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Immune Response Modifying Effects of Bee Venom Protein [Melittin]/ Autoclaved *L. donovani* complex in CD1 Mice: The Search for New Vaccine Adjuvants

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Abstract

Visceral leishmaniasis (VL) is a major cause of morbidity/mortality in remote areas of East Africa. Vaccines for VL can provide an effective control measure to help control/eliminate this fatal disease. To date there is no effective anti-leishmanial vaccine. There is an urgent need to develop effective adjuvants-potentiated anti-leishmanial vaccines. Bee venom protein, melittin is a natural substance that is reported to boost the immune system providing rapid non–specific defense against infections. This study aimed to determine the immune response modifying effects of melittin and melittin/Autoclaved *Leishmania donovani* [ALD] complex on Swiss CD1 Albino mice. One hundred and eighty five CD1 mice were divided into control [no vaccine] and vaccine groups [3 doses of ALD alone, melittin, melittin/ALD mixture or melittin-adsorbed ALD]. Whole blood cytokines levels [IL-10, IFN-γ and TNF-α] were measured using commercial ELISA kits. ALD alone group showed significant increase in mean levels of IL-10, IFN-γ and TNF-α compared to controls [p=0.00004, p=0.01 and p=0.00001 respectively]. The Melittin and Melittin/ALD mixture-vaccinated mice showed significant increase in IL-10 and IFN-γ mean levels [IL-10 p=0.00001, p=0.00003; IFN-γ p=0.03, p=0.035 respectively], while the mean levels of TNF-α decreased significantly [p=0.00009, p=0.001] compared to controls. Melittin-adsorbed ALD reduced significantly the mean levels of IL-10, IFN-γ and TNF-α [p=0.00001, p=0.00008 and p=0.000001 respectively]. In conclusion, melittin alone and Melittin/ALD complex affected significantly Th1 and Th2 immune responses in Swiss CD1 Albino mice. Meltttin could be a potentially effective adjuvant for future anti-leishmania vaccines.

Keywords Visceral leishmaniasis; Vaccines; Th1/Th2 immune responses

Abbreviations ALD: Autoclaved L.donovani; BV: Bee Venom; FBS: Foetal Bovine Serum; IL10: Interleukin 10; IFNγ: Interferon Gamma; PBS: Phosphate Buffered Saline; PHA: Phytohemagglutin; PTMs: Post Translational Modifications; sLA: Soluble Leishmania Antigen; sPLA2: Soluble Phospholipase A2; Th1: T Helper Cells; Th2: T Suppressor Cells; TNF-α: Tumor Necrosis Factor Alpha; VL: Visceral Leishmaniasis.

Introduction

Visceral leishmaniasis (VL) is a major public health problem that is widely prevalent in many parts of the world [1-3]. In Sudan, VL spreads over a wide belt extending from the Sudanese-Ethiopian border in the east to the White Nile state in Central Sudan. In eastern Sudan, Gedaref State is the main endemic area of VL. The mean yearly incidence was reported as 6.6-8.4 VL cases/1000 persons, with large variations between different areas. Currently, visceral leishmaniasis has become more widely distributed than before as it is reported from areas that were previously non-endemic such as White Nile State. Case detection and drug treatment is the most efficient method of control. Anti-leishmanial drugs pose serious problems due to cost and increased drug-related toxicities [4-9]. Development of a safe, effective and cheap vaccine seems to offer a real solution for controlling the disease. Vaccine adjuvants markedly enhance vaccines efficiency. Available parasitic vaccines adjuvants are far from optimum [10-13].

