Relapse after Treatment with Miltefosine for Visceral Leishmaniasis Is Associated with Increased Infectivity of the Infecting *Leishmania donovani* Strain

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ABSTRACT ***Leishmania donovani* is an intracellular protozoan parasite that causes leishmaniasis, which can range from a self-healing cutaneous disease to a fatal visceral disease depending on the infecting species. Miltefosine is currently the latest and only oral antileishmanial that came out of drug discovery pipelines in the past few decades, but recent reports indicate a significant decline in its efficacy against visceral leishmaniasis (also known as kala-azar) in the Indian subcontinent. This relapse rate of up to 20% within 12 months after treatment was shown not to be related to reinfection, drug quality, drug exposure, or drug-resistant parasites. We therefore aimed to assess other phenotypes of the parasite that may affect treatment outcome and found a significant association between the number of metacyclic parasites, parasite infectivity, and patient treatment outcome in the Indian subcontinent. Together with previous studies on resistance of *L. donovani* against pentavalent antimonials, these data suggest that the infectivity of the parasite, or related phenotypes, might be a more determinant factor for treatment failure in visceral leishmaniasis than drug susceptibility, warranting a reassessment of our current view on treatment failure and drug resistance in leishmaniasis and beyond.**

IMPORTANCE **The high miltefosine relapse rate poses a major challenge for the current Kala-Azar Elimination Program in the Indian subcontinent and other leishmaniasis control programs worldwide. This relapse rate could not be related to reinfection, drug-resistant parasites, or reduced treatment quality. Here we report that an increased infectivity of the parasite is associated with miltefosine relapse of visceral leishmaniasis (VL) patients. These results supplement those obtained with antimonial-resistant *L. donovani* where an increased infectivity was also observed. This challenges the current view of *Leishmania* drug susceptibility being the biggest parasitic factor that contributes to treatment failure in leishmaniasis. These selected more infectious parasites may pose an additional burden to leishmaniasis control programs, highlighting the importance of multifaceted control measures to achieve leishmaniasis elimination in the Indian subcontinent and other regions where leishmaniasis is endemic.**

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Visceral leishmaniasis (VL), or kala-azar, is a life-threatening disease caused by species of the *Leishmania donovani* complex. About 80% of the worldwide new VL cases are reported from the Indian subcontinent (ISC) (India, Bangladesh, and Nepal) (1). In the context of the current Kala-Azar Elimination Program in the Indian subcontinent, the increasingly failing treatment with pentavalent antimonials (sodium stibogluconate [SSG]) was replaced by oral treatment with miltefosine (MIL) in 2006 (2). However, 6.8% of the Indian patients redevelop symptoms of VL (relapse) within 6 months after treatment (3), and 10.8% or 20.0% of the Nepalese patients relapsed within 6 or 12 months after MIL treatment, respectively (4). This significantly undermines the current efforts to eliminate VL from the ISC.

While SSG treatment failure was associated with the presence of SSG-resistant parasites (5), the staggering MIL relapse rate of Indian and Nepalese patients is currently not linked to MIL resistance (4, 6). Moreover, the plasma MIL concentrations in cured and relapsed Nepalese patients were similar, indicating a similar exposure to MIL (4). Parasite fingerprinting of isolates obtained before and after treatment of the same patient suggested that MIL relapse was also not due to reinfection (4). The outcome of these studies therefore suggest that factors other than the drug resistance of the infecting parasite might be linked to the high MIL relapse rate that is currently observed in the ISC.

