Inadequacy of 12-Week Miltefosine Treatment for Indian Post-Kala-Azar Dermal Leishmaniasis

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Abstract. Post-kala-azar dermal leishmaniasis (PKDL) is a chronic dermatosis that generally occurs after apparent cure of visceral leishmaniasis caused by Leishmania donovani. In view of the prolonged treatment regimens necessary for PKDL, noncompliance is a major limitation; an optimal regimen is yet to be defined, but 12 weeks of therapy with miltefosine is generally recommended. We performed a single-arm open-label trial of miltefosine administered daily for 16 weeks in 27 patients in Kolkata with PKDL. After 4 weeks of treatment, nine patients were lost to follow-up because of unacceptable side effects, including severe abdominal pain, nausea, and vomiting. Of the 18 remaining patients, seven completed 12 weeks of therapy and 11 completed 16 weeks of therapy. Three of the seven who received 12 weeks of therapy and none of the 11 who received 16 weeks of therapy experienced disease relapse. Our results suggest that a 16-week course of miltefosine is required for reliable cure of PKDL. Further, they highlight the urgent need for a multicentric randomized controlled trial of 12 versus 16 weeks of treatment with miltefosine for PKDL so as to achieve the goal of elimination of leishmaniasis in south Asia.

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Post-kala-azar dermal leishmaniasis (PKDL), a chronic dermatosis appears in 5–10% and over 60% of apparently cured cases of visceral leishmaniasis (VL or kala-azar) in south Asia and east Africa, respectively. On the basis of their clinical features, patients with PKDL in south Asia can be broadly categorized into two variants namely, polymorphic, where hypopigmented macules, papules/plaques, and/or nodules are present, or macular, where patients primarily present with hypopigmented macules.1 The disease acquires greater clinical relevance in south Asia as, in the absence of a zoontic transmission, these patients are the proposed reservoirs for VL.2 To achieve the World Health Organization (WHO) target of elimination of VL by 2015 (annual incidence of less than one per 10,000 population) in south Asia, eradication of leishmaniasis has become a national priority (http://www.who.int/dtr/news/2012/vl_elimination/en/). Treatment of PKDL is a challenge for clinicians especially for macular PKDL, since the endpoint of therapy cannot be precisely defined making one dependent on resolution of clinical features.

Sodium antimony gluconate (SAG) was the mainstay of therapy of PKDL; however, the protracted treatment duration along with an increasing incidence of resistance to SAG3 has led to its steady decline. Alternatives include miltefosine and Amphotericin B, the former being recommended since 2012 by the WHO as the first line of therapy because of unacceptable side effects, including severe abdominal pain, nausea, and vomiting. Of the 18 remaining patients, seven completed 12 weeks of therapy and 11 completed 16 weeks of therapy. Three of the seven who received 12 weeks of therapy and none of the 11 who received 16 weeks of therapy experienced disease relapse. Our results suggest that a 16-week course of miltefosine is required for reliable cure of PKDL. Further, they highlight the urgent need for a multicentric randomized controlled trial of 12 versus 16 weeks of treatment with miltefosine for PKDL so as to achieve the goal of elimination of leishmaniasis in south Asia.

From 2008 onward, patients (N = 27) who presented with a strong clinical suspicion of PKDL were recruited from the Dermatology Outpatient Department, School of Tropical Medicine, Kolkata and Institute of Postgraduate Medical Education and Research, Kolkata. Diagnosis was based on the presence of symmetrically distributed erythematous papules/plaques/nodules and/or hypopigmented macules mostly occupying the face (muzzle area) followed by trunk and extremities, along with a suggestive history of cured VL. PKDL was confirmed by polymerase chain reaction (PCR) of internal transcribed spacer 1 (ITS1) region of Leishmania sp., rK39 strip test along with the presence of antileishmanial antibodies and Giemsa staining of lesion biopsies.6 Miltefosine (Capsule Impavid® 50 mg; Paladin Therapeutics, Canada) was administered orally in a daily single dose of 100 mg/day (body weight > 25 Kg) or 50 mg/day (body weight < 25 Kg) or 2.5 g/kg/day (aged 2–11 years). All patients were treated for 16 weeks unless treatment-emergent side effects limited further therapy; in case of polymorphic PKDL, therapy was terminated earlier, if there was complete resolution of papulo-plaques/nodules (N = 3). Punch biopsies from skin and peripheral blood were collected at disease presentation and thereafter every 4 weeks till completion of treatment; thereafter, biopsies were cultured every 3 months for 1 year (N = 11). The study received approval from the Institutional Ethics Committee of the School of Tropical Medicine, Kolkata, and Institute of Post Graduate Medical Education and Research, Kolkata; all treatment-emergent adverse events were treated.

Lesional parasite load was measured by real-time PCR (rPCR) using a standard curve, generated from serially diluted Leishmania donovani promastigotes (ranging from 1 × 10⁴ to 1) added to blood from a healthy control. DNA was extracted using a QIAmp DNA Mini kit and subjected to rPCR using the Applied Biosystem SYBR Green QPCR Master Mix in Applied Biosystem Step One Plus. A fragment of 116 bp of L. donovani kDNA was amplified by rPCR using a primer set (forward 5′-CCTATTTACACCCACCCGAT-3′ and reverse 5′-GCCTGAGGGCGGTTCTGCGAAA-3′).7 Template DNA (5 μL) was added to a 20 μL reaction mixture containing SYBR...
Green Master mix and 400 nM of each primer. The parasite load was extrapolated from the standard curve and quantified as number of parasites per μg genomic DNA used as template.

All the 27 patients with PKDL were included in this study, their median age being 30.0 (10.0–60.0) years and male female ratio, 24:3; majority (22/27, 81.48%) were polymorphic while five (18.5%) were macular, concordant with published data. All patients tested positive for ITS-1 PCR, rK39 strip test, enzyme-linked immunosorbent assay (ELISA) for antileishmanial antibodies, and Leishman-Donovan bodies were identified in Giemsa-stained sections of polymorphic PKDL.

After 4 weeks of treatment, 9/27 were lost to follow-up and were not considered for analysis; eight of them had polymorphic lesions and one had macular lesions (Table 1). They were contacted by telephone and counseled regarding the importance of complete treatment but declined therapy, owing to unacceptable side effects, namely, severe abdominal pain (N = 6), nausea, and vomiting (N = 3; Table 1).

In the 18 patients who were analyzable, 11 completed 16 weeks of therapy that translated into disappearance of papulo-plaques or nodules, whereas seven completed 12 weeks of therapy, of whom, three relapsed (Table 1). Among the 11 who completed 16 weeks of therapy, majority were polymorphic (N = 9) and a minority showed macular lesions (N = 2); till date, none have relapsed (Table 1). Among the seven who completed 12 weeks of therapy, five were polymorphic and two were macular; in the five with polymorphic PKDL, four showed complete resolution of symptoms, hence therapy was terminated; they are yet to show signs of relapse. The remaining one patient from this group developed a cerebrovascular accident (CVA) necessitating termination of treatment; this patient showed a flare-up of existing lesions after a quiescent period of 39 months. In two patients with macular PKDL, therapy was limited to 12 weeks as they developed intractable nausea, vomiting, and upper abdominal pain that could not be managed with proton pump inhibitors, sucralfate, and gastrokinetics. Both relapsed after 17 months characterized by the appearance of widespread hypopigmented macules. Kaplan-Meier survival plots were constructed for the two treatment arms (12 and 16 weeks) and compared by log-rank test; data of subjects (N = 9) who did not complete treatment were censored. There were no relapses with the 16-week treatment, whereas with 12-week treatment, there were three relapses, the median time to relapse exceeding the total observation period using log-rank test. The relapse experience was significantly different (P = 0.019) in favor of 16-week treatment (Figure 1).

Antileishmanial antibody titer is known to persist for years after cure and therefore antigen-based studies are accorded greater importance. Among the antigen-based studies, PCR is considered as more sensitive than microscopy. At active disease, all the 18 analyzable patients were positive but became negative for ITS1-PCR after 4 weeks of treatment. To validate this, we selected another region (116-bp fragment of the minicircle kDNA) of L. donovani and performed normal PCR and rtPCR (40 cycles), based on the kDNA copy number being higher in Leishmania parasites, the latter being considered more sensitive than ITS1-PCR; here too, PCR was negative after 4 weeks of treatment.

The three patients who relapsed after 12 weeks of therapy were diagnosed by PCR and ELISA for antileishmanial response and further treatment is planned with Amphotericin B.

Parasite load, that is, number of parasites per microgram of genomic DNA of these three patients at presentation were

![Figure 1](image.png)

**Figure 1.** Kaplan-Meier survival plots indicating relapse experience in the two treatment arms. There were no relapses in 11 cases that completed 16 weeks of treatment (stippled line) whereas 3/7 cases relapsed after 12 weeks of treatment (solid line).
10,887, 7,200, and 592 while at the time of relapse, it was 8,505, 2,845, and 36,666, respectively.

This case series highlights the fact that prolonged treatment with miltefosine is associated with gastrointestinal disturbances (abdominal pain, nausea, and vomiting) that led to a substantial number of patients not being accorded 16-week therapy. The CVA occurring in one patient was “possibly” because of miltefosine; causality association being determined by “Naranjo adverse drug reaction probability scale.” This single “severe” incidence (Level 5 on Hartwig’s severity assessment scale) should act as a whistle-blower for clinicians to monitor the risk factors for CVA while receiving miltefosine. Our study emphasizes the need to define the end point of therapy, which to date is yet to be precisely defined. It strongly advocates the urgent need for a multicentric trial of the existing 12 weeks versus 16 weeks of treatment with miltefosine, so as to achieve the goal of elimination of leishmaniasis in south Asia.

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REFERENCES


INADEQUATE MILTEFOSINE TREATMENT IN INDIAN PKDL
A male preponderance in patients with Indian post-kala-azar dermal leishmaniasis is associated with increased circulating levels of testosterone

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Abstract

Background Post-kala-azar dermal leishmaniasis (PKDL) is a neglected parasitic disease that occurs after apparent cure from visceral leishmaniasis (VL), and poses a challenge for elimination of VL, being its proposed reservoir. Several epidemiological studies have proposed that sex hormones may account for the increased susceptibility of males towards infectious diseases, including leishmaniasis; however, the role of testosterone and sex bias, if any, in PKDL has not been evaluated.

Methods The study population included 87 patients with PKDL and 39 with VL; levels of testosterone were measured by competitive enzyme-linked immunosorbent assay along with their levels of antileishmanial immunoglobulin and IgG. The association of testosterone, if any, was then correlated with age, gender, humoral response, lesional profile, disease duration, and lag period.

Results A male predominance was evident in PKDL, not in VL; importantly, this male bias was predominant postpubertal, strongly indicative of an association between sex hormone and disease progression. Male patients with PKDL had significantly higher levels of testosterone, which regressed significantly with miltefosine, not with sodium antimony gluconate. Additionally, a significant correlation was found between plasma testosterone and antileishmanial IgG.

Conclusion Taken together, our study has established a male dominance in PKDL, which showed a strong association with testosterone. This information should be taken into consideration for disease monitoring and control.

Introduction

An immune–endocrine balance has been proposed to account for gender-based susceptibility and resistance towards infectious and autoimmune diseases, but it is only recently that a definitive role for sex-specific hormones in the immune cell-specific transcriptome and functionality has been demonstrated. Interestingly, males are more susceptible towards intracellular pathogens than their female counterparts as evident in cutaneous leishmaniasis, tuberculosis, lepromatous leprosy, salmonellosis, leptospirosis, and hepatitis A; this bias has been attributed to sex hormones and/or sex specific behavior, as males have an increased risk of exposure along with their greater treatment seeking behavior. Additionally, the X chromosome contains more immune-related genes and due to the dosage effects of the X chromosome, females upon an antigenic challenge demonstrate a better immune response than males. Therefore, this X chromosome acts as a double-edged sword, making females more prone to autoimmunity disorders. On the other hand, males being heterogametic (XY) are more likely to develop infectious diseases by a single polymorphism or mutation in immune genes. As female sex hormones would contribute towards a more pronounced proinflammatory immune response, it may also facilitate their resistance towards infectious diseases such as leishmaniasis. Conversely, the immune response in males may be poorer as testosterone being immunosuppressive can translate into an enhanced susceptibility to infectious diseases. Leishmaniasis is endemic in 98 countries with over 350 million people being at risk for the disease, and in general, males are more affected than females. Post-kala-azar dermal leishmaniasis (PKDL), a chronic dermatosis, is
generally develops after apparent cure from visceral leishmaniasis (VL), and its occurrence is limited to two geographical pockets, East Africa and South Asia. The presence of parasite-rich nodules and papules with or without hypopigmented macules makes them the interepidemic reservoir for VL, necessitating a deeper understanding of their clinico-epidemiology. 

In leishmaniasis, the parasite *Leishmania* develops a non-mutual symbiotic relationship with host macrophages to ensure its growth and survival and accordingly, deviately modulates the host immune system by stimulating the secretion of anti-inflammatory cytokines [interleukin (IL)-4, IL-10, IL-13, transforming growth factor-β], which translates into an anti-inflammatory milieu, thus sustaining parasite growth. In cutaneous leishmaniasis, the increased susceptibility of males has been attributed to testosterone along with granulocyte colony stimulating factor and interferon-gamma. Although studies from Sudan (n = 134), India (n = 102), Nepal (n = 37), and Bangladesh (n = 185) have not revealed a sex bias in PKDL, studies undertaken by our group, and others have reported a male preponderance. We initially attributed this to males possibly being accorded preferential access to medical care, but had not explored the putative role of sex hormones. Accordingly, this study aimed to establish sexual dimorphism in Indian PKDL and its possible association with testosterone.

**Materials and methods**

**Study subjects**

The study population included patients diagnosed clinically as PKDL (n = 87, 2004-14) and patients with VL (n = 39, 2011-13) recruited from the dermatology outpatient department and the Department of Tropical Medicine respectively of the School of Tropical Medicine, Kolkata, India. Levels of testosterone were measured in 50 patients with PKDL recruited from 2011 to 2014, and 39 patients with VL recruited from 2011 to 2013. The diagnosis was based on clinical features (skin lesions for PKDL and hepatosplenomegaly with fever for VL) along with a previous history of VL (for patients with PKDL only) or if they were from an area endemic for VL. Diagnosis was confirmed by the rK39 strip test (In Bios International, Seattle, WA, USA), internal transcribed spacer sequence 1 (ITS1) polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay for antileishmanial antibodies. None of the patients had any secondary infections or any pre-existing disease. Age- and sex-matched healthy volunteers (n = 21; males 11 and females 10), were recruited from non-endemic areas for kala-azar/PKDL and were seronegative for antileishmanial antibodies.

**Results and discussion**

Among 87 patients with PKDL (Table 1), 69 were men and 18 female, thus entailing a clear male preponderance, the ratio of male/female being 3.8:1, and was in concordance with a recent study who reported a comparable
Table 1 Study population

<table>
<thead>
<tr>
<th>PKDL*</th>
<th>VL*</th>
<th>Healthy controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (IQR)</td>
<td>26 (18-35)</td>
<td>27 (19-41)</td>
</tr>
<tr>
<td>Sex ratio (M/F)</td>
<td>3.83 : 1</td>
<td>1.29 : 1</td>
</tr>
<tr>
<td>Number</td>
<td>87</td>
<td>39</td>
</tr>
<tr>
<td>Polymorphic/ macular lesions (no. of patients)</td>
<td>3.17 : 1</td>
<td>NA</td>
</tr>
<tr>
<td>Disease duration (years) (IQR)</td>
<td>3.0 (1.0-7.0)</td>
<td>NA</td>
</tr>
<tr>
<td>Interval between cure of VL and onset (years) (IQR)</td>
<td>3 (1.0-7.0)</td>
<td>NA</td>
</tr>
</tbody>
</table>

IQR, interquartile range; NA, not applicable; PKDL, post-kala-azar dermal leishmaniasis; VL, visceral leishmaniasis.

Although the male/female ratio of 4.18 : 1.19 is important, this sex difference was significant when the age group distribution of patients with PKDL was divided into pre- and postpuberal, the latter being the majority of patients (Table 2). However, in VL, there was no sex bias, as 22 of 39 patients were male and 17 female (Table 1). Until now, epidemiological studies from South Asia have not reported a sex bias in PKDL, possibly due to the broader age range of 2-68 years. Importantly, this sex difference was significant when the age group distribution of patients with PKDL was divided into pre- and postpuberal, the latter being the majority of patients (Table 2). However, in VL, there was no sex bias, as 22 of 39 patients were male and 17 female (Table 1). Until now, epidemiological studies from South Asia have not reported a sex bias in PKDL, possibly due to the broader age range of 2-68 years. In fact, a critical analysis of the 102 patients with PKDL in the study by Das et al. indicated that the prepubertal group constituted a smaller group (n = 31/102) who showed no male bias; however, the majority belonged to the postpubertal group (n = 71/102), where the ratio of male/female was >4, which is in concordance with our data (Table 2). In our study, the proportion of individuals <14 years was 13 of 87 (15.0%) akin to the study of Das et al., and showed no male bias. However, a larger proportion of patients with PKDL were >14 years (n = 74/87, 85%) and their male/female ratio was 5.17 : 1 (Table 2). Taken together, Indian PKDL primarily occurs in the postpubertal group, suggesting a role for sex hormones.