Bee venom (BV, apitoxin) is extracted from honey bees and is a well-known pharmacologically active product of the hive [14]. BV administration was reported to stimulate the immune system to affect the release of cortisol production which is a known natural anti-inflammatory agent [15]. BV contains several different peptides including Melittin, the major component, which binds to secretary phospholipase A2 (sPLA2) and inhibits its enzymatic activity [16,17]. The allergic response to these proteins in a sting victim varies from swelling, redness, pain, itching around the sting site and potentially life-threatening allergic effects that include anaphylactic shock. Secretary proteins of BV undergo a series of post-translational modifications (PTMs), such as phosphorylation, glycosylation and sulfation [18-20]. PTMs play a potential role in influencing IgE binding capacity, protein immunogenicity and antigenicity. Few proteins in BV such as melittin and icarapin are known to be phosphorylated, but phosphorylation is not well characterized [21-24]. Melittin (C 131 H 229 N 39 O 31) is the main component of BV, which accounts for 40-50% of the dried venom. Melittin polypeptide is made up of twenty-six amino acids [NH2-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH2] and has a molecular weight of 2.86 kD and high aqueous solubility [25-28]. In traditional medicine, BV is commonly used in the treatment of arthritic disorders, amyotrophic lateral sclerosis, liver fibrosis, colon cancer, skin diseases, metabolic disorders and diseases of the nervous system. Traditional therapy employs direct stings by bees to deliver BV, with a number of disadvantages, so it's necessary to design a suitable release system which provides a calculated, long-term and constant therapeutic dose

[28]. Low dose melittin has been proven to be safe in experimental animal models [29].

This study aimed to determine the immune response modifying effects of melttin and melittin/ALD complex on Swiss CD1 Albino mice. The study also looked at melittin as a potential adjuvant for anti-leishmanial vaccines [30].

Materials and Methods

Study design

This was an experimental and analytical study.

Ethical consideration

Swiss CD1 Albino mice were handled in accordance with the National Regulations for Experimentation on Animals and the European Manual of Ethics Committee for the use of laboratory animals.

Melittin preparation and purification

One gram of raw Bee Venom (China International Express (EMS) Company, Hong Kong, China) was dissolved in 5 ml of double distilled H2O in sterile fifty ml Falcon tubes in a water bath at 56°C for 2 h. The solution was centrifuged at 40,000 r.p.m for 40 min. The supernatant was filtered using 0.3 & 0.2 µm filters and divided to 1 ml aliquots in sterile 1.5 ml Eppendorf tubes and stored at -20°C until use.

Melittin precipitation by acetone

Cooled acetone [-20°C] to precipitate melittin in a ratio of 6:1 by volume in glass test tubes. The solution was vortexed and incubated in -20°C freezer for 2 h and centrifuged at 13,000-15,000 r.p.m for 15 min. The supernatant was discarded carefully so as not to dislodge the protein pellet. The pellet was washed three times in 100 µL cold 90% acetone and centrifuged at 13,000-15,000 r.p.m for 5 min. The pellet was dried at room temperature (overnight) and later dissolved in 1 ml normal saline. The concentration of Melittin in solution was determined spectrophotometrically [280 nm] using (Nanophotometer® P300, IMPLEN GmbH, Munich, Germany).

Autoclaved *Leishmania donovani* (ALD) preparation

Leishmania donovani parasites were donated by the Department of Clinical Pathology & Immunology, Institute of Endemic Diseases, University of Khartoum. Autoclaved *L. donovani* (ALD) was prepared from a parasite suspension (5-8 × 107 per ml) in complete media containing RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotics (Penicillin-Streptomycin 1%), from 3 to 4 days of culture by centrifugation at 4,000 r.p.m for 10 min. The parasite pellet was washed four times in cold phosphate buffered saline (PBS) in glass test tubes. The tubes were autoclaved in high pressure saturated steam at 121°C (249°F) for 15-20 min. Finally, ALD was mixed with 20 µg thimerosal per mL for preservation and stored at -20°C until used.

Soluble *Leishmania donovani* antigen (sLA) preparation

sLA was prepared as described by Gupta et al. [31]. Briefly: late log phase promastigotes were harvested from 3 to 4 days of culture by centrifugation at 4,000 r.p.m for 10 min; the pellet was washed four times in cold phosphate buffered saline (PBS). The pellet was lysed by repeated freeze-thawing 5-10 min. each using liquid nitrogen and 37°C water bath. The suspension was vortexed for 10 min. and centrifuged at 4,000 r.p.m for 10 min. The antigen in supernatant was aliquated and stored at -20°C until use for whole blood culture stimulation.

