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

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Objective: The rapid non–specific defense against infections. This study aimed to determine the immune response modifying effects of melittin and melittin/Autoclaved \textit{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. Melittin could be a potentially effective adjuvant for future anti-leishmanial vaccines.

Keywords: \textit{Vaccine Adjuvants}; \textit{Immune Response Modifying Effects}; \textit{Bee Venom}; \textit{Melittin}; \textit{Auto-claved L. donovani} complex

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 \textit{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. Melittin could be a potentially effective adjuvant for future anti-leishmanial vaccines.

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 rapid non–specific defense against infections. This study aimed to determine the immune response modifying effects of melittin and melittin/Autoclaved \textit{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. Melittin could be a potentially effective adjuvant for future anti-leishmanial vaccines.

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was sterile 1.5 ml Eppendorf tubes and stored at -20°C until use.

Leishmanial vaccines [30].

Normal saline.

Company, Hong Kong, China) was dissolved in 5 ml of double distilled H2O in sterile -20°C freezer for 2 h and centrifuged at 13,000-15,000 r.p.m for 15 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.

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 x 10^7 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 parasitic 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 aliquoted 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 x 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].

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

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

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-stimulated 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

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

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 0.00001 respectively). Spontaneous secretion of TNF-α was significantly higher compared to Group I \((p=0.02)\) (Table 4).

<table>
<thead>
<tr>
<th>Study groups</th>
<th>IL-10 Concentration pcm/ml</th>
<th>IFN-γ Concentration pcm/ml</th>
<th>TNF-α Concentration pcm/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Cells (No stimulation)</td>
<td>sLA Stimulation</td>
<td>Control Cells (No stimulation)</td>
</tr>
<tr>
<td>Group I ((n=30)) ([\text{no vaccination}])</td>
<td>159.7 ± 279.7</td>
<td>296.6 ± 390.0</td>
<td>6.9 ± 10.3</td>
</tr>
<tr>
<td>Group II ((n=10)) ([\text{ALD}])</td>
<td>565.2 ± 30.1*</td>
<td>522.7 ± 165.1*</td>
<td>24.5 ± 1.5*</td>
</tr>
<tr>
<td>Group III ((n=40)) ([\text{Melittin}])</td>
<td>488.3 ± 96.0*</td>
<td>444.5 ± 204.4*</td>
<td>26.0 ± 3.4*</td>
</tr>
<tr>
<td>Group IV ([\text{Melittin+ALD}]) ((n=40))</td>
<td>257.6 ± 195.3*</td>
<td>497.5 ± 320.0*</td>
<td>23.2 ± 1.6*</td>
</tr>
<tr>
<td>Group V ((n=65)) ([\text{Melittin-adsorbed ALD}])</td>
<td>0.00 ± 0.00*</td>
<td>125.1 ± 186.7*</td>
<td>3.3 ± 5.8*</td>
</tr>
</tbody>
</table>

Table 4: Means levels of IL-10, IFN-γ and TNF-α cytokines in the study groups \((\text{pcm/ml=picogram/ml; sLA}=\text{Soluble Leishmania Antigen; Positive control (Phytohaemagglutinin PHA) results [OKV]}\) 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 \(L.\hspace{1pt}donovani\) 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 \((\text{increased Th2 responses/susceptibility})\), with reduction in TNF-α \((\text{decreased healing abilities})\). 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 \((\text{affecting Th1/Th2 responses}\) compared to control mice, it is argued that a high IFN-γ production cannot be the sole factor that confers protection against \(L.\hspace{1pt}donovani\). 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 reduction induced 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-leishmanial 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 laboratory 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.

References