A similar male-biased sexual dimorphism was documented in American cutaneous leishmaniasis wherein boys 0-14 years, i.e., prepubertal were not afflicted by the disease and infection predominated at the adolescence phase, ≥15 years. However, in Sudan, where children in the age group of 4-8 years primarily develop PKDL, there was no such bias. This holds special significance in view of the spontaneous resolution of PKDL in Sudan, conspicuously absent in South Asian PKDL.

A previous history of VL was recorded in 83% (74 of 87) of the patients, with a predominance of the polymorphic variant, comparable with previous studies; however, there was no association between sex of the patient and the type of lesion, previous history of VL, lag period between treatment for VL and appearance of lesions (Table 2). However, females had a significantly shorter disease duration than males (P < 0.05, Table 2), possibly...
because as PKDL is a cosmetically debilitating disease, females are more likely to seek treatment earlier than males, as observed in vitiligo, a disease associated with hypopigmentation.1

Testosterone is mainly produced by the testicular Leydig cells and has potent immunosuppressive activity, via decreased synthesis of inducible nitric oxide synthase translating into the lowered generation of nitric oxide.1

Alongside, it also decreased levels of proinflammatory cytokines, tumor necrosis factor-α and interferon-γ concomitant with increased production of anti-inflammatory cytokines, IL-4, IL-5, IL-10, IL-13, and transforming growth factor-β.2-5

Figure 1 Disease profile and levels of testosterone in patients with PKDL. (a) Plasma testosterone levels among healthy controls (n = 21, ●) patients with PKDL at presentation (Pre t/t, n = 50, ■) and patients with VL at presentation (VL Pre t/t, n = 39, ▼). (b) Plasma testosterone levels among male healthy controls (n = 11, ●) patients with PKDL at presentation (Pre t/t, n = 39, ■) and patients with VL at presentation (VL Pre t/t, n = 22, ▲). (c) Plasma testosterone levels among female healthy controls (n = 10, ●) patients with PKDL at presentation (Pre t/t, n = 11, ■) and patients with VL at presentation (VL Pre t/t, n = 17, ▲). (d) Before and after plots of plasma levels of testosterone in patients with PKDL in SAG group (Pre t/t, ●, Post t/t, ♦, n = 15) and miltefosine (HePC) group (Pre t/t, ●, Post t/t, ▼, n = 16). (e) Correlation of plasma levels of testosterone with IgG levels in patients with PKDL (n = 50). Each dot represents one patient. (f) Plasma testosterone levels among male healthy controls (n = 11, ●), patients with macular PKDL at presentation (Pre t/t, n = 8, ■) and polymorphic PKDL at presentation (Pre t/t, n = 31, ▲). (g) Plasma testosterone levels among female healthy controls (n = 10, ●) patients with macular PKDL at presentation (Pre t/t, n = 4, ■) and polymorphic PKDL at presentation (Pre t/t, n = 7, ▲). Testosterone levels were measured by enzyme-linked immunosorbent assay as described in Materials and methods. (a–c, f, g) Kruskal–Wallis test followed by post Dunn test; (d) Wilcoxon signed rank test was performed; (e) Pearson's correlation method was applied. PKDL, post-kala-azar dermal leishmaniasis; SAG, sodium antimony gluconate; VL, visceral leishmaniasis.
growth factor-β. During Leishmania infection, testosterone induces anti-inflammatory T-helper 2 type immunity,44 furthermore, treatment of female hamsters with testosterone caused development of larger lesions,45 further endorsing our proposition that testosterone might well be considered as a double-edged sword, which protects males from autoimmune diseases, but enhances their propensity towards pathogen-mediated diseases.

To corroborate whether testosterone indeed affected the observed sex bias in PKDL, its level was estimated in all patients with PKDL irrespective of sex. Overall, levels of testosterone were significantly higher in patients with PKDL as compared to healthy controls, the median (interquartile range) being 4.44 (1.54–6.26) ng/ml versus 0.87 (0.16–2.98) ng/ml (P < 0.01; Fig. 1a) and remained high following treatment, 4.11 (2.70–7.00) ng/ml (P < 0.01, Fig. 1a). However, in patients with VL, testosterone levels were comparable with healthy controls, but significantly lower than patients with PKDL, being 2.15 (0.25–3.13) ng/ml (P < 0.01; Fig. 1a). On a subgroup analysis of male and female patients with PKDL, a similar pattern was evident, with levels of testosterone in males being significantly higher than healthy controls and their VL counterparts, 4.79 (2.99–7.11) versus 2.87 (1.81–4.46) versus 2.63 (1.18–4.79) ng/ml, P < 0.05 and P < 0.01 respectively (Fig. 1b). However, females with PKDL had testosterone levels comparable with female patients with VL and healthy controls, being 0.24 (0.17–0.49), 0.28 (0.21–0.46), and 0.16 (0.05–0.36) ng/ml respectively (Fig. 1c).

Although SAG (n = 15) did not affect upon levels of testosterone, 4.66 (2.52–8.73) ng/ml versus 4.73 (0.20–10.62) ng/ml (Fig. 1a,d), miltefosine (n = 16) significantly decreased levels of testosterone 4.70 (2.85–7.26) versus 3.75 (2.70–4.44) ng/ml, P < 0.01 (Fig. 1d). Antileishmanial chemotherapy, specifically miltefosine, has been demonstrated to have immunomodulatory activity in PKDL, as it significantly enhanced plasma levels of proinflammatory cytokines and decreased anti-inflammatory cytokines.27 In view of testosterone also having immunosuppressive properties, the observed lowering of testosterone levels could be a secondary action of the drug or a secondary phenomenon following disease resolution.

As patients with polymorphic and macular PKDL differ significantly in their humoral immune responses,18 we examined whether these two lesional variants differed in their testosterone levels. The testosterone levels correlated significantly with IgG (r = 0.36, CI = –0.15 to 0.42, P < 0.05; Fig. 1e), but not with immunoglobulin (r = 0.15, CI = 0.06–0.57). Male patients with polymorphic PKDL had significantly higher levels of testosterone than healthy controls, 5.14 (3.11–7.54) versus 2.87 (1.81–4.26) ng/ml (P < 0.05; Fig. 1f) but this was not evident in macular PKDL, being comparable with healthy controls, 3.72 (0.83–5.55) versus 2.87 (1.81–4.26) ng/ml (Fig. 1f). In female patients with PKDL, irrespective of the lesion, their levels of testosterone were comparable with healthy controls (Fig. 1g).

Conclusions

In view of the increased levels of testosterone in PKDL, it may be envisaged that testosterone via its ability to enhance the anti-inflammatory milieu, supported parasite persistence. This is the first study where a sex predisposition has been delineated in Indian PKDL and holds special significance in view of the World Health Organization mediated initiative of elimination of leishmaniasis targeted for 2015,55 wherein patients with PKDL are the strongest contenders for being the proposed reservoir for VL.46 We propose that male patients with VL, particularly postpubertal should be monitored with greater care, as they have a higher propensity towards developing PKDL. Additionally, this study also endorsed that immunomodulatory therapy such as miltefosine is critical for parasite elimination.46 55

Taken together, our study indicates that immune-endocrine interactions play a critical role in disease progression.

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References

A Defective Oxidative Burst and Impaired Antigen Presentation are Hallmarks of Human Visceral Leishmaniasis

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Abstract

Purpose Survival of the Leishmania parasite within monocytes hinges on its ability to effectively nullify their microbiocidal effector mechanisms. Accordingly, this study aimed to delineate this biological niche in patients with visceral leishmaniasis (VL).

Methods In monocytes, the redox status, antigen presenting capacity, expression of Toll-like receptors (TLRs), costimulatory molecules (CD80/86) and generation of intracellular cytokines (IL-8, IL-1β, IL-10 and LAP-TGF-β1) was measured by flow cytometry, levels of circulating cytokines (IL-1β, IL-6, TNF-α, IL-8, IL-4, IL-13, IL-10 and GM-CSF) by ELISA and arginase activity by spectrophotometry.

Results Within monocytes, generation of an oxidative burst was markedly attenuated as evident by decreased generation of nitric oxide and reactive oxygen species, concomitant with raised levels of thiols. This was accompanied by lowered frequency of TLR4+ monocytes, but the arginase activity remained unaltered. Pathogen persistence was enhanced by the predominance of anti-inflammatory cytokines within monocytes, notably IL-10. Alongside, development of adaptive immunity was severely attenuated as manifested by a pronounced impairment of antigen presentation and costimulation evident by down regulation of CD54, HLA-DR and CD86. Treatment corrected the redox imbalance and reversed the impaired antigen presentation.

Conclusions In VL, monocyte functions were severely impaired facilitating parasite persistence; anti-leishmanial chemotherapy mediated parasite elimination through modulation of the macrophage microenvironment by restoring its redox status and antigen presenting capacity.

Keywords Antigen presentation · arginase · co-stimulation · Leishmania donovani · nitric oxide · toll-like receptors · visceral Leishmaniasis

Introduction

Leishmaniasis constitutes a broad spectrum of diseases caused by Leishmania, a hemoflagellate protozoan parasite that depending on the infecting species and host immune status manifests in three divergent forms namely Cutaneous (CL), Mucocutaneous (MCL) and Visceral Leishmaniasis (VL) along with Post Kala-azar Dermal Leishmaniasis (PKDL), a sequel of VL. The visceral form is a major health concern being present in 66 countries and is potentially fatal with 58,227 cases annually reported worldwide and 202,200 to 389,100 people at risk [1]. This digenetic, obligate intracellular parasite resides as flagellated promastigotes within the sandfly vector and transforms into aflagellated amastigotes within mammalian mononuclear host phagocytes.

Innate effector cells are inherently programmed to destroy ingested prey and promote development of adaptive immnunity. Therefore for survival, it is mandatory that Leishmania establish strategies to evade the immune response and tilt the functional status of macrophages to sustain parasite persistence [2]. Accordingly, the robust parasite has developed diverse strategies and elegantly performs a well coordinated orchestra which directly or indirectly modulates the host-parasite interactions. The direct component involves attenuation of the oxidative burst within macrophages, thus
weakening its microbicidal armamentarium by preventing induction of pro-inflammatory molecules such as nitric oxide (NO), superoxide, HOCl etc. [3]. Collectively, the resultant redox imbalance increases the vulnerability of macrophages to intracellular pathogens and disease progression is achieved. However, the limited availability of circulating monocytes precludes detailed analysis of the redox status and available information is mainly from animal models of VL, that are not necessarily extraplatable.

The indirect survival strategies include suppression of cell mediated immunity wherein the cardinal features are a mixed Th1/Th2 response and impaired antigen specific lymphoproliferative responses [4, 5]. To allow parasites to multiply, a key player is the immunoregulatory cytokine IL-10 which by its ability to induce macrophage inhibitory factor renders macrophages to become unresponsive to activation signals leading to decreased generation of NO and TNF-α thereby allowing parasite survival [6].

Studies pertaining to human VL to date have primarily focused on immunopathogenesis with regard to T cell functions or the early interaction of *Leishmania* with host cells. In contrast, studies regarding monocyte functions, the primary host cell, remains limited, possibly due to the paucity of clinical material. This study aimed to address this lacuna and identify immunological biomarkers of innate and adaptive immunity in human VL, to allow for future development of immunomodulatory strategies targeting host macrophages.

**Methods**

**Reagents**

All immunological reagents were purchased from BD Biosciences (San Jose, CA, USA) except anti-CD54 Allophycocyanin (APC), anti-CD40 PE and ELISA kits for IL-4, IL-6, IL-8, IL-10, IL-13 and TNF-α (Immunotools, Friesoythe, Germany); GM-CSF ELISA kit, anti human IL-8 APC, IL-1β Alexa-fluor647, IL-10 APC, Latency-associated peptide-TGF-β1-APC or LAP-TGF-β1-APC and anti-CD14 Fluorescein Isothiocyanate (FITC) (BioLegend, San Diego, USA); anti-CD16 APC (eBioscience, San Diego, CA, USA); N-1 napthyl ethylenediamine dihydrochloride (NED, Loba Chemie Pvt. Ltd., Mumbai, India); sulphanilamide, trichloroacetic acid (TCA), L-arginine and Urea (Sisco Research Laboratories, Mumbai, India); 4,5-diaminofluorescein diacetate (DAF-2DA) (Cayman Chemical Company Ann Arbor, MI, USA); 2′,7′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) (Roche Applied Science, Penzberg, Germany); rk39 immunochromatographic test strips (In Bios International, Seattle, WA, USA); Isonitrosopropiophenone (Sigma Aldrich, St. Louis, MO, USA), polystyrene coated maxisorp strips (Nunc Immunomodules, Roskilde, Denmark); 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CMH2DCFDA) and 5-chloromethylfluorescein-diacetate (CMFDA), (Molecular Probes, Carlsbad, CA, USA); Monocyte separation medium HiSep 1073 (Himedia, Mumbai, India), the Quantikine Immunoassay kit for IL-1β (R&D systems, MN, USA); DNA PCR kit (Qiagen, Hilden, Germany).

**Study Population**

Patients clinically diagnosed with VL (*n*=27) were admitted to the Department of Tropical Medicine, School of Tropical Medicine, Kolkata. A diagnosis of VL was made based on clinical features such as fever and hepatosplenomegaly, which was corroborated by rk39 strip test and/or Internal Transcribed Spacer sequence 1 (ITS1) PCR [7]. Heparinized blood was collected from patients with VL, at presentation and on completion of treatment along with age and sex matched healthy volunteers from a non endemic zone for VL (*n*=10, rk39 and ITS1 negative). Treatment comprised either a single dose of liposomal Amphotericin B (7.5 mg/kg b.w., i.v.) followed by Miltefosine (2.5 mg/kg b.w., p.o. for 2 weeks) or only Amphotericin B (1 mg/kg b.w., i.v. for 20 days). At the end of treatment, clinical and parasitological cure was confirmed by ITS1 PCR, 6 months later, the absence of parasites was confirmed by ITS1 PCR. The study was approved by the Institutional Ethical Committee of School of Tropical Medicine, Kolkata and Institute of Post Graduate Medical Education and Research, Kolkata and informed consent was obtained from the patient or legally accepted representative.

**Isolation of Peripheral Blood Mononuclear Cells (PBMC)**

Peripheral blood (diluted 1:1 with phosphate buffered saline, PBS, 20 mM, pH 7.4) was layered over HiSep 1073 and centrifuged (400 g×30 min). The PBMC-rich interface was collected, washed twice with PBS and resuspended in PBS; cell viability was confirmed using trypan blue (>95%).

**Measurement of Cellular Oxidant Status**

Intracellular NO was measured in monocytes (5×10⁶) as previously described using DAF-2DA (2 μM), a non-fluorescent dye that fluoresces on reaction with NO [8]. This concentration of DAF-2DA selectively measures levels of NO in monocytes, as for measurement of levels of NO in parasites, a 20 fold higher concentration of probe is necessary [8]. Plasma nitrite, a stable radical generated from NO and representative of secreted NO was determined by a modified Griess assay [9] while
plasma arginase activity was measured in plasma as previously described [9].

To monitor the levels of reactive oxygen species (ROS) irrespective of their site of generation, PBMCs (5 × 10^5) were stained with CMH2DCFDA (5 μM, 30 min, 37 °C) and fluorescence of CMDCF acquired on a flow cytometer [10].

To measure the levels of non-protein thiols, PBMCs (5 × 10^5) were initially incubated at 37 °C for 30 min in an ATP depletion buffer (21 mM HEPES buffer, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH2PO4 and 20 mM sodium azide, 0.5 ml); cells were then washed with PBS, stained with CMFDA (50 nM, 15 min, 37 °C) and fluorescence of CMF was acquired on a flow cytometer [10].

Status of TLR2⁺ and TLR4⁺ Monocytes

PBMCs (5 × 10^5) were washed (400 g × 5 min) with PBS +2 % FBS and permeabilized with cytofix-cytoperm buffer (100 μl, 20 min at room temperature), washed with perm-wash buffer (100 μl), fixed and stained with a custom designed antibody cocktail containing anti human Peridinin Chlorophyll Protein (PerCP) labeled CD14, TLR2 PE and TLR4 FITC (BD Biosciences, Cat No. 622701 and 623128) for 15 min in the dark at room temperature. Cells were washed twice with perm-wash buffer and monocytes were gated initially on the basis of their morphology and subsequently based on CD14 positivity in a flow cytometer.