Mice injection

Hundred and eighty five Swiss CD1 Albino mice, ages 20-22 weeks and weights 25-33 grams were assigned randomly into a control group (Group I) and four vaccination groups. Mice were vaccinated on footpads and behind right ear on days 0, 7 and 14. Vaccination groups were vaccinated intradermally with 30 µg Melittin and Autoclaved *L. donovani* (5×10^7 "ALD"+Thimerosal 20 µg) alone or incubation as follows: Group II received ALD; Group III received melittin; Group IV received melittin+ALD with no incubation while Group V received melittin+ALD with mixture incubation for two days in -20°C (Table 1). During the experiment the followings variables were monitored: general condition [well, ill]; locomotion [active, immobile aggressive]; weight [increased, decreased, stable]; temperature [increased, decreased, stable]; Injection site [color "Normal, redness", size "Normal, swell", injury].

Vaccine Groups	Vaccine Dose	Dose No.	Route/Site
Group I [Control] (n=30)	-	-	-
Group II [ALD] (n=10)	50 million parasites/0.11 ml Normal Saline	3 doses	Intradermally: Footpad & ear pinna
Group III [Melittin] (n=40)	30 µg Melittin/0.11 ml Normal Saline	3 doses	Footpad & ear pinna
Group IV [Melittin+ALD] (n=40)	30 µg Melittin/50 million in 0.11 ml ALD	3 doses	Intradermally: Footpad & ear pinna
Group V [Melittin-adsorbed ALD] (n=65)	30 µg Melittin/50 million in 0.11 ml ALD	3 doses	Intradermally: Footpad & ear pinna

Table 1: Vaccination Schedule for different study groups (ALD=Autoclaved *L. donovani*).

Whole blood samples were collected from the control and the vaccine groups [a week of the third dose]. The blood was stimulated by 67 µg Phytohaemagglutinin "PHA" (positive control); 100 µg Souble Leishmania Antigen "sLA", 1 µg of Melittin/no additive (negative control) and incubated in a portable 37°C incubator [Cellestis, Melbourne, Australia] for 24 h. The supernatants were collected for measurement of IL-10, IFN-γ and TNF-α cytokines using commercial ELISA kits (Koma Biotech International, Seoul, Korea).

Statistical analysis

All data were analysed using Epidemiological Information (Epi Info) software version 7 and continuous variables were presented as mean ± SD. A 2-tailed t-test was used to compare mean levels of cytokines for control cells, soluble leishmania antigen and Phytohaemagglutinin-stimulated cells in control and vaccinated mice. P value<0.05 was considered significant.

Results

One hundred and eighty five Swiss CD1 Albino mice with mean ages and weights of 21 ± 1 weeks and 29 ± 4 gms respectively and a Male:Female ratio of 2:1. No change in movement patterns and no effect on injection site in all vaccinated mice groups (Group II-V) compared to control group (Group I). The weights of mice injected with three doses of melittin/melittin+ALD mixture and Melittin-adsorbed ALD were not significantly different from Group I (control group) during the follow up period (p>0.05) (Table 2). The temperature variation in vaccinated mice groups (Groups II-V) showed no statistically significant differences compared to Group I (control group) (p>0.05) (Table 3).

Weight (g)	Vaccine Groups				
	Group I Control (n=30)	Group II ALD (n=10)	Group III Melittin (n=40)	Group IV Melittin+ALD (n=40)	Group V Melittin-adsorbed ALD (n=65)
Initial Weight	27.2 ± 2.1	26.4 ± 2.0	28.4 ± 2.6	27.0 ± 2.5	29.2 ± 3.1
24 h	27.3 ± 2.0	26.6 ± 1.9	27.3 ± 2.5	27.0 ± 2.5	28.8 ± 3.0
Day 2	27.2 ± 2.1	26.7 ± 1.8	27.0 ± 2.3	27.0 ± 2.5	28.8 ± 3.0
Day 3	27.5 ± 2.2	26.8 ± 1.7	27.0 ± 2.1	27.0 ± 2.5	28.8 ± 3.0
Day 4	27.7 ± 2.4	26.9 ± 1.6	26.9 ± 2.1	27.0 ± 2.5	28.8 ± 3.0
Day 5	27.6 ± 2.4	27.1 ± 1.5	27.0 ± 1.9	26.9 ± 2.6	28.9 ± 3.0
Day 6	28.4 ± 2.5	27.3 ± 1.3	27.1 ± 1.8	27.0 ± 2.6	28.9 ± 2.9
Day 7	28.2 ± 2.5	27.4 ± 1.2	27.2 ± 1.8	26.9 ± 2.8	29.0 ± 3.1

Table 2: Weight followup after Vaccinated Doses (ALD=Autoclaved *leishmania donovani*, Continuous variables are expressed as Mean±SD).

