Immunochemotherapy of persistent post-kala-azar dermal leishmaniasis: a novel approach to treatment

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Immunochemothery of persistent post-kala-azar dermal leishmaniasis: a novel approach to treatment


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Summary Post-kala-azar dermal leishmaniasis (PKDL) is a recognized dermatosis that follows successful treatment of visceral leishmaniasis in the Sudan. This randomized and double-blind study aimed to assess safety, immunogenicity and curative potentials of a novel immunochemotherapy regimen in patients with persistent PKDL. Following informed consent, 30 patients were randomized to receive alum-precipitated autoclaved \textit{Leishmania major} (Alum/ALM) vaccine + Bacille Calmette-Guérin (BCG) and sodium stibogluconate (SSG) or vaccine diluent and SSG. The SSG+Alum/ALM+BCG proved safe with minimal local adverse events. In the SSG+vaccine group, 87\% of the patients were cured by day 60 compared with 53\% in the SSG alone group (SSG+vaccine efficacy = 71\%, 95\% CI for risk ratio 0.7—1.16). On day 90 of follow-up there were two relapses in the SSG alone arm and none in the SSG+vaccine arm. Pretreatment cytokines showed high IFN-\gamma or high IFN-\gamma/IL-10 levels and leishmanin skin test (LST) non-reactivity, while healing/clinical improvement were associated with LST reactivity and low
1. Introduction

Post-kala-azar dermal leishmaniasis (PKDL) is an increasingly recognized complication that follows successful treatment of visceral leishmaniasis (VL). It is one of the manifestations of the post-kala-azar leishmaniasises, which include post-kala-azar uveitis, conjunctivitis, blepharitis and laryngitis (El-Hassan et al., 1998; Nandy et al., 1997). PKDL occurs mainly in relation to kala-azar caused by *Leishmania donovani* and is characterized by skin macules, papules, ulcers and/or nodules (El-Hassan et al., 1992; Zijlstra and El-Hassan, 2001). Host immune response and ultraviolet (UVB) may play a role in its pathogenesis, and patients’ response to treatment is strongly correlated to leishmanin skin test (LST) reactivity (Ismail et al., 2006; Musa et al., 2002; Neogy et al., 1990; Zijlstra et al., 2000, 2003). Peripheral blood mononuclear cells (PBMCs) from the majority of Sudanese PKDL patients proliferate and produce IFN-γ and IL-10 in response to leishmanial antigens, indicating a mixed Th1/Th2 immune response (Gasim et al., 1998). IL-10 expressed simultaneously with IFN-γ in PKDL lesions is believed to block the action of IFN-γ, leading to disease chronicity (Ismail et al., 1999).

More than 50% of successfully treated VL patients in Sudan develop PKDL, with spontaneous healing in the majority within one year (Musa et al., 2002; Zijlstra et al., 1995). Treatment of persistent disease is protracted, expensive and requires the use of potentially toxic drugs (Hashim et al., 1996; Genaro et al., 1996; Machado-Pinto et al., 2002). Recently, the safety, immunogenicity and possible efficacy in persistent PKDL lesions for VL candidate vaccine [alum-precipitated autoclaved *L. major* (Alum/ALM) plus Bacille Calmette-Guérin (BCG)] were studied. The Alum/ALM+BCG was found to be safe, immunogenic and probably efficacious in patients with persistent PKDL lesions (Kamil et al., 2003; Khalil et al., 2005, 2006; Musa et al., 2005b).

This randomized study aimed to determine the safety, immunogenicity and the probable curative potentials of multiple doses of Alum/ALM+BCG in combination with sodium stibogluconate (SSG) (immunochemotherapy) in patients with persistent PKDL.

2. Materials and methods

2.1. Study design and protocol

This was a randomized, double-blind and placebo-controlled study. Study patients were admitted to the Tropical Diseases Hospital, Omdurman, Sudan during the period October 2003–December 2004 and monitored closely by trained medical doctors during the course of the study. Following informed consent from patients or their guardians, 30 patients with persistent PKDL who met specific selection criteria (see below) were randomly allocated to the two study arms. One group (*n* = 15) received four weekly doses of 100 µg Alum/ALM+BCG (one-tenth of the 0.1 ml dose used for TB vaccination), plus SSG i.m./i.v. daily for 40 d at the standard dose of 20 mg/kg body weight/d. The other group (*n* = 15) received four doses of the vaccine diluent (placebo) plus SSG in the same dose as the first group. The maximum SSG dose did not exceed 850 mg/d and all patients remained hospitalized for the duration of the study.

