels [11]. This emphasizes the necessity of including long-term *in vivo* protection assays as part of the prospective immunogen optimization studies. The death of a mice in the satellite group, two minutes after receiving a third injection of 0.9 mg of the immunogen,

suggests a possible development of an anaphylactic reaction.

The study results showed that, amongst the partially protected groups from Aah II toxin, group MAP1IFA-0.6 had the highest survival rates and had the better clinical tolerance following the injection of 2 LD50 challenge dose. This is explained by the more robust immune response with group MAP1IFA-0.6, confirmed by higher antibodies titres in ELISA assay, which can be attributed to IFA effect. The lower survival rates against 3 LD50 can be explained by insufficient antibodies to significantly neutralize toxin lethality. It should be noted that two studies using Aah II synthetic analogues achieved complete protection against the toxin. The use of the $(Abu)_8$AahII, a non-toxic synthetic analogue [11], or a recombinant Aah II-MBP fusion protein [12] allowed a complete protection against 3 LD50. In studies involving venom toxins of other scorpion species, the success of immunization approaches using toxin synthetic analogues in achieving a total protection was less consistent. The peptidomimetic heterodimer SP1 did not confer protection against 1 LD50 of Cn2 toxin of *Centruroides noxius*, despite inducing neutralizing antibodies and cross-reacting with the toxin [60]. However, a di-epitopic peptide conjugated to albumin allowed 100 % mice survival to 2.3 LD50 of *Tityus serrulatus* venom and 50 % to 2.8 LD50 [15]. Besides, 100 % survival was obtained against 2 LD50 of *Tityus serrulatus* Ts1 toxin in mice immunized with a recombinant immunogen of the toxin in a form of inclusion bodies [14]. Overall, published data indicated that the use of long peptide sequences, covering the entire toxin, as immunogens was associated with a total protection. Except for Duarte's study, which achieved total protection, di-epitopic peptides generated partial protection. The present study is consistent with these results. Other immunization approaches based on preparations derived from scorpion venoms have also been successfully developed. These methods were based on the use of detoxified venom, its detoxified toxic fractions or on the use of toxoids. Aah venom detoxified with gamma radiation conferred protection to immunized mice against 4 LD50 of native venom one month after immunization [24,52]. The G50 toxic fraction of *Tityus serrulatus* venom detoxified by conjugation to BSA with glutaraldehyde allowed protection to immunized mice against 2 LD50 of native venom [53]. Additionally, mice immunized with KAaH1 toxoid from *Androctonus australis* venom were protected against 2 LD50 of its toxic fraction. This was attributed to the cross-reactivity of anti-KAaH1 antibodies with Aah II toxin [13]. The major drawback of these methods is the difficulty of obtaining enough venom to produce adequate quantities of these preparations to vaccinate the populations at risk of envenomation. Despite the success of the immunization methods described above in animal studies, none of these concepts was taken to clinical trials.

The lower survival rates obtained with venom challenge doses compared to toxin challenge doses can be explained by the fact that generated anti-MAP1Aah2 antibodies were insufficient to neutralize the combined anti-mammal venom's toxins mortality. In fact, it is estimated that Aah II represents 50 % of the venom lethal activity [12,61,62]. However, Aah II antibodies do not cross-react with Aah group 1 toxins that are considerably involved in the venom lethality [23,41,62].

The superior neutralizing capacity of group MAP1IFA-0.6 mice sera over group MAP1Alu-0.6 is explained by higher specific antibodies titres. In the literature, immune serum generated in rabbits against the synthetic epitope [50–59]-BSA conjugate conferred complete protection against 14 LD50 of toxin injected intracerebroventricularly [30]. The immune serum generated in mice against the same epitope conjugated to KLH did not neutralize the toxin [59].

To conclude, the concept of using MAPs to induce protection against Aah II toxin effects has been established; however, the protective properties of the tested immunogen, MAP1Aah2, require optimization. Future research could be conducted to enhance the immunogen protective properties. The development of a MAPs containing two epitopes derived from the toxin sequence could provide protection with higher survival rates. The use of long toxin-derived sequence would allow optimal protection but could be costly and technically challenging. In order to achieve complete vaccine protection against Aah venom, two MAPs with sequences derived from each Aah toxin group could yield optimal results. Following the development of MAPs generating neutralizing antibodies against the different venom toxins, they could be tested to immunize serum-producing horses or to produce human anti-scorpion serum.

Ethics statement

All animal experiments carried out during this study were approved by the ethics committee of CHU Beni-Messous.

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CRediT authorship contribution statement

Safouane M. Benazzouz: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Nesrine Benlouahmia:** Investigation, Methodology. **Karima Bouhadida:** Investigation, Methodology. **Meriem Benlamara:** Investigation, Methodology. **Naziha Arezki:** Investigation, Methodology. **Oum El Kheir Sadeddine:** Investigation, Methodology. **Mourad Issad:** Methodology, Resources. **Nabila Attal:** Supervision, Validation, Visualization. **Kamel Mansouri:** Supervision, Validation, Visualization. **Fawzi Derrar:** Methodology, Resources. **Reda Djidjik:** Conceptualization, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2024.100503.

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