Group I: Control group (no injection) (n=30): The mean levels of IL-10, IFN-γ and TNF-α increased significantly in sLA-stimluated cells compared to non-stimulated ones (p=0.03, 0.01 and 0.00006 respectively) (Table 4).

Group II: ALD group (n=10): Spontaneous secretions of IL-10 and IFN-γ were significantly higher compared to Group I (p=0.00008, 0.0009 respectively). The mean levels of sLA-induced IL-10, IFN-γ and TNF-α were significantly higher compared to that produced by sLA-stimulated cells of the control group (Group I) (p=0.00004, 0.01 and 0.00001 respectively) (Table 4).

Temperature (°C)	Vaccine Groups				
	Group I Control (n=30)	Group II ALD (n=10)	Group III Melittin (n=40)	Group IV Melittin+ALD (n=40)	Group V Melittin-adsorbed ALD (n=65)
00 min	38.6 ± 0.4	38.4 ± 0.7	38.1 ± 0.9	38.5 ± 0.6	38.3 ± 0.6
30 min	38.5 ± 0.5	38.2 ± 0.6	36.9 ± 1.1	37.6 ± 0.6	37.7 ± 0.5
1 h	38.5 ± 0.4	38.8 ± 0.5	35.9 ± 1.3	36.5 ± 0.9	37.4 ± 0.5
2 h	38.5 ± 0.4	38.4 ± 0.5	35.1 ± 1.5	35.7 ± 0.7	37.3 ± 0.5
4 h	38.5 ± 0.4	38.3 ± 0.5	34.8 ± 1.6	36.4 ± 0.5	37.3 ± 0.6
6 h	38.6 ± 0.4	38.4 ± 0.5	35.5 ± 1.4	37.3 ± 0.4	37.3 ± 0.6
Day 1 to 7	38.6 ± 0.4	38.5 ± 0.4	37.3 ± 0.8	37.5 ± 0.4	38.0 ± 0.5

Table 3: Temperature Variation following Melittin/ALD injection (ALD=Autoclaved *leishmania donovani*, Continuous variables are expressed as Mean±SD).

Group III: Melittin group (n=40): Spontaneous secretion of IL-10 and IFN-γ were significantly higher compared to Group I (p=0.00002 and 0.001 respectively). The mean levels of sLA-induced IL-10 and IFN-γ were significantly higher than that secreted by sLA-stimulated cells in Group I (p=0.00001 and 0.03 respectively). While the mean level of sLA-induced TNF-α was significantly lower than that produced by sLA-stimulated cells in Group I (control group) (p=0.00009) (Table 4).

Group IV: Melittin+ALD mixture group (n=40): Spontaneous secretions of IL-10 and IFN-γ were significantly higher compared to Group I (p=0.00009 and 0.001 respectively). The mean level of sLA-induced IL-10 and IFN-γ were significantly higher than that induced in Group I (p=0.00003 and 0.035 respectively). While the mean level of sLA-induced TNF-α was significantly lower than that induced in Group I (control group) (p=0.001) (Table 4).

Group V: Melittin-adsorbed ALD group (n=65): Spontaneous secretion of IL-10 was negligible. The mean levels of sLA-induced IL-10, IFN-γ and TNF-α were significantly reduced compared to Group I (p=0.00001, 0.00008 and 0.000001 respectively). Spontaneous secretion of TNF-α was significantly higher compared to Group I (p=0.02) (Table 4).