At baseline (day 0) the distribution, appearance and severity of lesions were documented. The rash was graded according to distribution and severity. An individual with grade 1 PKDL had lesions mainly on the face and head, arms, chest and back. When the lesions affected other parts of the body, including the hands and feet, the case was considered grade 3. All other cases, with lesions on the head, scalp, forearms, upper legs and upper chest but not the hands and feet, were considered grade 2. Each grade was further sub-graded into 1, 2 and 3. Grade 1:1 is when the lesions are scattered; grade 1:2 is when the lesions are in close proximity; and grade 1:3 is when the lesions are dense and confluent. Grade 8:8 means no PKDL lesions (Musa et al., 2005; A.M. El-Hassan, personal observation). The vaccination site was covered before each check-up visit to maintain blinding.

2.2. Patient selection

Patients who fulfilled the following criteria were enrolled: males and females ≥7 to 60 years of age; PKDL skin rash of >6 months duration following a history of successful treatment for VL; normal ECG and positive serology for leishmaniasis (rK39 dipstick). Patients with concurrent or chronic illnesses (iridocyclitis, TB, diabetes mellitus, leprosy, epilepsy, hypertension, suspected kala-azar, etc.), pregnancy or lactation were excluded. Known allergy to any of the vaccine components (e.g., BCG, alum), allergic conditions requiring medical treatment (especially steroids and levamisole) and known immunological deficiency (including HIV) were additional exclusion criteria. Patients were not/should not be involved in any other drug or vaccine trial during the study period, and have no known or planned vaccination within one month before the study period.

2.3. Safety assessment of SSG+Alum/ALM+BCG

Safety was assessed by daily monitoring for 60 d and then at day 90. Ten milliliters of venous blood was taken at baseline and on days 21 and 60 for assessment of hematological and biochemical profiles and PBMC harvesting for in vitro...
culture. The safety assessment was based on local and systemic adverse events and changes in laboratory values.

2.4. Immunogenicity assessment and endpoints

Significant conversion/increase in LST induration, significant increase in IFN-γ and reduced or absent production of IL-10 by PBMCs were taken as endpoints.

LST was performed at screening (day 3) and again on days 21, 40, 60 and 90. LST induration was measured using the ballpoint pen technique, as described previously (Musa et al., 2002; Sokal, 1975). IFN-γ and IL-10 levels were measured on days 0, 21 and 60 from harvested PBMC culture supernatant. Soluble Leishmania antigens at a concentration of 10 μg/well were used to stimulate PBMCs. Wells containing cell culture medium plus PBMCs or culture medium plus PBMCs plus 12.5 μg/ml phytohemagglutinin (PHA) were used as negative and positive controls, respectively. Cultures were harvested after 24 h from PHA wells and after 48 h from soluble L. donovani antigen wells. Levels of IFN-γ and IL-10 produced were measured in aliquots of cell-free supernatants by a double sandwich ELISA commercial kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany).

2.5. Treatment outcome assessment

Photographs of the skin lesions were taken on days 0, 21, 40, 60 and 90. Treatment progress was assessed on days 30, 40, 60 and 90 by clinical evaluation of the distribution and severity of the skin lesions. SSG treatment was stopped for patients who were cured by day 40. For those who attained marked improvement (as measured by a downgrading from grade 3:2 to 2:1 or 2:2 to 1:1, etc.) on day 40, SSG treatment was stopped for 20 d and continued in the follow-up to day 90. In the final analysis, these cases were considered as failures.

2.6. Statistical analysis

Data were analyzed on an intention-to-treat basis: safety endpoints were compared between the two arms. LST induration conversion/increase and cytokine patterns in relation to cure were assessed and compared between arms. Clinical outcome data were used for assessment of risk ratio (RR) and associated 95% CI. Risk ratios, differences in proportions and means and their associated tests of statistical significance (χ² and Student’s t-tests) were used to assess and compare the effect of SSG+vaccine and SSG+placebo. Epinfo software version 3.3.2 (CDC, Atlanta, GA, USA) was used for statistical computations. Data were managed by a team from the University of Khartoum, Sudan and WHO/TDR, Geneva.