Immunophenotyping of Surface Markers on Monocytes

PBMCs (5 × 10^5) after being washed with PBS were stained with anti human CD40 PE, CD54 APC, HLA-DR PE, CD14 FITC, CD16 APC, CD14 PerCP, CD80 PE or CD86 FITC (15 min, room temperature); cells were washed twice with PBS and fluorescence was acquired on a flow cytometer.

Measurement of Cytokines

Levels of circulating cytokines (IL-1β, GM-CSF, IL-4, IL-6, IL-8, IL-10, IL-13 and TNF-α) were measured in plasma of patients with VL and healthy controls by sandwich ELISA according to manufacturer’s instructions. Intracellular cytokines were detected in PBMCs as previously described with some modifications [11]. Briefly, PBMCs (1 × 10^6/ml) were initially harvested, resuspended in PBS-FBS (150 μl) and incubated with anti human CD14-FITC for 15 min at room temperature. Cells were then fixed and permeabilized in Cytofix-Cytoperm buffer (100 μl) at room temperature for 20 min, after which fluorochrome-conjugated anti cytokine antibodies and their respective isotypes were added and incubated for 15 min; cells were washed and acquired on a flow cytometer.

Flow Cytometry

Cells (5 × 10^5 or 1 × 10^6) from different experimental groups were monitored for their cell-surface or intracellular fluorescence on a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA) equipped with an argon-ion laser tuned to 488 nm and red-diode laser tuned to 635 nm. Fluorescence of the triazolic product of DAF (DAF-2T) or CMDCF or CMF or FITC was collected in the FL1 channel, equipped with a 530/30 nm band pass filter, PE in the FL2 channel having a 585/42 nm band pass filter, PerCP in the FL3 channel having 670 nm long pass filter and APC or Alexa fluor647 in the FL4 channel having 661/16 nm band pass filter. Fluorescence was measured in the log mode using CellQuest Pro software (BD Biosciences, San Jose, CA, USA) and expressed as geometrical mean fluorescence channel, i.e. average or central tendency of fluorescence of analyzed particles (GMFC) for probes and % positivity for receptors or cytokine frequency. For acquisition and analysis, 3000 monocytes were gated on the basis of their characteristic linear side vs. forward scatter and then on CD14 positivity in a flow cytometer.

Statistical Analysis

Data were analyzed between groups by Kruskal-Wallis test followed by Dunn’s multiple comparison tests (for > two group of patients) or Mann Whitney tests (for two group of patients) for non-parametric data for analysis of variance. All data are expressed as mean ± SD; additionally age and circulatory cytokines were also expressed as median and inter-quartile range. Correlation was done using Spearman rank correlation test for non-parametric data and data were analyzed using Graph Pad Prism software (version 5.0, GraphPad software Inc., La Jolla, CA, USA), p<0.05 being significant.

Results

Study Population

Among patients with VL, 16 of 27 were male (Table 1). All patients were either rk39 positive and/or ITS1 PCR positive at diagnosis; at the end of treatment, they were clinically and parasitologically cured and remained ITS1 PCR negative at 6 months. Fever (mean duration being 4.05 months) was present along with hepatosplenomegaly, the mean liver and spleen size being 3.04 and 10.18 cm respectively and was accompanied with a significant degree of anemia and leukopenia (Table 1). Owing to the characteristic leukopenia in VL, 10 ml blood generally yielded 5 × 10^6 cells; as 5 × 10^5 – 1 × 10^6
cells were required per analysis, all markers could not be evaluated in each patient. In terms of geographic distribution, they hailed mainly from Bihar (n=17) followed by West Bengal (n=4), Jharkhand (n=4) and Uttar Pradesh (n=2). The patients from Bihar, in terms of endemicity, were mostly from a meso-endemic region (8/17, 47%) whereas in terms of their responsiveness to antimony, 11/17 (65%) were from an antimony resistant zone [12], justifying the usage of Amphotericin B or a combination of Amphotericin B and Miltefosine.

Impairment of the Oxidant Status in Monocytes of Patients with VL

Nitric oxide (NO) is the principal antimicrobial molecule generated within monocytes from L-arginine via the enzyme, inducible nitric oxide synthase (iNOS). In Leishmaniasis, parasite survival necessitates evasion of its antimicrobial functions and intracellular levels of NO in monocytes of patients with VL were significantly decreased as compared to healthy individuals, GMFC being 74.34±70.91 vs. 306.30±160.20 (p<0.001, Fig. 1a); with treatment, levels reverted to normal, GMFC being 356.70±50.67 (p<0.001, Fig. 1a). Plasma nitrite levels showed no alterations, levels at presentation being comparable with healthy individuals (6.78±3.34 vs. 4.50±2.22 μM); anti-leishmanial chemotherapy effected no changes (6.54±6.34 μM).

Reactive oxygen species (ROS) also contribute to the antimicrobial function of monocytes and includes all reactive radicals generated as a byproduct of metabolism of oxygen [13]. In patients with VL, the levels of ROS were significantly dampened as compared to healthy controls, GMFC being 687.60±266.10 vs. 2563.00±794.90 (p<0.001, Fig. 1b).

Treatment enhanced generation of ROS but levels did not revert to baseline (1256.00±719.90, Fig. 1b).

Among non-protein thiols, glutathione (GSH) in its reduced form (γ-L-glutamyl-L-cysteinyl-glycine) is considered as the principal cellular antioxidant by virtue of its free radical scavenging activity and reduction of peroxides generally in conjunction with glutathione-S-transferase (GST) and glutathione peroxidase [14]. In patients with VL, levels of thiols increased 2.2 fold as compared to healthy controls, GMFC being 2280.00±1373.00 vs. 1007.00±468.80 respectively (Fig. 1c); treatment caused a significant reduction in GMFC as compared to levels at presentation (515.00±337.50, p<0.05; Fig. 1c). At disease presentation, the down regulation of ROS negatively correlated with upregulation of GSH within monocytes (r=−0.67), collective evidence of an overall anti-oxidant milieu.

In VL, though the redox status was skewed towards an anti-oxidant milieu (Fig. 1a–c), frequency of the pro-inflammatory CD14+CD16+ subset of monocytes remained comparable with healthy controls (30.19±15.87 vs. 28.81±20.84 %); treatment too effected no change (19.67±10.90 %). Additionally, there was no alteration in the frequency of anti-inflammatory CD14+CD16− subset of monocytes as compared to healthy controls at presentation (27.42±22.42 vs. 40.05±32.62 %) and upon completion of treatment (47.88±10.37 %).

Lowered Levels of Intracellular NO were Not Associated with Increased Arginase Activity

As generation of NO within monocytes occurs via iNOS whose substrate is L-arginine, the observed lowering of intramonocytic level of NO is attributable to either a decrease in iNOS or/and an enhanced arginase activity [15]. We observed no alterations in arginase activity as compared to healthy individuals (456.30±156.20 vs. 365.80±88.75 IU/L); chemotherapy too caused no changes (465.60±131.90 IU/L).

Lowered Frequency of TLR2+ and TLR4+ Monocytes was Associated with Reduced Production of NO

As the generation of NO occurs downstream to activation of TLRs, we studied the frequency of TLR2+ and TLR4+ in CD14+ monocytes, wherein both were significantly down

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### Table 1  Study population

<table>
<thead>
<tr>
<th>Features</th>
<th>Healthy controls (n=10, non-endemic for VL)</th>
<th>Patients with VL (n=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Age, years, Median (inter-quartile range)</em></td>
<td>30.36±7.03 (25.75–34.00)</td>
<td>38.45±16.65 (37.50, 23.50–52.25)</td>
</tr>
<tr>
<td>Male: Female</td>
<td>3:2</td>
<td>3:2</td>
</tr>
<tr>
<td><em>Duration of fever, months</em></td>
<td>NA</td>
<td>4.05±5.36</td>
</tr>
<tr>
<td><em>WBC count, 10⁶ cells/μl</em></td>
<td>7.15±1.45</td>
<td>2.98±1.51</td>
</tr>
<tr>
<td><em>Hb level, g/dL</em></td>
<td>13.07±1.30</td>
<td>7.84±1.41</td>
</tr>
<tr>
<td><em>Size of spleen, cm</em> (NA)</td>
<td>10.18±6.66</td>
<td>10.18±6.66</td>
</tr>
<tr>
<td><em>Size of liver, cm</em> (NA)</td>
<td>3.04±1.18</td>
<td>3.04±1.18</td>
</tr>
</tbody>
</table>

*a Values are mean ± SD, *p < 0.01 and **p < 0.001 significantly different from healthy controls. NA: Not applicable.*
regulated at disease presentation (35.77±25.58 vs. 74.55±9.45 %, \( p<0.05 \) and 19.18±13.72 vs. 41.99±12.05 %, \( p<0.05 \); Fig. 2a–c respectively). However, after treatment the frequency of CD14\(^+\) monocytes expressing TLR2 and TLR4 was marginally upregulated, being 58.81±21.13 and 40.62±13.04 % respectively (Fig. 2a and c). The lowered frequency of CD14\(^+\)TLR4\(^+\) monocytes correlated with a reduced fluorescence of the 4,5-triazolic product of diaminofluorescein, DAF-2T, representative of NO (\( r=0.78 \), Fig. 2d).

**Fig. 1** Redox status of monocytes in patients with VL. a. Measurement of intracellular NO in PBMCs (5×10\(^5\)) from controls [1] (n=6), patients with VL at presentation [2] (n=15) and on completion of treatment [3] (n=10). b. Detection of intracellular ROS in PBMCs (5×10\(^5\)) from healthy controls [1] (n=6), patients with VL at presentation [2] (n=11) and after treatment [3] (n=7). *p<0.001 significantly different from healthy controls, †p<0.001 significantly different from patients with VL at presentation.

Impaired Antigen Presentation by Monocytes of Patients with VL

Monocytes along with dendritic cells and B cells are major antigen presenting cells whose interaction with T-cells dictates the activation of T-cell mediated immune responses. CD54 is an adhesion molecule on monocytes necessary for interaction with T-cells for antigen presentation and their frequency was significantly lowered at disease presentation (70.53±14.52 vs. 90.52±8.30 %, \( p<0.01 \); Fig. 3a and b); treatment significantly restored the frequency of CD54 (89.47±6.47 %, \( p<0.001 \); Fig. 3a and b).

Similarly, the population of CD54\(^+\)HLA-DR\(^+\) monocytes was significantly down regulated at disease presentation as compared to healthy controls (57.22±14.52 vs. 86.14±10.19 %, \( p<0.01 \); Fig. 3a and c) and was effectively restored upon treatment (83.49±7.12 %, \( p<0.01 \); Fig. 3a and c). Concomitantly, the frequency of HLA-DR\(^+\) monocytes too was significantly decreased during active disease (65.83±14.75 vs. 87.09±9.02 %, \( p<0.05 \); Fig. 3a and d) and reverted to baseline values with treatment (81.47±16.67 %).

Status of Co-Stimulatory Molecules on Monocytes of Patients with VL

Co-stimulatory molecules are pivotal secondary signals during antigen presentation essential for strengthening interaction with T-cells [16]. In CD14\(^+\) monocytes of patients with VL, a significant up regulation of CD80\(^+\) monocytes was evident as compared to healthy controls (6.58±8.60 vs. 0.93±1.37 %, \( p<0.05 \); Fig. 4a and c), which decreased slightly with chemotherapy (4.63±3.59 %, Fig. 4a). However, the frequency of CD86 was diametrically opposite being significantly downregulated as compared to healthy controls (21.69±13.10 vs. 57.49±10.12 %, \( p<0.01 \); Fig. 4b and c); post-treatment showed a moderate increase (36.68±19.80 %, Fig. 4b). The frequency of CD40\(^+\) monocytes, another important co-stimulatory molecule remained unaltered (data not shown).

Patients with VL Showed a Mixed Cytokine Response

Intracellular microbes shape the macrophage phenotype by modulating the cytokine response which in turn influence
downstream host responses. Accordingly, we measured circulating levels of cytokines which regulate activation of monocytes and/or are produced by monocytes, namely four pro-inflammatory cytokines (IL-6, IL-1β, IL-8 and TNF-α), three anti-inflammatory cytokines (IL-4, IL-10 and IL-13) along with GM-CSF. Among the pro-inflammatory cytokines, levels of IL-6 and TNF-α were comparable with healthy controls while IL-1β was not detectable in both groups (Table 2). However, IL-8, a chemotactic factor for neutrophils, was significantly enhanced in active disease as compared to healthy controls (256.10±290.50 vs. 32.01±32.43 pg/ml, p<0.01, Table 2) but remained unchanged with treatment (225.80±161.30 pg/ml, Table 2).

Amongst the anti-inflammatory cytokines, patients with VL during active disease showed a 2.87 fold higher level of IL-4 as compared to healthy controls (201.90±79.62 vs. 70.41±19.24 pg/ml, p<0.01, Table 2) which remained unaltered with treatment (181.10±134.30 pg/ml, Table 2). The levels of IL-10 dramatically increased by 18.77 fold as compared to healthy controls (48.06±17.97 vs. 2.56±2.45 pg/ml, p<0.001, Table 2); here again, treatment effected minimal change (27.33±22.84 pg/ml, Table 2). Similarly, IL-13 showed a significant 6.06 fold increase at disease presentation (407.70±368.30 vs. 67.31±19.24 pg/ml, p<0.001, Table 2) which decreased 3.20 fold with treatment (127.20±90.01 pg/ml, Table 2). Taken together, at active disease, there is a pronounced upregulation of IL-4, IL-10 and IL-13, evidence of an enhanced anti-inflammatory response; levels of GM-CSF remained unchanged (Table 2).

Intramonocytic Status of Cytokines in Patients with VL

In circulation, a mixed Th1/Th2 profile with Th2 bias occurs in human VL but the contribution of monocytes has not been studied. Accordingly, we aimed to evaluate the dynamic events of cytokine production that reflects the in vivo baseline immune responses in human VL, and therefore investigated the intracellular cytokine profiles specifically in the absence of any exogenous stimulus. Pro-inflammatory (IL-1β and IL-8) and anti-inflammatory (IL-10 and LAP-TGF-β1) cytokines were studied, as their primary source was monocytes. Among CD14+ monocytes, decreased frequency of IL-1β+ and IL-8+ cells were evident as compared to healthy controls being...
32.45±10.98 vs. 69.08±10.34 %, p<0.05 and 14.77±8.30 vs. 63.46±14.63 %, p<0.05 respectively (Fig. 5a and b). Treatment caused no changes being 48.14±9.94 % for IL-1β and 24.92±10.86 % for IL-8 (Fig. 5a and b).

The number of IL-10+ cells among CD14+ monocytes was 2.98 fold higher in active VL as compared to controls (9.79±7.32 vs. 3.28±1.86 %, p<0.05, Fig. 5a and c). TGF-β1 was measured in terms of LAP-TGF-β1, an inactive form of TGF-β1 [17] and showed a significantly lowering at disease presentation as compared to controls (23.54±9.37 vs. 42.09±10.03 %, p<0.05, Fig. 5a and c), implying levels of active TGF-β1 may be raised. In lymphocytes, there was no change in the frequency of LAP-TGF-β1+ as compared to healthy controls (37.82±7.46 vs. 23.45±7.77 %). Post-treatment, no changes were evident in the intramonicytic frequencies of IL-10 or LAP-TGF-β1 being 15.24±15.94 and 23.61±8.39 % respectively (Fig. 5a and c).

Discussion

Intracellular pathogens like Leishmania have evolved strategies to reside within the hostile environment of monocytes, conventional key regulators of the innate immune response, courtesy their ability to generate an oxidative burst and present antigens to T helper cells [18, 19]. In experimental models of VL, a differential regulation of macrophage activation has been reported wherein raised IL-10 promotes parasite survival and facilitates disease progression, while enhancement of IL-12p40 caused parasite elimination [19]. Accordingly, the study of effector functions of monocytes in a clinical setting is necessary which this study aimed to address.

A major weapon in the macrophage antimicrobial arsenal is the phagocytotic oxidative burst which the Leishmania parasite attenuated as evident by the lowered intracellular levels of the principal microbicidal molecule NO and gets reversed by...
Miltefosine and Amphotericin B (Fig. 1a), corroborating previous reports [8]. However, the plasma nitrite levels failed to reflect the intramonocytic scenario, possibly because circulating levels of nitrite can be contributed by multiple sources [3].