Another parasite factor that might be of interest is the parasite's virulence, as it was shown to be related to SSG treatment failure (though as a factor secondary to SSG resistance) (7, 8). One important parameter of virulence is metacyclogenesis, the cellular process whereby trypanosomatid parasites differentiate from noninfective procyclic promastigotes to infective (metacyclic)

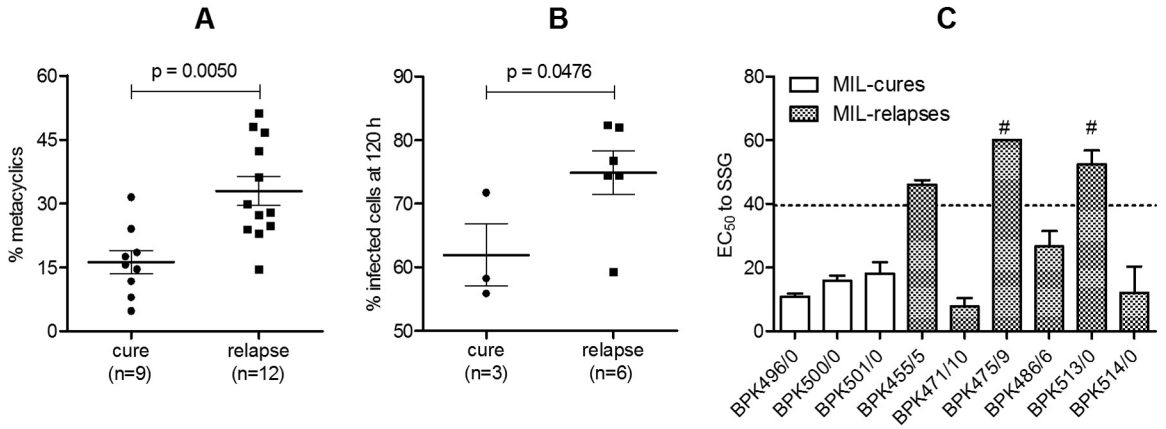

FIG 1 (A) Proportion of metacyclic parasites in stationary-phase promastigote cultures. (B) Percentage of *in vitro* infected macrophages 120 h postinfection. (C) Parasite susceptibility to SSG. Error bars indicate the standard errors of the means, and *P* values were calculated with a Mann-Whitney U test. Each symbol represents the value for an individual patient. The horizontal bars in panels A and B indicate the mean values for the groups. The broken line in panel C indicates the cutoff to determine whether a strain is SSG sensitive or SSG resistant. The # symbol indicates that the EC_{50} of this strain sometimes exceeded the maximum concentration (60 μg/ml) to which it was exposed, and in this case, the EC_{50} of that replicate was fixed to 60 μg/ml.

promastigotes that are preadapted for survival in the host (9). Thus, this is a crucial developmental process of *Leishmania*, since only metacyclic parasites will be able to infect host cells. The main aim of this study was therefore to evaluate whether metacyclogenesis might also play a role in the recent MIL treatment failure observed in the ISC.

Confirmed VL patients (fever for more than 2 weeks with splenomegaly), were recruited at the B. P. Koirala Institute of Health Sciences (BPKIHS) in Nepal from 2010 to 2012. Written informed consent was obtained from each patient. Ethical clearance was obtained from the institutional review boards of the Nepal Health Research Council, Kathmandu, Nepal, and the University of Antwerp, Antwerp, Belgium. Nine patients were considered a "definite cure" as no signs or symptoms were apparent at the end of treatment or at any of the follow-up exams until 12 months after treatment. Twelve patients showed a "relapse," defined by an apparent cure at the end of treatment but reappearance of clinical symptoms with 12 months after treatment: (i) 10 of these 12 patients were recruited in our study, received MIL treatment at BPKIHS, and relapsed within 12 months (prospective relapse); (ii) 2 had received MIL treatment elsewhere and were recruited at the time of the retrospective relapse and received a second treatment at BPKIHS.

Parasites were isolated from bone marrow aspirates of confirmed VL patients and typed as described elsewhere (4). Routine cultures consisted of M199 (Sigma-Aldrich) supplemented with 100 μM adenosine, 0.5 mg/liter hemin, 0.35 g/liter $NaHCO_3$, and 20% fetal calf serum that was inoculated with 5×10^5 parasites per ml and incubated at 26°C until the 2nd to 3rd day of stationary phase.