2.7. Ethical considerations

The study was conducted in adherence to GCP guidelines and received expert clinical trial monitoring from the WHO/TDR and follow-up by the Data and Safety Monitoring Board (DSMB) of the Institute of Endemic Diseases, University of Khartoum, Sudan.

3. Results

3.1. Baseline characteristics of patients

The mean ages of the study arms were not significantly different (P = 0.98). The increased M:F ratio (3:1) is not by design, but male patients tend to be more willing to travel for treatment compared with females. The mean ± SD LST induration at screening was 2.3 ± 3.2 mm and 1.9 ± 3.8 mm for the SSG+Alum/ALM+BCG and SSG+placebo arms, respec-

### Table 1

<table>
<thead>
<tr>
<th>ID no.</th>
<th>IFN-γ/IL-10 (pg/ml)</th>
<th>LST (mm)</th>
<th>PKDL grade</th>
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</thead>
<tbody>
<tr>
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<td>Day 60</td>
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<td>274/456</td>
<td>874/35</td>
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<td>3178/406</td>
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<tr>
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<td>2428/21</td>
<td>2261/164</td>
<td>865/08</td>
</tr>
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<td>2505/16</td>
<td>3284/232</td>
<td>438/22</td>
</tr>
<tr>
<td>111</td>
<td>99/00</td>
<td>723/103</td>
<td>45/05</td>
</tr>
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<td>00/33</td>
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<td>41/61</td>
</tr>
<tr>
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<td>1016/33</td>
<td>3072/177</td>
<td>968/81</td>
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<td>117</td>
<td>00/16</td>
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<tr>
<td>118</td>
<td>427/12</td>
<td>3284/52</td>
<td>85/36</td>
</tr>
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<td>121</td>
<td>00/00</td>
<td>3178/59</td>
<td>531/22</td>
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<tr>
<td>123</td>
<td>101/04</td>
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<tr>
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<td>342/19</td>
<td>3390/33</td>
<td>270/40</td>
</tr>
</tbody>
</table>

tively ($P = 0.7$). PKDL grades of the study patients are shown in Tables 1 and 2. The rK39 serology test was positive in all patients at screening.

### 3.2. Adverse events during hospitalization

Hematological and biochemical profiles were not significantly different at screening and follow-up visits in the two study arms ($P > 0.05$). The adverse events were minimal and were confined to the vaccine injection site in forms of local indurations and ulcers, which resolved spontaneously. One patient in the SSG alone arm experienced myalgia, which responded to paracetamol (1.0 g three times per day for 7 d).

### 3.3. Treatment outcome

Of the patients who received SSG+Alum/ALM+BCG, 87% (13/15) healed completely within 60 d and the remaining 13% (2/13) showed considerable improvement (from grade 3:3 to 2:2 ID #110 and from grade 3:2 to 2:1 ID #129; Table 1). However, 53% (8/15) of the patients who received SSG+placebo healed completely within 60 d, one patient (7%) showed considerable improvement (from grade 3:2 to 2:1 ID #130), and the remaining 40% (6/15) remained the same (Table 2). The RR (vaccine/placebo) was 0.29 (95% CI 0.7—1.16) with the associated efficacy (1 — RR) of 71%.

### 3.4. Immune responses

#### 3.4.1. SSG+vaccine arm (Table 1)

Eight patients (8/15; 53.3%; ID #102, 111, 114, 117, 121, 126, 128, 129) had low IFN-γ and low IL-10 at day 0. On day 21, the immune response changed to a predominantly Th1 immune response in seven patients with significant IFN-γ production ($P = 0.009$). The IFN-γ levels dropped to baseline (day 0) levels on day 60. One patient (ID #102) showed a mixed Th1/Th2 immune response with high IFN-γ and high IL-10 on day 21 and a non-reactive LST, which changed to predominantly Th1 on day 60 with LST conversion. All eight patients (8/8; 100%) were cured by day 60, with significant LST induration ($P = 0.004$).