In monocytes-macrophages, levels of NO are regulated by a critical interplay between iNOS and arginase who compete for the substrate L-arginine, the former through an enhanced presence of pro-inflammatory cytokines IFN-γ and TNF-α causing increased availability of NO. However, the latter via an enhanced Th2 response promotes parasite persistence via reduced synthesis of NO and additionally, facilitates synthesis of critical polyamines essential for parasite growth [15].

![Figure 4](image)

**Fig. 4** Frequency of CD14+ monocytes expressing co-stimulatory molecules CD80 and CD86. a. Representative profile of % of CD14+CD80+ monocytes from a healthy control, a patient with VL at presentation and on completion of treatment with corresponding isotypes. b. Representative profile of % of CD14+CD86+ monocytes from a healthy control, a patient with VL at presentation and on completion of treatment with corresponding isotypes. c. The % of CD14+ monocytes expressing CD80 and CD86 in healthy controls [●] (n=6), patients with VL at presentation [□] (n=13) and on completion of treatment [▪] (n=9). *p<0.05 and **p<0.01, significantly different from healthy controls.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Healthy controls (n=10)</th>
<th>VL (Presentation) (n=12)</th>
<th>VL (Post treatment) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (pg/ml)</td>
<td>Mean ± SD (pg/ml)</td>
<td>Mean ± SD (pg/ml)</td>
</tr>
<tr>
<td>IL-6</td>
<td>50.02±15.72 (50.63, 40.82–64.88)</td>
<td>58.44±14.18 (47.35, 24.08–89.80)</td>
<td>52.40±37.39 (36.80, 24.10–78.19)</td>
</tr>
<tr>
<td>IL-8</td>
<td>32.01±32.43 (31.81, 5.26–33.98)</td>
<td>256.10±290.50 **(165.60, 61.62–273.40)</td>
<td>225.80±161.30 *(181.60, 100.60–395.40)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>114.00±151.60 (75.63, 0.0–149.80)</td>
<td>292.60±346.50 (203.50, 63.54–386.40)</td>
<td>228.70±250.00 (97.21, 53.48–389.10)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>4.89±2.02 (5.82, 2.75–6.48)</td>
<td>6.57±4.19 (5.69, 3.24–9.56)</td>
<td>7.85±6.60 (6.13, 2.87–14.57)</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.56±4.25 (4.25, 0.00–4.91)</td>
<td>48.06±17.97 *** (48.95, 31.64–61.49)</td>
<td>27.33±22.84 *(25.20, 9.45–36.70)</td>
</tr>
<tr>
<td>IL-4</td>
<td>70.42±2.16 (72.19, 43.70–107.80)</td>
<td>201.90±79.62 **(222.30, 130.20–258.30)</td>
<td>181.10±134.10 (188.70, 56.00–298.60)</td>
</tr>
<tr>
<td>IL-13</td>
<td>67.31±19.24 (67.51, 51.73–88.02)</td>
<td>407.70±368.30 *** (210.70, 162.60–553.10)</td>
<td>127.20±90.01 (123.50, 48.55–182.80)</td>
</tr>
</tbody>
</table>

Levels of plasma cytokines were measured in healthy controls along with patients with VL at presentation and after completion of treatment as described in Materials and methods. Values are expressed as mean ± SD; Median and inter-quartile ranges are given in parentheses. *p<0.05, **p<0.01, ***p<0.001, significantly different from healthy controls; †p<0.05 significantly different from disease presentation. ND: Not detectable.
the reduction in intracellular NO (Fig. 1a) can be attributed to a decreased expression of iNOS and/or increased activity of arginase, establishing the status of these immunomodulatory enzymes was necessary. In experimental CL and VL as also in patients with PKDL, increased arginase activity has been reported [9, 20–22] but differed from Indian VL where activity was unaltered. This unchanged activity of arginase tilted the axis towards decreased expression of iNOS playing a predominant role. However, detection of iNOS has logistic limitations being non-detectable in human macrophages [23] and consequently it’s lowering, if any, in VL was not measurable.

In Leishmaniasis, Toll like receptors, TLR2 and TLR4 hold special importance as the parasite interacts with these receptors [24] and as they are established regulators of iNOS, their down regulation could potentially translate into decreased generation of NO. Indeed, this was corroborated by the significant decrease in percentage of monocytes expressing TLR2/4 (Fig. 2a–c). Furthermore, the strong positive correlation between TLR4 and intracellular NO (Fig. 2d) endorsed its role [25, 26]. Treatment caused a modest increase in TLR2/4 suggesting that their reappearance occurs later, in concordance with other studies [26].

In experimental VL, the parasite’s ability to attenuate generation of ROS within monocytes-macrophages is well established [18, 27]. In human VL, this deactivated state of monocytes has been indirectly evaluated in vitro in terms of decreased activity of NADH oxidase, myeloperoxidase and NADPH oxidase etc. [28, 29]. However, this study measured the intracellular status of ROS exclusively within monocytes (Fig. 1b) and as there was no external stimulus, it provided a more accurate representation of the in vivo scenario. Treatment raised levels of ROS but not as prominently as NO, suggesting the latter’s contribution was possibly greater.

Another contributor towards generation of an anti-inflammatory milieu is GSH, as by reacting with...
hydroperoxides, it protects cells from oxidative damage. In Leishmaniasis, the status of GSH is restricted to studies on *Leishmania* parasites wherein antileishmanial drugs like Miltefosine depleted GSH causing a pro-oxidant, parasiticidal effect [30]. At disease presentation, the raised levels of GSH (Fig. 1c) was evidence of the parasites successful attenuation of the oxidative burst corroborated by its inverse correlation with levels of ROS (r = −0.67). Treatment resulted in a substantial decrease in levels of thiols that corroborated with in vitro studies [30]. Although the proportions of pro-(CD14^−CD16^+ ) and anti-inflammatory monocytes (CD14^+CD16^−) were unaltered in VL, it would be worthwhile to evaluate the redox status in these subsets, to understand their individual contribution towards changes in the macrophage-monocyte milieu and the subsequent control of infection.

The prevention of adaptive immunity is essential for parasite survival and a common approach adopted is downregulation of MHC Class II molecules and antigen presentation, to evade recognition by CD4^+ T-cells. Important molecules expressed on antigen presenting monocytes include CD54 or ICAM-1 (Fig. 3a and b) and TLRs, cell adhesion and co-stimulatory pathways [42]. Additionally, as CD54 is a downstream event following TLR activation [32], its measurable decrease (Fig. 3a and c) reinforced the capability of *Leishmania* parasites to impede macrophage activation. Similarly, the lowered expression of HLA-DR (Fig. 3a and d) akin to studies with *L. chagasi* [33], LCL [34] and Sicilian VL [35] endorsed that in Leishmaniasis, impaired antigen presentation is a consistent feature, irrespective of the geographic region.

Additional signals for T-cell activation include co-stimulatory molecules CD80 (B7.1) and 86 (B7.2) [36]. Although the decrease in CD86^+ monocytes (Fig. 4b and c) corroborated with the associated immunosuppression, the unexpected but significant increase in CD80^− monocytes (Fig. 4a and c) possibly contributed by virtue of their higher affinity towards CTLA-4, the inhibitory ligand on T-cells as reported from CL patients [37]; however, no change in CD80 has been reported in PKDL, LCL and DCL [9, 38].

The conventional strategy of macrophages upon encountering a pathogen includes phagocytosis, followed by formation of the microbicidal phagosolysosome for eventual elimination of the engulfed pathogen. However, the engulfed *Leishmania* parasite thwarts or delays this maturation process by a series of complex interactions with host macrophages. This enhances them to maneuver the immune system towards a Th2 profile primarily by interfering with key macrophage regulatory signaling pathways [4, 39]. Unlike experimental VL, where resistance and susceptibility correlate with distinct patterns of Th1 or Th2 cytokine production respectively, clinical VL shows a mixed Th1-Th2 profile [40, 41] with increased circulating levels of pro-inflammatory (IFN-γ, TNF-α, IL-6, IL-8) and anti-inflammatory (IL-10, IL-4 and IL-13) cytokines [42]. Although reports of raised pro-inflammatory cytokines in circulation have been reported in human VL and CL [42–44], this study indicated that in Indian VL, only levels of circulatory IL-8 were raised (Table 2) which was biologically relevant as it can delay the spontaneous apoptosis of neutrophils [45], allowing them to function as a temporary host for parasites prior to their entry into macrophages [45]. Although, there is severe impairment of crucial host defense mechanisms, VL is usually not associated with severe co-infections, that are expected if both antigen presentation and microbial killing are profoundly impaired. This is probably due to neutrophil functions remaining unchanged which are impaired in malnourished children suffering from VL [46].

In VL, data regarding circulating cytokines shows a wide variation [42, 47] attributable to a geographical etiology or the methodological approaches applied, underlying the importance of studying cytokine responses in the absence of antigen specific stimulation. Possibly, the most precise approach would be evaluating the intramonocytic status of cytokines by consciously excluding the component of antigen stimulation, so as to truly reflect dynamic in vivo events. As frequency of CD14^- monocytes expressing IL-8 and IL-1β were decreased (Fig. 5a and b), it indicated that in human monocytes, the parasite generated an anti-inflammatory milieu. Although levels of GM-CSF, a maturation factor for monocytes correlate with cytopenia in VL [42], we observed no changes (Table 2).

*Following internalization of amastigotes via* phosphatidylserine receptors on monocytes, an enhanced secretion of IL-10 and TGF-β follows which then blocks induction of iNOS resulting in decreased production of NO [48]. A modest increase in intracellular IL-10 within monocytes (Fig. 5a and c) along with a significant decrease in LAP-TGF-β1 (Fig. 5a and c) endorses the role of anti-inflammatory cytokines in suppressing the microbicidal activity of macrophages in VL. This is in agreement with Saha et al., [49] who reported increased levels of TGF-β in Indian VL.

**Conclusion**

Owing to limited availability of monocytes, studies on their functions in VL are limited in the clinical setting, yet rational designing of chemotherapy requires that their functional impairment be delineated. Our study has conclusively established that in human VL, the *Leishmania* parasite has evolved a multipronged approach to cause impairment of monocyte functions, in terms of a lowered pro-oxidant status and decreased frequency of TLRs, cell adhesion and co-stimulatory molecules, along with an enhanced presence of Th2 cytokines, all of which are collectively conducive for the parasite. Anti-leishmanial treatment effectively restored
majority of monocyte functions indicating that these compounds are both parasiticidal and immunomodulatory. Our findings have provided invaluable insight into the microenvironment of host cells which can facilitate development of novel immunomodulatory targeted strategies against Leishmaniasis.

Acknowledgments Financial assistance was provided by Indian Council for Medical Research (ICMR), Council for Scientific & Industrial Research (CSIR) and Department of Science and Technology (DST), Govt. of India. S.R. is recipient of a Senior Research fellowship from CSIR, D.M. and S.G. are recipients of a Senior Research fellowship from ICMR, Govt. of India while S.M. is a recipient of an INSPIRE-Senior Research fellowship from DST, Govt. of India.

Conflict of Interest The authors report no conflict of interest and are responsible for the content and writing of the paper.

References


Evaluation of antileishmanial activity of artemisinin combined with amphotericin B or miltefosine in \textit{Leishmania donovani} promastigotes

Musifikur Rahaman, Susmita Ghosh, Lopamudra Dhar Chowdhury, Mitali Chatterjee*

INTRODUCTION

Leishmaniasis is a vector borne disease caused by the protozoan parasite \textit{Leishmania} sp. mainly manifested as cutaneous, mucocutaneous and visceral leishmaniasis (VL); in addition, a few patients in the Indian subcontinent and East Africa upon recovery from VL develop Post Kala-Azar dermal leishmaniasis. About 350 million people are at risk of developing the disease in 88 countries world wide with India, Bangladesh, Brazil, Nepal, and Sudan accounting for 90\% cases of VL.\textsuperscript{1}

Sodium stibogluconate (SSG) remains the first line treatment against VL all over the world; however, in India, the increasing incidence of unresponsiveness to SSG has limited its use.\textsuperscript{2,3} The newer drugs, include amphotericin B and its liposomal preparations, which though less toxic, is expensive. Miltefosine, initially developed as an anti-cancer drug, is orally effective, but its teratogenicity, low therapeutic index and potential for developing resistance pose limitations.\textsuperscript{4} Taken together, the current treatment options are far from adequate and the need for combination chemotherapy has been tested in the laboratory\textsuperscript{6} and clinical setting with encouraging outcomes where they have proved efficacious and safe, thereby increasing patient compliance and reducing emergence of drug-resistant parasites, important considerations for the treatment of VL in the Indian subcontinent.\textsuperscript{7}

Artemisinin, a sesquiterpene lactone, obtained from plant \textit{Artemisia} sp., is an established anti-malarial drug used mainly for falciparum malaria.\textsuperscript{8} At present, artemisinin based combination therapy involving either artesunate-mefloquine or arteether-lumefantrine is recommended for treatment of uncomplicated falciparum malaria to prevent recrudescence as well as emergence of drug resistance.\textsuperscript{9} Artemisinin has shown significant anti-leishmanial activity in experimental models\textsuperscript{10-12} and considering its effectiveness

ABSTRACT

The increasing incidence of drug resistance in Leishmaniasis necessitates evaluation of combination chemotherapy. Miltefosine and amphotericin B are established anti-leishmanial drugs, while artemisinin has shown significant leishmanicidal activity in experimental models. In this study, we have evaluated the additive/synergistic effect of artemisinin with amphotericin B or miltefosine.

Methods: \textit{Leishmania} parasites were isolated from the bone marrow aspirate of a patient with visceral leishmaniasis. Parasites were typed as \textit{Leishmania donovani} by restriction fragment length polymorphism of internal transcribed spacer 1 region of \textit{Leishmania} genome. Promastigotes were incubated in a fixed ratio combination of artemisinin (0-500 \textmu M) and amphotericin B (0-100 nM) or miltefosine (0-100 \textmu M) and cell viability was assessed. An isobologram was constructed to evaluate the additive/synergistic effect, wherein it was considered additive if the mean sum fractional inhibitory concentration (mean \Sigma FIC) at the IC\textsubscript{50} level was <2, but \geq 1 and synergism, if the mean \Sigma FIC was <1.

Results: The isobologram showed an additive effect for three combinations of artemisinin-amphotericin B and artemisinin-miltefosine, the mean \Sigma FICs ranging from 1.02 to 1.44 and 1.08 to 1.33 along with a synergistic effect with one combination, the mean \Sigma FICs being 0.58 and 0.81 respectively.

Conclusions: This study supports the combination use of artemisinin-amphotericin B and artemisinin-miltefosine, worthy of future pharmacological consideration.

Keywords: Antileishmanial activity, Artemisinin, Amphotericin B, Miltefosine, Promastigotes
METHODS

Reagents

All chemicals were obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA) except phenazine methosulfate (PMS, Sisco Research Laboratories, Mumbai, India), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS), inner salt (Promega, Madison, WI, USA). Artemisinin and amphotericin B were dissolved in dimethyl sulfoxide to prepare 100 mM and 1 mM stock solutions, respectively. Miltefosine was freshly prepared in M199 medium (2 mM).

Parasite isolation and transformation

Leishmania parasites (amastigotes) were isolated from a bone marrow aspirate of a patient with VL. The aspirated material was collected and diluted with Schneider’s insect medium 1:1, supplemented with 20% heat inactivated fetal calf serum, penicillin G (50 IU/mL) and streptomycin (50 µg/ml) in a tissue culture flask (25 cm²). After incubation at 24°C, culture growth was evident after 6 days. After transformation from amastigotes to promastigotes, they were gradually adapted into M199 supplemented with 10% fetal calf serum, penicillin G (50 IU/ml), and streptomycin (50 µg/ml) and subcultured every 72 hrs, inoculum being 1×10^5/ml.

Species specific typing of L. donovani strains

The parasites were typed as L. donovani using restriction fragment length polymorphism (RFLP) of the amplified internal transcribed spacer 1 (ITS1) region. DNA was isolated from L. donovani promastigotes by QIAamp DNA mini kit (Qiagen, Hilden, Germany) and eluted in 200 µl elution buffer. The ITS1 region was amplified using primers LITSR (5’-CTGGATCATTTTCCGATG-3’) and L5.8S (5’-TGATACCACTTATCGCACTT-3’). Amplification reactions were performed in 25 µl reaction volume and PCR conditions followed as previously described. The negative and positive control was distilled water and DNA of a World Health Organization reference L. donovani (MHOM/IN/1980/DD8) strain respectively. The amplified ITS1 region was digested using Hae III (Fermentas, Glen Burnie, MD, USA); briefly, reactions were carried out using 1U of Hae III, 1X buffer, 5 µl of the ampiclon (approximately 100 µg of DNA) and incubated at 37°C for 3 hrs. The digested product was analyzed by electrophoresis (3% agarose, 5 V/cm for 1.5 hrs) along with a GeneRuler™ low range DNA ladder (Fermentas, Glen Burnie, MD, USA) and visualized in G-BOX gel doc (Syngene, Cambridge, UK) using Gene Tools Software (version 4.01.04, La Jolla, CA, USA).