Study groups	IL-10 Concentration pcm/ml		IFN-γ Concentration pcm/ml		TNF-α Concentration pcm/ml	
	Control Cells (No stimulation)	sLA Stimulation	Control Cells (No stimulation)	sLA Stimulation	Control Cells (No stimulation)	sLA Stimulation
Group I (n=30) [no vaccination]	159.7 ± 279.7	296.6 ± 390.0	6.9 ± 10.3	19.9 ± 10.5	22.0 ± 34.9	78.8 ± 30.9
Group II (n=10) [ALD]	565.2 ± 30.1*	522.7 ± 165.1*	24.5 ± 1.5*	25.1 ± 2.8*	20.0 ± 19.1	169.3 ± 390.4*
Group III (n=40) [Melittin]	488.3 ± 96.0*	444.5 ± 204.4*	26.0 ± 3.4*	24.2 ± 1.7*	30.5 ± 25.0	36.1 ± 47.6*
Group IV (n=40) [Melittin +ALD]	257.6 ± 195.3*	497.5 ± 320.0*	23.2 ± 1.6*	22.9 ± 1.5*	20.0 ± 24.9	18.0 ± 24.8*
Group V (n=65) [Melittin-adsorbed ALD]	0.00 ± 0.00*	125.1 ± 186.7*	3.3 ± 5.8*	6.3 ± 9.3*	57.3 ± 39.3*	41.1± 90.9*

Table 4: Means levels of IL-10, IFN-γ and TNF-α cytokines in the study groups (pcm/ml=picogram/ml; sLA=Soluble Leishmania Antigen; Positive control (Phytohaemagglutinin PHA) results [OK√] not shown; *p value=Significant difference).

Discussion:

The present study is the first direct, head-to-head comparison of the immune response modifying effects of melittin or melittin/Autoclaved *L.donovani* complex on Swiss CD1 Albino mice, no mice challenge was conducted because the study is not attend to test leishmania vaccine efficacy. The study is justified due to lack of effective anti-parasite vaccines and adjuvants [30,32,33]. Bee venom protein, Melittin is introduced as a potential adjuvant. Although Bee venom has been extensively used in traditional oriental medicine, detailed effects on Th1 and Th2 immune response have not been delineated. It has also been shown that increasing the number of Bee stings is not advantageous. Measuring the exact amount of melittin can be a scientific way to calibrate and standardize Bee venom doses used for treatment [15,28].

ALD vaccinated mice affected both Th1/Th2 immune responses as evidenced by increased levels of IFN-γ, IL-10 and TNF-α. This could probably affect susceptibility and healing abilities of CD1 mice in the course of infection with leishmania parasite. It is therefore assumed that ALD alone is not a good candidate to be taken further as a potential vaccine.

On the other hand, injection with melittin alone/Melittin+ALD mixture affected Th1 and Th2 immune responses leading to increased IFN-γ and IL-10 levels (increased Th2 responses/susceptibility), with reduction in TNF-α (?decreased healing abilities). This could limit their use as future anti-leishmanial vaccine candidates.

Melittin adsorbed ALD seems a good option to be taken forward for anti-leishmanial vaccine studies because it abolishes spontaneous IL-10 secretion in injected mice. Although, melittin adsorbed ALD reduced sLA-induced IL-10, IFN-γ and TNF-α secretion [affecting Th1/Th2 responses] compared to control mice, it is argued that a high IFN-γ production cannot be the sole factor that confers protection against *L. donovani*. In addition, a markedly low IL-10 could be beneficial and favours melittin-adsorbed mixture as a potential vaccine for leishmaniasis. It is also well documented that high IL-10 levels inhibit IFN-γ production by macrophages and vice versa. So, it is expected that IL-10 reductioninduced by melittin-adsorbed ALD can shift the balance towards Th1 immune responses and protection [34-36].

Conclusion

Bee Venom protein melittin modulates both Th1 and Th2 immune responses in Swiss CD1 Albino mice. Melittin-adsorbed ALD is a potentionally good candidate to be taken for future protective anti-leishmania vaccine studies based on its marked inhibitory effects on Th2 immune responses.

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Author's Contribution

WSES and EAGK conceived the idea did the experimental work, the laborataory and data analysis. WSES and EAGK prepared and approved the manuscript.

Disclosure

None

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Competing Interests

The authors declare that they have no competing interests.

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