Seven patients (7/15; 46.7%) had a predominantly Th1 immune response on day 0, four of them were LST non-reactive, two of those converted by day 21, and all four were LST-reactive on day 60. Five patients (5/7, 71%) had a mixed Th1/Th2 immune response on day 21 that changed to a predominantly Th1 pattern on day 60. The IFN-γ dropped to baseline levels in the remaining two patients (2/7; 29%). On day 60, five patients were cured, with significant LST induration ($P = 0.004$), while two patients (ID #110 and 129) showed considerable improvement.

#### 3.4.2. SSG+placebo arm (Table 2)

Six patients (6/15, ID #101—109 + 130) had a predominantly Th1 immune response on day 0 with high IFN-γ levels and non-reactive LST (induration $<$5 mm). On day 21, four of these patients (4/6) had a mixed Th1/Th2 immune response with non-reactive LST; the fifth patient (1/6; ID #109) continued to have a predominantly Th1 immune response and a non-reactive LST. The sixth patient was lost to follow-up (1/6; ID #130). On day 60, the five followed-up patients had a predominantly Th1 immune response, 3/5 (60%) patients showed significant conversion in LST (induration $>$5 mm) ($P < 0.001$). The skin lesions cleared completely by day 60 in 3/5 patients, with no change in the remaining two patients (ID #101 and 108).

Eight patients (8/15; ID #113—127) had low IFN-γ and low IL-10 at day 0; six of them (6/8, 75%) showed no LST reactivity, while the rest (2/8, 25%) had significantly large indurations (ID #113 and 115). The immune response changed to a mixed immune response (Th1/Th2) in 3/8 (ID #115, 122, 125) on day 21, and changed to a predominantly Th1 response by day 60 in two of them. In 4/8 patients the immune responses were predominantly Th1 on day 21,

<table>
<thead>
<tr>
<th>ID no.</th>
<th>IFN-γ/IL-10 (pg/ml)</th>
<th>LST</th>
<th>PKDL grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 21</td>
<td>Day 60</td>
</tr>
<tr>
<td>101</td>
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<td>1102/300</td>
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<td>1202/393</td>
<td>1298/12</td>
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<td>2193/00</td>
<td>2681/536</td>
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</tr>
<tr>
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<td>813/120</td>
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NA: not applicable.
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and cytokine levels dropped to baseline levels by day 60. One patient (1/8, ID #124) did not show any change in the immune responses throughout the follow-up periods. The fifteenth patient (1/15; ID #112) had a mixed Th$_1$/Th$_2$ immune response and no LST reactivity on day 0, which changed to a predominantly Th$_1$ response with LST conversion on day 60. LST conversion was seen in 9/15 (60%) patients in the SSG+placebo arm, with complete skin lesion healing in the majority of these (8/9, 89%).

3.5. Day 90 follow-up

All 30 patients were seen in their home villages in eastern Sudan. Two patients from the SSG+placebo arm, who were cured by day 60, relapsed with florid skin lesions. One had a reactive LST, while the other was LST non-reactive. They were referred to a field hospital for further treatment with AmBisome (Gilead). The LST remained strongly reactive in all patients (100%) who received SSG+vaccine (LST induration = 9.7 ± 1.8 mm) and in 11/15 (73%) patients who received SSG+placebo (LST induration = 8.7 ± 2.1 mm).

4. Discussion

Over the last few years, the pathology of PKDL has been well elucidated, but its pathogenesis is far from clear. It is well documented that a mixed Th$_1$/Th$_2$ response dominates the picture in the skin of most patients. LST reactivity is a good correlate to immune responses and cure in patients with PKDL.