Drug combinations

The dilutions of artemisinin, amphotericin B and miltefosine were prepared in fixed ratios (Tables 1a and 1b), wherein the starting ratio of artemisinin (µM):amphotericin B (nM) was 0:100, 100:80, 200:60, 300:40, 400:20, and 500:0, respectively and for artemisinin (µM):miltefosine (µM) was 0:100, 100:80, 200:60, 300:40, 400:20, and 500:0, respectively (solutions 1-6). The solutions were then serially diluted (Tables 1a and 1b) and cell viability assay was performed.

Evaluation of anti-promastigote activity

The anti-leishmanial activity of artemisinin, miltefosine and amphotericin B was established in promastigotes, cell viability being measured by the MTS assay. Briefly, log phase promastigotes (1×10^10 cells/200 µl/well) were incubated with artemisinin (0-500 µM), amphotericin B (0-100 nM), miltefosine (0-100 µM) alone and in combination (Tables 1a and 1b) for 48 hrs at 24°C. At the end of 48 hrs, 20 µl of a solution comprising MTS (2.0 mg/ml) and PMS (0.92 mg/ml) in a ratio of 5:1 was added per well. The plates were then incubated further for 3 hrs at 37°C and absorbances of the resultant formazan measured at 490 nm using a plate reader (BioRad, California, USA). MTS is converted to formazan by mitochondrial dehydrogenases of viable parasites in presence of an electron coupler PMS. Therefore, the amount of formazan produced and thereby the intensity of color change was considered to be a measure of cell viability. The mean percent viability was calculated as:

Mean specific absorbance of drug treated parasites ×100
Mean specific absorbance of untreated parasites

Specific absorbances were determined by subtracting the background absorbance of medium. For each combination (1-6), its inhibitory concentration 50 (IC₅₀) i.e. the concentration that inhibited 50% cell growth, was enumerated by graphical extrapolation using GraphPad Prism software (version 4.01.04, La Jolla, CA, USA). From the IC₅₀ obtained of each combination, its fractional inhibitory concentration (FIC) was derived using the following formula:

\[
\text{FIC} = \frac{\text{Concentration of drug in combination}}{\text{Concentration of drug alone required to produce IC}_{50}}
\]

Preparation of isobologram

An isobologram was constructed with mean FIC to determine the interactions between drug artemisinin and amphotericin B or artemisinin and miltefosine. The sum FIC (ΣFIC) value

Table 1a: Ratio of artemisinin (µM) and amphotericin B (nM).

<table>
<thead>
<tr>
<th>Combinations</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>Serial dilutions</td>
<td>0:100</td>
<td>100:80</td>
<td>200:60</td>
<td>300:40</td>
<td>400:20</td>
<td>500:0</td>
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<tr>
<td>0:50</td>
<td>50:40</td>
<td>100:30</td>
<td>150:20</td>
<td>200:10</td>
<td>250:0</td>
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<tr>
<td>0:25</td>
<td>25:20</td>
<td>50:15</td>
<td>75:10</td>
<td>100:5</td>
<td>125:0</td>
<td></td>
</tr>
<tr>
<td>0:12.5</td>
<td>12.5:10</td>
<td>25:7.5</td>
<td>37.5:5</td>
<td>50:2.5</td>
<td>62.5:0</td>
<td></td>
</tr>
<tr>
<td>0:6.25</td>
<td>6.25:5</td>
<td>12.5:3.75</td>
<td>18.75:2.5</td>
<td>25:1.25</td>
<td>31:25</td>
<td></td>
</tr>
<tr>
<td>0:3.12</td>
<td>3.12:2.5</td>
<td>6.25:1.87</td>
<td>9.37:1.25</td>
<td>12.5:0.62</td>
<td>15.62</td>
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</table>

Table 1b: Ratio of artemisinin (µM) and miltefosine (µM).

<table>
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<th>Combinations</th>
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<tr>
<td>0:50</td>
<td>50:40</td>
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<td>150:20</td>
<td>200:10</td>
<td>250:0</td>
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<tr>
<td>0:25</td>
<td>25:20</td>
<td>50:15</td>
<td>75:10</td>
<td>100:5</td>
<td>125:0</td>
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<tr>
<td>0:12.5</td>
<td>12.5:10</td>
<td>25:7.5</td>
<td>37.5:5</td>
<td>50:2.5</td>
<td>62.5:0</td>
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<tr>
<td>0:6.25</td>
<td>6.25:5</td>
<td>12.5:3.75</td>
<td>18.75:2.5</td>
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<td>9.37:1.25</td>
<td>12.5:0.62</td>
<td>15.62</td>
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</table>

for each of the six combinations was determined to classify the drug-drug interaction as \( \Sigma \text{FIC} = \text{FIC (artemisinin)} + \text{FIC (amphotericin B)} \) or \( \text{FIC (miltefosine)} \) where \( \Sigma \text{FIC} <1 \) represented synergism, \( \Sigma \text{FIC} \geq 1 \), but <2 represented additive interaction and \( \Sigma \text{FIC} \geq 2 \) indicated antagonism.

RESULTS

The parasite isolate showed a ITS1 PCR-RFLP pattern similar to the reference strain MHOM/IN/80/DD8 and accordingly was typed as *Leishmania donovani*. The IC\(_{50}\) of artemisinin, amphotericin B and miltefosine were 108.2 µM, 18.75 nM and 18.67 µM respectively, corresponding to previous studies.\(^{10,15}\)

Regarding the combination study, two sets of five dose response curves were obtained from each replicate of the combination assay (Tables 1a and 1b) with each set representing four combination solutions and one curve for a drug alone (Figures 1 and 2). Combination dose-response curves were prepared to obtain IC\(_{50}\) of each drug in combination. Extrapolating IC\(_{50}\) of one drug in combination, it has been assumed that the other drug has not contributed to the anti-leishmanial effect and vice versa, and the FIC\(_{50}\) of each combination was accordingly calculated. The mean FIC\(_{50}\) values were plotted for six drug combinations to obtain an isobologram of each combination experiment (Figure 3).

Interaction between artemisinin and amphotericin B

The mean FIC of each combination was calculated at different fixed ratios and the isobologram was constructed. Combination 2 showed synergy (\( \Sigma \text{FIC} <1 \)), while combinations 2, 3 and 4 showed additive interactions (\( \Sigma \text{FIC} <2 \) but \( \geq 1 \)). No antagonistic interaction was found (Table 2a). Figure 3a shows the graphical representation of the interaction.

Interaction between artemisinin and miltefosine

The isobologram prepared from the fixed ratio values showed synergism between the two drugs in combination \( \Sigma \text{FIC} <1 \), whereas combinations 2, 3, 4 showed additive interactions (\( \Sigma \text{FIC} <2 \) but \( \geq 1 \)). No antagonistic interaction was found (Table 2b, Figure 3b).

DISCUSSION

Artemisinin (Chinese-qinghaosu), a sesquiterpene lactone, is an established anti-malarial drug, but has also shown good leishmanicidal activity in experimental models.\(^{11}\) Amphotericin B and miltefosine both are known anti-leishmanial drugs, but have dose limiting toxicity; more over miltefosine is susceptible to rapid development of resistance owing to its long half-life.\(^{4}\)

Artemisinin and miltefosine have been reported to generate free radicals within the *Leishmania* parasites, which possibly contributes toward its leishmanicidal activity.\(^{10,11,19}\) Amphotericin B inhibits ergosterol biosynthesis; *Leishmania* parasites possess a high ergosterol content and intracellular accumulation through aqueous pores causes their cell lysis.\(^{4}\)

Artemisinin has a shorter duration of action when compared to miltefosine or amphotericin B, so the combination may be valuable in ameliorating emergence of drug resistance as recommended in treatment of falciparum malaria.

Taken together, this study supports the combinational use of artemisinin-amphotericin B or artemisinin-miltefosine
Table 2a: Interaction between artemisinin and amphotericin B against *L. donovani* promastigotes.

<table>
<thead>
<tr>
<th>Combination</th>
<th>*Mean FIC50±SD</th>
<th>Mean ΣFIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisinin</td>
<td>Amphotericin B</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0 ± 0.038</td>
<td>1.023</td>
</tr>
<tr>
<td>2</td>
<td>0.147 ± 0.012</td>
<td>1.293</td>
</tr>
<tr>
<td>3</td>
<td>0.332 ± 0.017</td>
<td>1.110</td>
</tr>
<tr>
<td>4</td>
<td>0.563 ± 0.025</td>
<td>0.873</td>
</tr>
<tr>
<td>5</td>
<td>0.374 ± 0.004</td>
<td>2.075</td>
</tr>
<tr>
<td>6</td>
<td>1.023 ± 0.034</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Promastigotes (1×10^5/200 µl/well) were incubated with artemisinin in combination with amphotericin B as described in materials and methods. Each value is the mean of at least three experiments in duplicate. SD: Standard deviation, FIC: Fractional inhibitory concentration

Table 2b: Interaction between artemisinin and miltefosine against *L. donovani* promastigotes.

<table>
<thead>
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<th>Combination</th>
<th>*Mean FIC50±SD</th>
<th>Mean ΣFIC</th>
</tr>
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<tbody>
<tr>
<td>Artemisinin</td>
<td>Miltefosine</td>
<td></td>
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<td>1</td>
<td>0.0 ± 0.053</td>
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</tr>
<tr>
<td>2</td>
<td>0.197 ± 0.021</td>
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<tr>
<td>3</td>
<td>0.389 ± 0.048</td>
<td>0.687</td>
</tr>
<tr>
<td>4</td>
<td>0.757 ± 0.038</td>
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<tr>
<td>5</td>
<td>0.631 ± 0.025</td>
<td>0.183</td>
</tr>
<tr>
<td>6</td>
<td>1.001 ± 0.036</td>
<td>0.0</td>
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</table>

*Promastigotes (1×10^5/200 µl/well) were incubated with artemisinin in combination with miltefosine as described in Materials and methods. Each value is the mean of at least three experiments in duplicate. SD: Standard deviation, FIC: Fractional inhibitory concentration

**Figure 1:** Drug concentration-response curves of artemisinin (a) and amphotericin B (b) in different combinations of artemisinin-amphotericin B (each point represents the mean of at least three experiments in duplicate).

**Figure 2:** Drug concentration-response curves of artemisinin (a) and miltefosine (b) in different combinations of artemisinin-miltefosine (each point represents the mean of at least three experiments in duplicate).

**Figure 3:** Isobolograms showing interaction between artemisinin-amphotericin B (a) and artemisinin-miltefosine (b). The straight line joins both axes at combination one and six of the mean fractional inhibitory concentration values.

in VL, and understandably should be validated in vivo. These combinations may have special relevance in the Indian subcontinent, where drug resistance is a significant problem and the therapeutic armamentarium to date is limited.

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6. Seifert K, Croft SL. In vitro and in vivo interactions between

Coinfection of Leptomonas seymouri and Leishmania donovani in Indian Leishmaniasis

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Leishmania donovani is considered the causative organism of visceral leishmaniasis (VL) and post-kala-azar dermal leishmaniasis (PKDL). Testing of 4/29 DNA samples from VL and PKDL patients as well as 2/7 field isolates showed an aberrant internal transcribed spacer 1 (ITS1) restriction fragment length polymorphism (RFLP) pattern, which upon sequencing strongly matched Leptomonas seymouri, thus confirming its presence in Indian leishmaniasis.

Visceral leishmaniasis (VL) is a vector-borne disease caused by replication of parasites of the Leishmania donovani complex (L. donovani and L. infantum) within the macrophage-phagocytic system. In the Indian subcontinent and parts of Africa, its transmission is anthropogenic (2), with post-kala-azar dermal leishmaniasis (PKDL) being a sequel of VL, and is characterized by a macular, maculo-papular, or nodular rash (11).

Generally, in the Indian subcontinent, patients presenting with clinical features suggestive of VL/PKDL have the diagnosis confirmed by the presence of parasites in Giemsa-stained smears and/or culture positivity, serological diagnosis (enzyme-linked immunosorbent assay [ELISA] or rK39 strip test), and, rarely, by molecular approaches. The causative parasites are assumed to be L. donovani, although in recent years, studies have shown L. donovani causing cutaneous leishmaniasis in Sri Lanka (18). Additionally, a lower trypanosomatid, Leptomonas seymouri, has been detected in isolates from patients with VL, but not in clinical specimens (19).

Molecular diagnosis of leishmaniasis is often by PCR that typically targets the internal transcribed spacer 1 (ITS1), separating the genes coding for small subunit (SSU) rRNA and 5.8S rRNA (7). Additionally, isolates have been characterized by restriction fragment length polymorphism (RFLP) analysis of the ITS1 region (9) or the gene fragment encoding the 70-kDa heat shock protein (hsp70) (13), the latter being among the first kinetoplastid genes to be cloned and characterized due to their conserved nature (10). Upon routine diagnosis of patients with suspected VL or PKDL by ITS1 PCR, a different band pattern was reported that did not match the classical L. donovani WHO reference strain DD8 (MHOM/IN/1980/DD8) (7). Accordingly, this study was undertaken to study the RFLP patterns of clinical specimens sourced from patients with VL or PKDL along with archived parasite isolates from a different patient population.

The study population included 29 patients from 1 January 2010 to 31 January 2012 who were admitted to the School of Tropical Medicine, Kolkata, India, with clinical features of VL (n = 23) or PKDL (n = 6). Clinical materials included peripheral blood from patients with VL or lesional skin biopsy specimens from patients with PKDL after obtaining informed consent. The diagnosis of VL/PKDL was confirmed by rK39 strip test (20), ELISA for anti-leishmanial antibodies, and PCR of the ITS1 region of Leishmania sp (7). The study received approval from the Institutional Ethical Committee of the School of Tropical Medicine, Kolkata, India, and Institute of Postgraduate Medical Education and Research, Kolkata, India.

In addition, our study included archived Leishmania isolates (n = 7; VL to V5, P1, and P2), obtained from patients with VL (n = 5) or PKDL (n = 2); all except V5 presented at the School of Tropical Medicine between 2006 and 2011. In patients with VL, parasites were isolated from spleen/bone marrow aspirates (7), while for PKDL, a 3-mm punch biopsy specimen from a nodule was collected in medium 199 (M199) supplemented with 20% heat-inactivated fetal calf serum (FCS), penicillin G (30 IU/ml), and streptomycin (50 μg/ml). The material was passed through a 230-μm sterile iron mesh and finally resuspended in 1.5 ml of the same medium, and after incubation at 24°C, culture growth was evident after 5 to 10 days. After transformation from amastigotes to promastigotes, they were gradually adapted into M199 supplemented with 10% FCS, penicillin G (50 IU/ml), and streptomycin (50 μg/ml) and subcultured every 2 to 3 days, the inoculum being 1 × 10⁶/ml. When parasites reached the range of 10⁷, they were cryopreserved (approximately 1 × 10⁷ parasites per cryo vial) in freezing medium (M199 containing 30% FCS and 7.5% dimethyl sulfoxide [DMSO]).

All of the archived strains were typed by ELISA using species-specific L. donovani monoclonal antibody (5) and PCR-RFLP (9). For PCR, DNA following isolation from peripheral blood, skin biopsy specimens, and isolates (QIAamp DNA minikit; Qiagen, Hilden, Germany) was eluted in 200 μl elution buffer. Different parts of Leishmania were amplified, namely (i) ribosomal ITS1 (9) and (ii) hsp70 (13). Amplification reactions were performed in 25 μl of mixture (JumpStart REDTaq ReadyMix reaction mix; Sigma-Aldrich Chemicals, St. Louis, MO) in a Master cycle (Eppendorf, Hamburg, Germany). The amplified ITS1 and hsp70 regions were digested using HaeIII (Fermentas, Glen Burnie, MD); briefly, reactions were carried out using 1 U of HaeIII, 1 × buffer, and 5 μl
of the amplicon (approximately 100 μg of DNA) and incubated at 37°C for 3 h (for ITS1) or overnight (for hsp70). The digested product was analyzed by electrophoresis (3% agarose, 5 V/cm for 1.5 h) along with a 100-bp DNA ladder or GeneRuler low-range DNA ladder (Fermentas, Glen Burnie, MD) and visualized in a G-BOX Gel Doc system (Syngene, Cambridge, United Kingdom) using Gene Tools software (version 4.01.04).