Treatment of patients with persistent lesions poses a great challenge and a considerable financial burden. A simple and relatively cheap treatment could be the only attractive choice to convince patients to seek treatment. Alum/ALM vaccine was shown to be safe and immunogenic in healthy volunteers and PKDL patients with significant LST conversion. Combining the vaccine with SSG seems an attractive option to modulate the immune response with a shift to a pure Th$_1$ response and induction of healing in a relatively short period. The results of a pilot study carried out by this group (Musa et al., 2005b) demonstrated that the curative potentials of the SSG+Alum/ALM+BCG surrogate markers of healing included a change in LST reactivity and an increase or a drop in IFN-γ, depending on the stage of healing (dichotomy of immune responses). This dichotomy between LST induration and IFN-γ levels was previously documented in healthy volunteers who received Alum/ALM+BCG vaccine (Khalil et al., 2005). Healing of skin lesions probably starts at the cellular level by antigen presentation, followed by secretion of IL-2 followed by IL-12, which facilitates expansion of the Th$_1$ population (The Leishmaniasis Research Group/Sudan, unpublished data). The expansion will be followed by IFN-γ and TNF-α secretion. Effective parasite clearance is probably due to the effect of nitrogen and oxygen reactive intermediates (NRI and ORI), which are produced in macrophages under the effect of IFN-γ. IFN-γ secretion starts sometime before the start of treatment and could be an attempt at spontaneous healing by the immune system (Musa et al., 2002).

Elimination of the parasite will give room for the healing processes and complete resolution by reducing the parasite antigen load. Memory cells will evidently be produced, as clearly shown by the persistence of LST conversion at day 90. It is well documented that LST conversion is probably a lifelong effect (Khalil et al., 2002).

The present data showed that LST is a simple immune correlate for an efficacious immune response that predicts healing in persistent PKDL lesions. The role of LST as a simple in vivo marker of healing in PKDL patients is well documented and is further confirmed by this study, in which almost all LST-reactive patients in the SSG+vaccine and the majority in the SSG+placebo were cured (Musa et al., 2002, 2005b; Zijlstra et al., 2000).

The recent data confirm earlier findings that IFN-γ production preceded LST conversion in most patients and tended to wane with time. Only one patient in the SSG+vaccine group did not heal by day 60 despite LST reactivity. IFN-γ and IL-10 levels of this patient were still high at day 60 (mixed Th$_1$/Th$_2$ immune response), but his skin lesions healed completely by day 90, indicating that he is probably a slow responder. Slow responders were more common in the SSG alone group, where the majority had a mixed Th$_1$/Th$_2$ immune response and a non-reactive LST. These patients responded completely to AmBisome treatment, with complete clearance of the rash and conversion in the LST.

These recent findings showed that the immune responses that induce healing in persistent PKDL patients are stereotyped and that the differences between the SSG+Alum/ALM+BCG and SSG alone are differences in magnitude and timing. It is evident that the SSG+Alum/ALM+BCG is superior to the SSG+placebo in augmenting IFN-γ production, tipping the balance toward a Th$_1$-type immune response and healing. This is highlighted by the fact that the number of patients who completely healed in the SSG+Alum/ALM+BCG arm was significantly higher compared with those in the SSG+placebo arm. However, the patients who showed clinical improvement in their lesions had high baseline IFN-γ, with no significant change in IFN-γ and/or IL-10 levels during follow-up ($P=0.4$ in both cases). In non-healers, IFN-γ levels did not increase significantly ($P=0.3$), while IL-10 levels did increase significantly ($P=0.009$). The high IL-10 levels probably block the production and impair the action of IFN-γ, leading to persistence of skin lesions and disease chronicity.

In conclusion, SSG+Alum/ALM+BCG is safe and immunogenic in patients with persistent PKDL lesions, with healing potentials that are superior to SSG alone. The healing process is probably due to modulation of the patients’ immune system tipping the Th$_1$/Th$_2$ immune response to a pure Th$_1$ response and induction of healing. The LST is a simple and cheap test that could be used to predict the outcome of treatment of PKDL lesions.

Authors’ contributions: AMM, EAGK, FM, AME and HWG designed the study protocol, were responsible for patient recruitment, management and follow-up, and design of the Case Record Form, and drafted and revised the manuscript; FAM, AMYE and MHA were responsible for clinical management and follow-up of patients; SHHE was responsible for routine laboratory investigations, the cytokine assay, and data entry and cleaning; SN was responsible for design of the Case Record Form, data entry, cleaning, analysis and interpretation, and contributed to the writing and revision of the manuscript.
of the manuscript. All authors read and approved the final manuscript. AME, FM and EAGK are guarantors of the paper.

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