For sequencing of archived Leishmania isolates, PCR products of the ITS1 region were purified (QiAquick gel extraction kit; Qiagen, Hilden, Germany) and then cloned into the pJET1.2 vector by blunt end ligation (CloneJET PCR cloning kit; Fermentas, Glen Burnie, MD). Recombinant plasmid DNA was used to transform *Escherichia coli* DH5α; eight colonies with an ITS1 insert were selected for each sample. Plasmid DNA was purified from colonies using a Qiagen plasmid minikit (Qiagen, Hilden, Germany) and sequenced (BigDye Terminator v3.1 cycle sequencing kit; Applied Biosystems, Foster City, CA) on an automated DNA sequencer (ABI Prism 3130, Foster City, CA). DNA sequence editing and analysis were performed using Seqscape V2.5 software (Applied Biosystems, Foster City, CA).

The reference sequences of the ITS1 gene from several trypanosomatid species were retrieved from GenBank and aligned with the sequence determined in this study (http://www.ncbi.nlm.nih.gov/GenBank/index.html) using ClustalW software and a phylogenetic tree constructed by the neighbor-joining method using MEGA version 5.0 (21).

Blood was sourced from patients with VL (n = 23) and leishional skin biopsy specimens from patients with PKDL (n = 6) (Table 1); 58.62% of patients hailed from Bihar, India (17/29), and among them, 11 (64.70%) were from zones with antimonial resistance (17). Of the remaining 12 patients, 11 were from West Bengal, India, and one was from Chhattisgarh, India, whose areas of antimonial resistance and three (V4, V5, and P2) were from an area having no antimonial resistance (17). The remaining two archived isolates (V1 and V3) were isolated from a patient each from West Bengal and Assam, respectively, whose patterns of antimonial resistance, if any, are yet to be defined.

All archived strains showed strong binding with D2, an *L. donovani* species-specific monoclonal antibody (12), and the absorbances obtained were comparable with that obtained with DD8 (MHOM/IN/1980/DD8), the *L. donovani* reference strain; accordingly, they were typed as *L. donovani*. To further characterize these archived isolates, we performed ITS1 RFLP and found two variations in the PCR products (Fig. 1, inset) that were verified by HaellI digestion. RFLP data showed that pattern A was dominant, being present in 5/7 isolates (71.4%), and pattern B was present in 2 isolates (28.6%) (Fig. 1), akin to the profile obtained in clinical specimens. This lack of digestion by HaellI has not been reported previously in leishmaniasis and suggests unusual variations in the sequence of the ITS1 region among *Leishmania* strains. Although the ITS1 region amplified from *Leptomonas seymouri* and *L. donovani* isolates showed a single 418-bp band of DD8 and (ii) dual bands of 320 and 418 bp in 13.8% (4 patients with VL). Examination of the RFLP pattern of the 320-bp product revealed a pattern similar to that of DD8, having 3 fragments with sizes of 191, 75, and 54 bp, defined as “pattern A.” With regard to the 4 samples having a dual band pattern, each band was gel extracted, purified, and digested separately with HaellII; the 320-bp product had an RFLP profile similar to that obtained with DD8 (i.e., pattern A), while the larger PCR product of 418 bp remained undigested by HaellII and was defined as “pattern B.”

Among the seven archived isolates studied, five were obtained from bone marrow/splenic aspirates of patients with VL (V1 to V3), while two were from dermal tissue of patients with PKDL (P1 and P2). The majority of these patients (5/7, except V1 and V3) hailed from Bihar, the main zone of endemcity for VL in India; among them, two (V2 and P1) were from areas of antimonial resistance and three (V4, V5, and P2) were from an area having no antimonial resistance (17). The remaining two archived isolates (V1 and V3) were isolated from a patient each from West Bengal and Assam, respectively, whose patterns of antimonial resistance, if any, are yet to be defined.

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**TABLE 1 Clinical features of the study population**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Result for patients with:</th>
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<tbody>
<tr>
<td></td>
<td>VL (n = 23)</td>
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<tr>
<td>Age (yr)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>16/7</td>
</tr>
<tr>
<td>History of VL (%)</td>
<td>NA</td>
</tr>
<tr>
<td>Interval between cure of VL and onset (yr)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
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<tr>
<td>Spleen size (cm)</td>
<td>Mean ± SD</td>
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<td>Range</td>
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<td>Liver size (cm)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>Range</td>
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* NA, not applicable.
some sequence variations in the ITS1 region between strains of *L. donovani* have been reported, (9, 16), a difference of 100 bp in the PCR product has not been reported to date.

To substantiate our findings, we performed hsp70 PCR-RFLP with our archived isolates. Once again, two patterns emerged: i.e., 5 isolates matched the *L. donovani* reference strain, whereas 2 isolates (V5 and P2 which showed pattern B for ITS1 RFLP) showed another pattern (data not shown). The latter pattern was not comparable with any other *Leishmania* species (13), but it was similar to that reported in 9 Indian isolates (19). In the clinical specimens, the hsp70 PCR did not yield any product (data not shown).

In order to identify the organism from which this aberrant ITS1 gene was being amplified, the nucleotide sequence of the 418-bp fragment was determined, wherein we selected DD8 and P2 as representatives of patterns A and B, respectively; P2 was selected as it had undergone very few passages following transformation. We aligned two sequences denoting one sequence, “LdA,” for *L. donovani* DD8, representative of pattern A and denoting the other, “LdB,” for P2, representative of pattern B; the ClustalW alignment of the two sequences showed several mismatches and deletions (score, 73), indicating there were significant differences between them (Fig. 2A). To determine whether these sequence variants were due to Taq polymerase errors, two colonies of the variant were selected for a second round PCR using the same primers and resequenced; they were all identical to the original sequence, confirming that the observed sequence variant was not a technical error. Furthermore, two sequences were BLAST searched independently, where LdB showed a strong match with *Leptomonas seymouri* (accession no. EU623433.1). Based on the sequence of the ITS1 region of P2 and DD8, a neighbor-joining tree was constructed that included 33 *L. donovani* Indian isolates, available at National Centre for Biological Information (http://www.ncbi.nlm.nih.gov), and an extended set of organisms (Fig. 2B). The tree showed that P2 was most closely related to *Leptomonas seymouri* (score, 99.0) (Fig. 2B) along with 21 *L. donovani* Indian isolates. The remaining 12 *L. donovani* Indian isolates were phylogenetically closely related to DD8 (Fig. 2B) (http://www.ncbi.nlm.nih.gov/nuccore/EU364830). Additionally, sequence analysis of these 33 isolates showed that the size of the ITS1 PCR product of the 21 isolates that matched *Leptomonas seymouri* was 418 bp, while the size of the 12 Indian archived isolates that matched with DD8 was 320 bp. Importantly, no HaeIII restriction site was present in the 418-bp sequence, corroborating our observations (Fig. 1).

To confirm the presence of *Leptomonas seymouri* in the clinical specimens, we designed a reverse primer from a 30-bp portion (nucleotide positions 197 to 227) unique to this organism. It was selected based on it being the inserted sequence present in the ITS1 region of P2 following alignment of P2 and DD8 (Fig. 2A). The forward primer (5’TCTGGATCATTTCCTGACTATATC3’) was designed from the common sequence (bp 1 to 25) between P2 and DD8, while the reverse primer was 5’TGCCCTCTCTCA CACAGCA3’; a partial ITS1 region was amplified at an annealing temperature of 60°C for 30 s. Among the archived isolates, P2 and V5, which showed pattern B gave a 210-bp product, confirming these strains were *Leptomonas seymouri*. We propose that after parasite transformation, *Leptomonas seymouri* outgrew *L. donovani* as *Leptomonas* species have been reported to grow faster than *L. donovani* (19). In patient DNA, 4 samples appeared coinfected with *L. donovani* and *Leptomonas seymouri* as they gave the
210-bp *Leptomonas* ITS1 PCR product; additionally, 2 more samples from patients with VL showed a 210-bp product (data not shown); none of the patients with PKDL showed a 210-bp band.

Based on this analysis, we conclude that clinical specimens (4/29) isolated from patients with VL/PKDL were concomitantly infected with *Leptomonas seymouri* as also were two archived culture isolates among seven studied; importantly, they phylogenetically clustered more closely to the monoxenous parasite *Leptomonas seymouri*. The occurrence of insect trypanosomatids in humans is exceptional, but reports are available that HIV-positive patients are additionally infected with nonpathogenic insect trypanosomatids (6). In Brazil, Pacheco et al. (15) described a flagellate,
ently a monoxenous trypanosomatid, in a 35-year-old HIV-positive male who presented with symptoms of VL. Hybridization analyses, against a panel of many different trypanosomatids, revealed that the unknown flagellate had kinetoplastid DNA (kDNA) cross-homology only with *Leptomonas pulexsimulantis*, a parasite of a dog flea (18). However, the presence of lower trypanosomatids in immunocompetent individuals is a matter of greater concern (4). Our patients had no evidence of HIV infection (testing negative for HIV), yet four of them were coinfected with *Leptomonas seymouri* and *L. donovani*. Additionally, *Leishmania* coinfections, including with HIV (3), *Plasmodium vivax* (1), or *Mycobacterium tuberculosis* (8), have been reported. Therefore, it may be envisaged that VL in an immunocompetent host in areas of antimonial resistance, it raises the possibility that growing incidence of unresponsiveness to antimonials reported in India as well as worldwide raises questions about the clinical relevance of this pathogen. However, to date, studies pertaining to the pathobiology of these opportunistic lower trypanosomatids infecting humans have been limited.

As this study had a substantial number of patients coming from zones of antimonial resistance, it raises the possibility that *Leptomonas* strains are possibly less sensitive to antimony. The *in vitro* susceptibility toward antimony of both monoxenous trypanosomatid field isolates P2 and V5 was lower than that of the other 5 strains (M. Chatterjee, personal communication), which raises the possibility of the potential contribution of *Leptomonas* to the growing incidence of unresponsiveness to antimonials reported from the Indian subcontinent; however, this must be substantiated in a larger study group to conclude whether *Leptomonas* infections influence the epidemiology, pathology, or case management of VL. Taking these findings together, this study emphasizes the importance of estimating the extent of opportunistic pathogens in leishmaniasis.

**Nucleotide sequence accession number.** The sequence determined in this study has been submitted to GenBank and is available under accession no. JN848802.

**ACKNOWLEDGMENTS**

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Leishmania strains causing self-healing cutaneous leishmaniasis have greater susceptibility towards oxidative stress

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Abstract

The survival of Leishmania parasites within macrophages is influenced by generation of free radicals. To establish whether generation of free radicals influenced chemotherapeutic response, promastigotes from isolates causing self-healing or delayed/non-self-healing cutaneous leishmaniasis (CL) or visceral leishmaniasis (VL) were evaluated for their susceptibility to nitric oxide (NO), antimony and miltefosine. In a self-healing CL strain of Leishmania major (5ASKH), susceptibility to NO and antimony was higher than other species. Likewise, a Leishmania amazonensis strain, M2269, showed greater susceptibility to NO and antimony than other species but no such correlation was observed with miltefosine. Additionally, 5ASKH and M2269 showed poorer free radical scavenging capacity as also their thiol levels were lower than species causing VL. Collectively, our study suggests that self-healing isolates tend to be more susceptible to oxidative stress.

Keywords: anti-leishmanial, antimony resistance, leishmaniasis, nitric oxide, oxidative stress, promastigotes

Introduction

Leishmaniasis is a group of diseases caused by the protozoan parasite of genus Leishmania having a disease spectrum ranging from a self-limiting cutaneous lesion to the near fatal visceral form. Leishmaniasis is endemic in 98 countries with an estimated 350 million people at risk [1]. The commonest presentation of cutaneous leishmaniasis (CL) is one or more skin lesions, which often heal spontaneously; however, CL with persistent lesions has been reported and shown to be associated with non-responsiveness to conventional chemotherapy [2].

Leishmania species show significant variations in their sensitivity to established and experimental drugs, which possibly accounts for the variation in their clinical response to antimonials [3]. These variations have been attributed to biochemical and molecular differences [4]. Promastigotes of Leishmania amazonensis (L. amazonensis) were found to be more sensitive to antimonials than L. donovani, L. mexicana and L. infantum [5]. In limited controlled clinical trials that have compared the sensitivity of lesions caused by different species towards antimonials, L. braziliensis caused lesions had a significantly higher cure rate than lesions caused by L. mexicana [6].

As the Leishmania parasite resides within macrophages, nitric oxide (NO) is considered as a critical molecule [7] that exerts leishmanicidal actions in macrophages, which include inhibition of mitochondrial respiration, inactivation of peroxidases, increased susceptibility to oxidant damage, inhibition of glycolysis, S-nitrosylation, ADP-ribosylation, tyrosine nitration of proteins, disruption of Fe-S clusters, zinc fingers or heme groups and peroxidation of membrane lipids [8]. Resistance to NO has also been described in Escherichia coli and Mycobacterium tuberculosis, wherein resistant isolates are
associated with a more severe outcome of disease than NO-sensitive strains [9]. Similarly, Leishmania spp. resistant to NO showed a positive correlation with disease severity, suggesting that levels of NO in parasites can influence their chemotherapeutic outcome [10]. The parasite’s redox biology contributes to drug resistance evidenced by the fact that anti-mononcyt resistant parasites displayed elevated IC_{50} values for the NO donors, sodium nitrite (NaNO₂), S-nitroso-N-acetylpenicillamine (SNAP) and 2,2-(hydroxynitrosohydrazono)-bis-ethanamine (DETA/ NONOate), compared to Sb-responsive strains [11]. However, it remains to be seen whether this resistance to NO is due to elevated levels of trypanothione (T\text{[SH]}_2) or other underlying antioxidant mechanisms.

Recent studies have shown that isolates of L. amazonensis and L. Viannia braziliensis, responsible for self-healing CL, are more susceptible to nitrosative stress [10]. Accordingly, this study aimed to establish whether susceptibility of different Leishmania species to pro-oxidants like antimony, miltefosine and NaNO₂ correlated with their ability to handle oxidative stress.

Materials and methods

Reagents

All chemicals were of analytical grade and obtained from Sigma–Aldrich Chemicals (St. Louis, MO, USA) except 5-chloromethylfluorescein-diacetate (CMFDA, Molecular Probes, Carlsbad, CA, USA). Stock solution of CMFDA (0.5 mM) and DCFDA (2 mM) were prepared, stored at −20°C and diluted immediately before use.

Parasite culture

Promastigotes representing several Leishmania species, responsible for visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL), from the Old and the New World were included (Table 1). Promastigotes were routinely cultured at 24°C in Medium 199 supplemented with 10% heat inactivated foetal calf serum (FCS), penicillin G (50 IU/ml) and streptomycin (50 µg/ml), referred to as Medium A. For experimental purposes, log phase promastigotes were subcultured every 72–96 hours, inoculum being 1×10^6/ml [12].

Evaluation of susceptibility towards antimony, miltefosine and NO in Leishmania promastigotes

Susceptibility towards antimony, miltefosine and NaNO₂ (a NO-generating agent at pH 5.0) was determined by measuring the viability of promastigotes using a modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based cell viability assay [13]. Briefly, log phase L. donovani promastigotes (5×10^4/well) were incubated in a final volume of 50 µl Medium A with [Sb(III), 0–100 µg/ml, pH 7.2], miltefosine (0–50 µM, pH 7.2) or NaNO₂ (0–20 mM, pH 5.0). After 48-hour incubation at 24°C, MTT [5 mg/ml stock in phosphate-buffered saline (0.02 M, pH 7.2, PBS), 10 µl] was added and plates incubated overnight at 24°C. To dissolve crystals, 50 µl of sodium dodecyl sulphate (10% SDS) along with isopropyl alcohol (50%) were added and further incubated at 37°C for 6 hours. After microscopically confirming dissolution of crystals, the optical density at 550 nm (OD_{550}) was determined. The specific OD_{550} for each well was calculated by subtracting the OD_{550} of medium containing Sb(III), miltefosine or NaNO₂. The mean per cent viability was calculated as:

\[
\text{Specific OD}_{550} = \frac{\text{Specific OD of Sb(III)/miltefosine/NaNO}_2 \text{treated parasites \times 100}}{\text{Specific OD of control parasites}}
\]

The results were expressed as the IC_{50}, that is, the concentration that inhibited 50% cell growth as enumerated by graphical extrapolation using GraphPad Prism software (version 4).

Flow cytometric measurement of cell viability in the presence of antimony was measured by uptake of propidium iodide (PI). Briefly, log phase promastigotes (1×10^6/ml) following incubation with Sb(III) (100 µg/ml, 3 hours, 37°C) were washed with PBS; just prior to acquisition of fluorescence on a flow cytometer, cells were incubated with PI (0.05 µg/ml) for 2 minutes.

Measurement of reactive oxygen species (ROS) in Leishmania promastigotes

The generation of reactive oxygen species (ROS) was measured as described by Saha et al. 2009 [14]. Briefly, log phase promastigotes (1×10^6/ml), following incubation with Sb(III) (100–300 µg/ml, 3 hours, 37°C) and H_2O_2 (0–1000 µM, 30 minutes, 37°C), were washed with PBS and then incubated with 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA, 50 µM) for 45 minutes at 37°C after which the fluorescence of 2,7-dichlorofluorescein (DCF) was acquired on a flow cytometer.

Measurement of non-protein thios in Leishmania promastigotes

Parasites were washed twice in HEPES-buffered saline (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH_2PO_4 and 20 mM glucose, pH 7.4, HBS); they were initially depleted of ATP by incubating in glucose-free HBS but containing sodium azide (20 mM, 4×10^(-7)/ml) for 30 minutes at 24°C. Cells were then washed, resuspended in PBS containing...
CMFDA, (5.0 µM) for 15 minutes at 37°C and analysed for fluorescence as previously described [15].

Measurement of superoxide dismutase activity in Leishmania promastigotes

Superoxide dismutase (SOD) activity was assayed by determining its ability to inhibit autoxidation of pyrogallol [16]. Briefly, the assay mixture contained 0.2 mM pyrogallol equilibrated in air, triis-cacodylic acid buffer (50 mM, pH 8.2) and ethylenediaminetetraacetic acid (1 mM). The rate of autoxidation was obtained by monitoring the increase in absorbance at 420 nm in the presence and absence of parasite lysate (50 µg); protein was determined using Folin–Ciocalteu’s phenol reagent [17].

Flow cytometry

Cells (1 × 10⁶/ml) from different experimental groups were monitored for their intracellular fluorescence on a flow cytometer (FACS Calibur, Becton Dickinson, CA, USA) equipped with an argon-ion laser (15 mW) tuned to 488 nm. Fluorescence of the DCF and thioether trypanothione methylfluorescein (TSMF) were monitored for their intracellular fluorescence on a flow cytometer (FACS Calibur, Becton Dickinson, CA, USA). The fluorescence was measured in the log mode using CellQuest Pro software (BD Biosciences, CA, USA) and expressed as geometrical mean fluorescence channel (GMFC), that is, average or central tendency of fluorescence of analysed particles. Acquisition was performed on 10,000 gated events and data analysed using CellQuest Pro software (BD Biosciences, CA, USA).

Statistical analysis

Each experiment was performed at least thrice in duplicates and results expressed as mean ± SEM. Statistical analysis was evaluated by T test or one way ANOVA followed by Tukey’s multiple comparison test (wherever applicable) and strength of linear dependence between two variables by Spearman’s correlation coefficient using Graph Pad Prism software, version 4 (GraphPad Software Inc, San Diego, CA, USA). p < 0.05 was considered as statistically significant.

Results and discussion

Study population

Promastigotes representing several Leishmania species responsible for VL and CL from the Old and the New World were studied (Table I). They were grouped as (a) strains causing VL namely L. donovani (DDB), L. infantum (IPT1) and L. chagasi (PP75) (b) strains causing self-healing CL, namely L. major (5ASKH) [18] and a strain of L. amazonensis (M2269) [19], that caused local CL along with (c) strains causing delayed or non-self-healing CL namely L. tropica (K27) and L. mexicana (MIC).

Isolates causing self-healing CL are more sensitive to antimony

Viscerализing species are inherently more resistant to antimony, as treatment for CL requires an intraregional dose of sodium antimony gluconate, SAG (20 mg/kg b.w.) for 10–20 days [20], whereas for VL, an IM dose of SAG (20 mg/kg b.w.), for 28–30 days is necessary [21]. We tested the sensitivity of strains to trivalent antimony since promastigotes cannot convert the pentavalent form to the trivalent form [Sb(III)]. The antimony sensitivity profiles of promastigotes appeared to reflect the same, as among the seven strains, the IC₅₀ of Sb(III) in the two self-healing CL causing species, 5ASKH and M2269 was lowest, mean ± SEM being 5.73 ± 4.33 and 11.71 ± 1.25 µg/ml respectively (Table I). With regard to 5ASKH, the IC₅₀ of Sb(III) was consistently lower than the VL causing species DDS, IPT1 and PP75, the fold difference being 12.05 (p < 0.001), 6.28 and 8.28 (p < 0.05) respectively (Table I). Similarly, when compared to the delayed or non-self-healing CL strains, the IC₅₀ of 5ASKH was 2.97- and 6.04-fold lower than K27 and MIC respectively (Table I).

The scenario was similar with M2269 as its IC₅₀ of Sb(III) was lower than VL causing species DDS, IPT1 and PP75 by 5.9- (p < 0.001), 3.07- and 4.05- (p < 0.05) fold respectively (Table I). Upon comparison with delayed or non-self-healing CL strains, M2269 had comparable IC₅₀ values with K27 but was 2.95 fold lower than the other delayed or non-self-healing strain, MIC. Taken together, parasites causing self-healing CL are more susceptible to antimony vis a vis other species.

To examine whether parasites respond in a similar pattern with miltefosine, the only orally effective modality against Indian Leishmaniasis [22], their sensitivity to miltefosine was examined. Among the strains, the effect of miltefosine was least against L. tropica (K27), IC₅₀ being 30.97 µM and most sensitive against L. mexicana (MIC), IC₅₀ being 1.92 µM (Table I); the other strains had an IC₅₀ that ranged between 3.26–8.73 µM. Taken together, no correlation was evident between sensitivity of strains to antimony vs. miltefosine. This is possibly due to the leishmanicidal activity of miltefosine being via its ability to inhibit phosphatidylcholine (PC) biosynthesis choline transport [23], whereas antimony killed parasites by enhanced generation of oxidative stress. This reflects the clinical scenario where strains are
generally sensitive to miltefosine, irrespective of their responsiveness to antimony [22].

Isolates from self-healing CL are more sensitive to nitric oxide

As NO generated in macrophages and parasites has been demonstrated to be a principal effector molecule responsible for mediating intracellular killing of *Leishmania* parasites. Guidice et al. (2007) [24] evaluated the effect of NO on strains of CL, namely *L. (V.) braziliensis* and *L. (L.) amazonensis*. They demonstrated that *Leishmania* amastigotes resistant to NO showed a positive correlation with lesion size, a clinical measure of disease severity. Keeping this in mind one could envisage that compounds capable of increasing the generation of NO in parasites may well have good leishmanicidal activity.

To confirm whether inherent susceptibility towards NO was a feature of strains causing self-healing CL, we tested their sensitivity towards acidified NaNO$_3$, a compound that releases NO (at pH 5.0) [25]. Although at acidic pH, promastigotes can convert to axenic amastigotes; microscopical examination after 48-hour incubation (pH 5.0) indicated the presence of elongated promastigotes rather than rounded axenic amastigotes. This is possibly because for the conversion of promastigotes to axenic amastigotes, a specialized nutrient rich medium and modified incubation period (initial 24 hours at 24°C and then 72–96 hours at 37°C, [26]) are required. Both self-healing CL strains (5ASKH and M2269) were more sensitive to NO, their IC$_{50}$ being 1.26 ± 0.26 mM and 1.62 ± 0.48 mM, respectively (Table I). With regard to 5ASKH, its IC$_{50}$ was consistently lower than the VL causing strains, DD8 and PP75 by 2.11- and 3.85-fold, respectively; however, its IC$_{50}$ was comparable with another VL causing strain, IPT1. 5ASKH, when compared with the delayed or non-self-healing strains of CL (K27 and MIC), showed a similar trend in that its IC$_{50}$ was lower by 3.85- and 2.57-fold, respectively (Table I). The other self-healing strain, M2269 also had a lower IC$_{50}$ than two VL causing strains, DD8 and PP75, by 1.64- and 2.62-fold, respectively, as also had a lower IC$_{50}$ than the delayed or non-self-healing CL strains, K27 and MIC, by 3.0- and 2.0-fold, respectively (Table I). Taken together, our study strongly supports the hypothesis that enhanced susceptibility to NO contributes towards the spontaneous cure observed in self-healing strains of CL.

Generation of reactive oxygen species by Sb(III) requires lower concentrations in strains causing CL

Mehta and Shaha [27] have established the pro-apoptotic potential of antimony following generation of ROS, as it triggered depolarization of mitochondrial membrane potential and uncoupling of oxidative phosphorylation. To examine whether increased generation of ROS accounted for the enhanced susceptibility of self-healing CL causing strains to Sb(III) and NaNO$_3$ (Table I), their oxidative status (intracellular free radicals) was measured by flow cytometry using H$_2$DCFDA. This is a lipid soluble, membrane permeable compound whose oxidation by a wide range of intracellular ROS produces a fluorescent compound DCF [28,29]; therefore, the fluorescence of DCF serves as a surrogate marker for the amount of ROS, that is, free radicals generated.

In previous studies, it has been established that in strains causing VL, a significant amount of ROS was generated by Sb(III) at 100–300 µg/ml for 3 hours [30], the parasite viability remaining >80% as measured by the uptake of PI. In this study, Sb(III) [100 µg/ml, 3 hours] caused no significant generation of ROS in WHO reference strains of VL (DD8, IPT1 and PP75) and delayed/non-self-healing CL (K27 and MIC, Table II) as also the parasite viability remained >95% (data not shown), indicating that this concentration could not generate ROS or impede cell viability. However, this very same concentration of Sb(III) decreased generation of ROS in the two self-healing isolates of CL, 5ASKH and M2269. This

### Table I. IC$_{50}$ of Sb(III), miltefosine and NaNO$_3$ in *Leishmania* promastigotes.

<table>
<thead>
<tr>
<th>Species (code)</th>
<th>Designation</th>
<th>Disease</th>
<th>IC$_{50}$ Sb(III) in µg/ml</th>
<th>IC$_{50}$ Miltefosin in µM</th>
<th>IC$_{50}$ NaNO$_3$ in mM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. donovani</em> (DD8)</td>
<td>MHOM/BZ/1972/PP75</td>
<td>VL</td>
<td>5.92 ± 0.48</td>
<td>8.7 ± 1.26</td>
<td>4.26 ± 0.84</td>
</tr>
<tr>
<td><em>L. infantum</em> (IPT1)</td>
<td>MHOM/BZ/1972/PP75</td>
<td>VL</td>
<td>3.23 ± 0.12</td>
<td>8.5 ± 0.96</td>
<td>2.68 ± 0.51</td>
</tr>
<tr>
<td><em>L. chagasi</em> (PP75)</td>
<td>MHOM/BZ/1972/PP75</td>
<td>VL</td>
<td>3.41 ± 0.23</td>
<td>6.0 ± 0.53</td>
<td>2.12 ± 0.38</td>
</tr>
<tr>
<td><em>L. braziliensis</em> (5ASKH)</td>
<td>MHOM/SU/1973/5ASKH</td>
<td>Self-healing CL</td>
<td>5.73 ± 0.33</td>
<td>6.80 ± 0.26</td>
<td>1.26 ± 0.26</td>
</tr>
<tr>
<td><em>L. amazonensis</em> (M2269)</td>
<td>MHOM/BZ/1972/5ASKH</td>
<td>Self-healing CL</td>
<td>11.71 ± 1.05</td>
<td>8.50 ± 0.60</td>
<td>1.62 ± 0.48</td>
</tr>
<tr>
<td><em>L. major</em> (K27)</td>
<td>MHOM/AZ/1974/SAF-K27</td>
<td>Delayed/non-self-healing CL</td>
<td>17.07 ± 3.27</td>
<td>39.20 ± 3.40</td>
<td>4.86 ± 0.74</td>
</tr>
<tr>
<td><em>L. mexicana</em> (MIC)</td>
<td>MHOM/MX/2006/MEX/MIC</td>
<td>Delayed/non-self-healing CL</td>
<td>3.63 ± 0.53</td>
<td>1.92 ± 0.14</td>
<td>0.24 ± 0.61</td>
</tr>
</tbody>
</table>

*VL*: visceral leishmaniasis and CL: cutaneous leishmaniasis. *Promastigotes* (5 × 10$^5$) were seeded in 96-well plates with Sb(III), miltefosine or NaNO$_3$, for 48 hours, and cell viability was measured using a modified MTT assay as described in Materials and methods. The data represents the mean ± SEM value of at least three experiments in duplicate.
occurred as Sb(III) adversely affected their cell viability, reflected in their higher uptake of PI of 83.71% and 89.18%, respectively, indicating that these two strains are far more susceptible to Sb(III) than their visceralizing counterparts (Figure 1). For M2269, a mere 3-hour incubation at 37 °C was toxic as 21.13% cells became PI positive (Figure 1). However, this concentration of Sb(III) was non-toxic to the VL strain DD8 as its baseline PI positivity increased marginally from 1.02 to 3.82% (Figure 1).

To determine the optimum non-toxic concentration of Sb(III) that caused generation of ROS in 5ASKH and M2269, they were exposed to Sb(III) (0–200 µg/ml), a shorter duration (1–3 h) and variable temperature (37 °C or 24 °C). With 5ASKH, Sb(III) beyond 100 µg/ml was toxic; using lower concentrations of Sb(III), cells remained viable but showed no measurable increase in fluorescence. Therefore, in 5ASKH, we were unable to identify the critical concentration of Sb(III) at which a measurable amount of ROS is generated. In case of M2269, Sb(III) was even more toxic, as viability was hampered beyond 50 µg/ml of Sb(III) (37 °C, 3 hours). However, a shorter incubation period of 1 hour at 24 °C caused the GMFC to increase to 351.71 versus 269.28 (baseline). Taken together, the data corroborated that self-healing strains are more susceptible to Sb(III) than delayed/non-self-healing or VL causing strains.

Scavenging of ROS is poor in strains of CL

The variable proportion of ROS scavenged by antimonial sensitive and antimonial resistant strains causing VL has previously been studied [30]. No detectable fluorescence was evident in VL causing strains when H$_2$O$_2$ was added in the range of 1 –100 µM, suggesting that L. donovani parasites irrespective of their chemosensitivity profiles, effectively scavenge this amount of H$_2$O$_2$. In a previous study [30] an antimony sensitive strain of VL, H$_2$O$_2$ (100 µM) generated an 11-fold increase, mean GMFC being 550; furthermore, increasing the concentration of H$_2$O$_2$ (500 and 1000 µM) translated into a dose-dependent increase in GMFC of 1065 and 1571 respectively [30]. In this study, WHO reference strains were treated with H$_2$O$_2$ (1000 µM, 30 minutes), wherein increased generation of ROS was observed, except in two self-healing strains (5ASKH and M2269, Table II). This

<p>| Table II. Levels of ROS in different Leishmania strains treated with antimony or H$_2$O$_2$. |
|----------------------------------|----------------------------------|</p>
<table>
<thead>
<tr>
<th>Strains</th>
<th>Sb(III) [100 µg/ml, 3 hours]</th>
<th>H$_2$O$_2$ [1 mM, 30 minutes]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD8</td>
<td>924.40 ± 5.38</td>
<td>919.40 ± 39.41</td>
</tr>
<tr>
<td>IPT1</td>
<td>324.20 ± 12.77</td>
<td>328.30 ± 15.72</td>
</tr>
<tr>
<td>PP75</td>
<td>530.10 ± 26.06</td>
<td>585.50 ± 10.55</td>
</tr>
<tr>
<td>SASKH</td>
<td>197.5 ± 25.50</td>
<td>50.50 ± 11.50</td>
</tr>
<tr>
<td>K27</td>
<td>98.15 ± 14.77</td>
<td>159.50 ± 35.20</td>
</tr>
<tr>
<td>M2269</td>
<td>269.28 ± 11.17</td>
<td>60.25 ± 13.97</td>
</tr>
<tr>
<td>MIC</td>
<td>125.90 ± 7.94</td>
<td>117.80 ± 9.59</td>
</tr>
</tbody>
</table>

Promastigotes (1 × 10$^6$/ml) were treated with Sb(III) or H$_2$O$_2$, stained with H$_2$DCFDA and analysed in a flow cytometer as described in materials and methods. The data represents as mean GMFC ± SEM value of at least three experiments in duplicate. *p<0.05 as compared to baseline.

Figure 1. Cell viability of Leishmania promastigotes. Log phase promastigotes (1 × 10$^6$/ml) of SASKH, M2269 and DD8 were incubated in absence (A) or presence (B) of Sb(III) (100 µg/ml, 3 hours, 37°C), stained with propidium iodide and analysed by flow cytometry as described in Materials and methods. The figure is a representative profile of at least three experiments.
concentration of $H_2O_2$ was extremely toxic for these two self-healing isolates (as measured by $>$90% PI uptake, data not shown). Subsequently, we tested lower concentrations of $H_2O_2$ in these two self-healing strains. Addition of 250 $\mu$M of $H_2O_2$ generated a mere 1.67-fold increase in fluorescence and thereafter higher concentrations of $H_2O_2$ caused cell death, indicating their inability to scavenge ROS. With M2269, concentrations above 5 $\mu$M $H_2O_2$ triggered cell death, indicating that both these strains have a poor antioxidant system that contributed towards their greater susceptibility to $H_2O_2$, which corroborated with Sb(III) sensitivity.

**CL causing species have lower amounts of cellular thiols**

The antioxidant mechanism operative in trypanosomatidae is unique and relatively weak in its being exclusively based on the bis-glutathionylspermidine conjugate T[SH]$_2$, and the flavoenzyme trypanothione reductase [31,32]. It replaces the nearly ubiquitous glutathione (GSH)/glutathione reductase (GR) system, protecting parasites from oxidant damage, toxic heavy metals, xenobiotics and delivering the reducing equivalents for DNA synthesis [32,33]. Although thioredoxin reductases an important group of antioxidant components, catalase and glutathione peroxidase, the Leishmania parasite is rendered more vulnerable to free radical mediated toxicity [36]. Antimony impairs the parasite’s intracellular thiol buffering capacity and the thiol redox state, thereby making them susceptible to oxidative stress and resultant cell death [37]. In laboratory raised antimonial-resistant *L. tarentolae*, the degree of drug resistance correlated with raised levels of thiols [38]. Similarly, in antimony resistant field isolates of VL, levels of intracellular non-protein thiols were consistently higher [15,30] and was accompanied by an increase in the T[SH]$_2$ dependent antioxidant system [39]. Accordingly, we studied whether the levels of non-protein thiols in self-healing CL causing strains 5ASKH and M2269 influenced their observed increased sensitivity to Sb(III).

Measurement of non-protein thiols in seven *Leishmania* species indicated that the two self-healing isolates, 5ASKH and M2269, have lower amounts of non-protein thiols, mean GMFC ± SEM being 134.20 ± 64.82 and 152.80 ± 34.93 respectively (Figures 2A and B) as compared to the visceralizing species (DD8, IPT1 and PP75). The levels of non-protein thiols of 5ASKH were 3.68- ($p<0.001$), 2.40- ($p<0.05$) and 4.95- ($p<0.001$) fold lower, respectively. However, levels of non-protein thiols of 5ASKH were comparable with the two delayed or non-self-healing CL species, K27 and MIC (Figures 2A and B).

As parasites are devoid of two important antioxidant components, catalase and glutathione peroxidase, the *Leishmania* parasite is rendered more vulnerable to free radical mediated toxicity [36]. Antimony impairs the parasite's intracellular thiol buffering capacity and the thiol redox state, thereby making them susceptible to oxidative stress and resultant cell death [37]. In laboratory raised antimonial-resistant *L. tarentolae*, the degree of drug resistance correlated with raised levels of thiols [38]. Similarly, in antimony resistant field isolates of VL, levels of intracellular non-protein thiols were consistently higher [15,30] and was accompanied by an increase in the T[SH]$_2$ dependent antioxidant system [39]. Accordingly, we studied whether the levels of non-protein thiols in self-healing CL causing strains 5ASKH and M2269 influenced their observed increased sensitivity to Sb(III).

**Figure 2. Flow cytometric measurement of basal levels of non-protein thiols in different *Leishmania* species.** A: Representative histogram profile of log phase promastigotes (1 x 10$^5$/ml) from a self-healing CL causing isolate 5ASKH, (---) and a VL causing isolate (PP75, —) that were incubated with CMFDA (5.0 $\mu$M) for 15 minutes as described in Materials and methods. B: Log phase promastigotes (1 x 10$^5$/ml) from VL causing isolates (DD8, IPT1 and PP75) and CL causing isolates (5ASKH, M2269, K27 and MIC) were labelled with CMFDA (5.0 $\mu$M), and fluorescence analysed as described in Materials and methods. Data are expressed as mean GMFC ± SEM of at least three independent experiments in duplicate.
Collectively, the data suggests that self-healing *Leishmania* strains (5ASKH and M2269) have inherently poorer antioxidant systems, which contribute to their higher susceptibility to Sb(III) (Table I).

**SOD activity was comparable amongst *Leishmania* strains**

SOD is an enzyme, which catalyses the dismutation of toxic superoxide radical ($O_2^-$) to molecular oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$). It exists in three different forms (Cu/Zn-SOD, Mn-SOD and Fe-SOD), of which Fe-SOD is the main form demonstrated in *Leishmania* [40]. SODs dismutate $O_2^-$ before it can exacerbate pro-oxidant damage by participating in the formation of even more toxic species, namely hydroxyl radicals and peroxynitrite. As antimony kills promastigotes via enhanced generation of free radicals [15,30], we measured the SOD activity in seven different *Leishmania* species and no significant differences were observed (Figure 3) indicating that SOD is possibly not an important factor in anti-leishmanial activity and plays a minimal role in detoxification of $O_2^-$ in *Leishmania* parasites.

**Correlation between sensitivity of Sb(III) and NaNO$_2$ with non-protein thiols**

Amastigotes survive within macrophage phagolysosomes, a hostile environment for microbes as they continuously encounter ROS and reactive nitrogen intermediates (RNI). Sodium antimony gluconate, the main stay of treatment for leishmaniasis [41], generates ROS and RNI within parasites [30,42]. A recent study by Souza et al. (2010) [10] suggests that resistance to NO can be correlated with unresponsiveness to antimony in American tegumentary leishmaniasis. Therefore, it is possible that other antimonial resistant *Leishmania* strains may also show a similar degree of resistance to NO. However, in our study population, a poor correlation existed between sensitivity to antimony and susceptibility to NO ($r = 0.16$). This is possibly because the antileishmanial activity of antimony is contributed by an overall increase in free radicals, NO being one of the contributory leishmanicidal molecules generated by antimony [12].

Antioxidant defences for *Leishmania* parasites rely upon a relatively less efficient, unique T[SH]$_2$ dependent antioxidant system [43] along with Fe-SOD, which only marginally protect them from free radical mediated damage [44]. In antimony resistant *Leishmania donovani* field isolates, levels of intracellular non-protein thiols were consistently higher [15,30]. Furthermore, in the visceralizing species (DD8, IPT1 and PP75), along with four additional strains of VL (AG83, 2001, NS2 and 41) [15], a positive correlation ($r = 0.64$) was present between the levels of non-protein thiols and their sensitivity to Sb(III) (Figure 4). A recent study by Souza et al. (2010) [10] suggests that resistance to NO can be correlated with unresponsiveness to antimony in American tegumentary leishmaniasis. Therefore, it is possible that other antimonial resistant *Leishmania* strains may also show a similar degree of resistance to NO. However, in our study population, a poor correlation existed between sensitivity to antimony and susceptibility to NO ($r = 0.16$). This is possibly because the antileishmanial activity of antimony is contributed by an overall increase in free radicals, NO being one of the contributory leishmanicidal molecules generated by antimony [12].

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**Conclusions**

As amastigotes are the biologically relevant form of *Leishmania* that cause disease in the host, measurement of any biological parameter in *Leishmania* amastigotes is more appropriate than in promastigotes. However, experiments with amastigotes are labour intensive and require expertise. Furthermore, as the effect of trivalent antimony in promastigotes correlates with antimony sensitivity in amastigotes [15,26], they were used in this study. Our data collectively...
suggests that higher susceptibility of self-healing CL-causing strains is possibly linked to their inability to handle oxidative and/or nitrosative stress, which impacts on their chemotherapeutic response.

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**Declaration of interest**

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**References**


Leishmania strains and susceptibility to oxidative stress


Leishmaniasis in India resulting from infection of the hemoflagellate protozoan parasite Leishmania donovani manifests in two different forms, visceral leishmaniasis (VL; also known as kala-azar) and its dermatologic sequel post-kala-azar dermal leishmaniasis (PKDL; discussed in ref. 1 and references therein). PKDL was first described by Brahmachari in 1922 in cured VL patients with eruptions and plaques in the skin, it was confirmed by demonstration of Leishman–Donovan bodies (LD) in slit skin smears, and it was termed dermal leishmanoid (discussed in ref. 1 and references therein). Later, the disease was renamed PKDL, because eruptions follow the visceral form, commonly called kala-azar. PKDL manifests in a variety of clinical forms ranging from hypopigmented macules to infiltrated plaques and nodules. Diagnosing PKDL has always been a challenge, because its geographical pocket is endemic for leprosy, a disease that closely mimics PKDL. A previous history of VL is often sought for making a diagnosis but can be misleading at times, because a history of VL was found in 87.5% of patients in a recent study with PKDL. Because PKDL is the proposed reservoir, especially during the interepidemic periods of VL, diagnosing PKDL is of paramount importance to prevent further epidemics (discussed in ref. 1 and references therein). In more recent years, several diagnostic approaches have been developed ranging from serological tests and immunohistochemistry to polymerase chain reaction (PCR). Among the serological tests, the immunochromatographic strip test using recombinant kinesin 39 (rK39) is considered as a rapid, convenient, and useful test for diagnosis of Indian leishmaniasis. The rK39 epitope is highly conserved in the visceralizing species of Leishmania and was used to develop a diagnostic test for VL and PKDL. In cases of VL, the rK39 nitrocellulose-based dipstick test showed high sensitivity and specificity and therefore, has gained substantial popularity for its case of use, especially in the field setting. For detection of polymorphic PKDL (comprising both macular and papulo-nodular skin lesions), the sensitivity of the rK39 strip test is 95.6%, and for macular PKDL, the sensitivity is 86.3%. This report deals with three cases where the rK39 strip test failed to diagnose two cases of post–kala-azar dermal leishmaniasis and one case of visceral leishmaniasis. However, a strong clinical suspicion prompted further evaluation by polymerase chain reaction (PCR), which established the etiology. The present case series highlights the usefulness of PCR in the diagnosis of leishmaniasis.

Ethical Committee, and peripheral blood or skin biopsy was collected after obtaining informed consent from the parent/guardian of the minors.

CASE 1

A 9-year-old boy from the Godda district of Jharkhand, India, presented with multiple hypopigmented macules occupying the face and upper torso as well as the upper and lower limbs (Figure 1). Five years ago, he had suffered from VL, for which he was treated with sodium stibogluconate (SSG). He complained of photosensitivity but had no sensory abnormality and no thickened or tender peripheral nerves. Cervical and axillary lymphadenopathy was present; however, no mucosal or genital lesions were present, and also, no systemic abnormality was found. Because the rK39 strip test was negative, he was not considered as a case of PKDL. Slit skin smear using modified Ziehl–Neelsen (ZN) stain and Giemsa was also negative for Acid Fast Bacilli (AFB) and LD body, respectively. However, on the grounds of a strong clinical suspicion of PKDL, indirect enzyme-linked immunosorbent assay (ELISA) was performed using crude Leishmania antigen as the coating antigen. The presence of antileishmanial antibody in serum was detected using horseradish peroxidase-conjugated Protein-A (Sigma-Aldrich Chemicals, St. Louis, MO), which reacted with substrate 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Roche Diagnostics, Mannheim, Germany), and optical density was measured at 405 nm (OD405) by a microplate reader (Model 680; Bio-Rad, Hercules, CA). The titer was considered as positive when the OD405 of the suspected case was at least twofold higher than the composite mean of 15 non-endemic controls (mean ± standard deviation [SD] was 0.08 ± 0.06). Because the OD405 was 0.26 and 3.3-fold higher, it was considered positive. We further confirmed the result by PCR (i.e., tested for parasite DNA in the skin biopsy sample [3-mm punch biopsy] using Leishmania-specific primers LITSR [5′-CTGGATCATTTCCGGATG-3′] and L5.8S [5′-TGATACCACTTATCGCATGCT-3′]) using Platinum Taq polymerase (Invitrogen, Carlsbad, CA). PCR buffer, and deoxyribonucleotide triphosphates (dNTPs; Fermentas, Glen Burnie, MD) in a Master cycler (Eppendorf, Hamburg, Germany). The PCR products were visualized by agarose gel electrophoresis (1.3%) and analyzed in G-BOX gel doc (Syngene, Cambridge, UK) using Gene Tools (version 4.01.04) software. DNA was isolated from both peripheral blood and skin biopsy by the QIAamp DNA mini kit (Qiagen, Hilden, Germany) and eluted in 200 µL elution buffer. For the PCR assay, a positive control...
(DNA isolated from *L. donovani* promastigotes obtained from a patient with PKDL [SS10]) and a negative control (water) were used. The PCR product length of the skin biopsy sample was 306 bp, which was comparable with the PCR product of the PKDL isolate (product length = 312 bp) (Figure 2). PCR was also performed in a peripheral blood sample obtained from a non-endemic human control, and it gave no band. The patient received SSG (20 mg/kg body weight per day intramuscularly), because the patient was not from an area associated with resistance. Outcome of the treatment was assessed after 4 months and showed considerable improvement.

**CASE 2**

A 15-year-old girl from Murshidabad district of West Bengal, India, presented with multiple hypopigmented patches that were restricted to the face (Figure 3). The lesions were asymptomatic, and there was no sensory abnormality; importantly, they appeared 1 month after cure from VL. She had previously suffered from VL 1.5 years ago and had been treated with SSG. She gave no history of atopy, and peripheral nerves were not thickened. The slit skin smear did not reveal any AFB or LD body, and because rK39 was negative, the results posed a diagnostic dilemma; diagnosing PKDL solely on the basis of a positive history of VL was unjustified, especially because her lesions were restricted to the face. Indeterminate leprosy presenting with multiple lesions was also unlikely, and accordingly, the patient was offered ketoconazole shampoo and topical emollient, because seborrheic dermatitis was considered the most plausible cause. She was asked to return after 1 month but instead, returned after 2 months when the lesions had increased in size and number; again, there was no sensory abnormality or peripheral nerve thickening noted. Histopathology was performed, and it showed non-specific perivascular mononuclear infiltrate with no evidence of perineural/periappendageal localization that would be suggestive of leprosy.

With a strong clinical suspicion of PKDL, we performed indirect ELISA; the antileishmanial antibody titer was negative, OD$_{405}$ was 0.05, and the composite mean OD$_{405}$ of the control group was 0.08. PCR was done as previously described from a skin biopsy (3-mm punch biopsy) and was positive (having two bands of length of 318 and 415 bp) (Figure 2). Understandably, this was a case of false-negative rK39 strip.

![Figure 1. A 9-year-old boy who presented with multiple macular lesions.](image1)

![Figure 2. Polymerase chain reaction (PCR) assay with clinical samples of post-kala-azar dermal leishmaniasis (PKDL) and visceral leishmaniasis (VL). Lane M = 100-bp ladder. Lane 1 = PCR control (water). Lane 2 = VL isolate (DD8). Lane 3 = VL isolate (NS2). Lane 4 = VL isolate (YR08). Lane 5 = PKDL isolate (SS10). Lane 6 = non-endemic control (peripheral blood). Lane 7 = PKDL skin biopsy (case 1). Lane 8 = PKDL skin biopsy (case 2). Lane 9 = VL peripheral blood (case 3).](image2)

![Figure 3. A 15-year-old girl with few hypopigmented patches present primarily on the face.](image3)
The observed variation can, therefore, be attributed to a sequence variation known to occur between strains of *L. donovani*. The patient received a daily injection of amphotericin B (0.75 mg/kg body weight) for 4 weeks.

In case 1, it was very important to clinically differentiate it from lepromatous leprosy, because infiltration of peripheral nerves is symmetrical and asymptomatic, making the diagnosis difficult. Other tell-tale signs like photosensitivity and lymphadenopathy were important parameters to clinically differentiate PKDL from leprosy. In case 2, because both rk39 and indirect ELISA were negative, it suggests that the patient may have been uncompromised (however, the patient tested negative for HIV) and therefore, only an antigen- or DNA-based test would be effective. In case 3, the clinical presentation prompted us to additionally evaluate by ELISA, PCR, and for the most definitive evidence, parasite transformation.

These three cases emphasize the need for relying on a high index of suspicion and clinical acumen in dealing with patients of VL and PKDL. The laboratory tests are always a supplement to clinical methods but relying too much on tests can be counterproductive. Slit skin smear, for detection of both AFB and LD bodies, lacks sensitivity; and here, the predictive value of a negative test is low. The rk39 is undoubtedly a useful test, especially in a field setting; however, if the rk39 is negative but clinical suspicion is high, one may consider performing a DNA-based test. In the Indian perspective, eradication of VL is presently a national priority. Because it is widely accepted that PKDL serves as the disease reservoir, it would, therefore, be pertinent to consider development of a referral system, where cases of PKDL can receive appropriate attention, to achieve the goal of eliminating VL.

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