The MIL susceptibility of 22 *L. donovani* isolates was assessed using an *in vitro* promastigote susceptibility assay as described and validated elsewhere (4, 10). The infectivity of promastigotes to *in vitro* macrophages and the *in vitro* amastigote SSG susceptibility assay was performed on a subset of strains (see File S1 in the supplemental material), using standardized assays that have been described in detail elsewhere (8). At 24 h postinfection (p.i.) for the controls and 120 h p.i. for the controls and the SSG-exposed cultures, *in vitro* amastigote survival was evaluated by counting at least 100 macrophages per well. A strain was considered SSG resistant (SSG-R) if the 50% effective concentration (EC_{50}) of the respective strain exceeded 39.48 μg/ml, which is 3× the average EC_{50} of the SSG-sensitive reference strain BPK206/0cl10 that was included in all SSG susceptibility assays. Mouse care and experimental procedures were performed with approval of the Animal Ethic Committee of the Institute of Tropical Medicine (PAR-020).

Since SHERP (small hydrophilic endoplasmic reticulum-associated protein) and META1, proposed molecular markers for metacyclogenesis in many *Leishmania* species, have previously proven not to be valid for interstrain comparison of *L. donovani* strains (7), we assessed the level of metacyclogenesis through morphology, another commonly used method (7, 11), in combination with *in vitro* infections as functional assays. The procyclic parasites possess large cell bodies with a flagellum of similar length, while the metacyclic parasites are short slender cells with a flagellum length that is considerably longer than the cell body length. Parasite growth was carefully monitored by microscopic evaluation, and smears of cultures were prepared during the stationary growth phase of 21 clinical isolates. Giemsa-stained smears were photographed at a magnification of ×40 using an Olympus BX41 microscope and Cell^D software (version 2.7). The same software program was used to record the flagellum length (*F*), cell body length (*L*), and cell body width (*W*) of at least 100 promastigotes per smear. In Excel, the *F*/*L* ratio and the cell body area ($W \times L$) were calculated. These data were entered in R, version 2.15.2, and the *k*-means method was implemented to classify the parasite population into three clusters with minimal "within-group" variance and maximal "between-group" variance. The percentage of parasites contained in each of these clusters, representing procyclic, transitional, and metacyclic parasites, respectively, were calculated. The exact script and a detailed explanation of the workflow is available in File S2 in the supplemental material.

Data are reported as the means ± standard errors of the means (SEMs) of the cure and relapse groups. Differences between groups were statistically evaluated using a Mann-Whitney U test in GraphPad Prism 5. All data are available in File S1 in the supplemental material.

Previous studies did not yet detect MIL resistance in patients

with MIL relapse (4, 6). This is also apparent in our data set, where the *in vitro* promastigote susceptibility of strains isolated from MIL-treated cured (6.284 ± 1.379 μM; $n = 9$) and MIL-treated relapsed patients (5.163 ± 0.9931 μM; $n = 12$) proved similar ($P = 0.4996$). Morphometric analysis showed more metacyclic parasites in stationary-phase cultures of MIL-treated relapsed strains (32.97 ± 3.372%; $n = 12$) than in strains from MIL-treated cured patients (16.24 ± 2.694%; $n = 9$) (Fig. 1A). This correlated well with the observed significantly increased percentage of *in vitro* infected macrophages at 120 h p.i. by MIL-treated relapse parasites (74.88 ± 3.433%; $n = 6$) compared to MIL-treated cure parasites (61.95 ± 4.941%; $n = 3$) (Fig. 1B; see File S3 in the supplemental material). Similarly, increased metacyclogenesis has also been observed in the context of SSG resistance (7). This urged us to determine whether the increased metacyclogenesis in MIL-treated relapse strains was not due to the occurrence of SSG-resistant parasites. The tested strains from the MIL-treated cured group were all sensitive to SSG, while 3 out of 6 tested strains from the MIL-treated relapse group were SSG-R (Fig. 1C).

The increased metacyclogenesis is the first parasite-related factor that shows an association with the recently observed MIL treatment failure in the Indian subcontinent. A higher parasite load may indeed be more difficult to treat, but there are also several other ways by which the higher capacity to better infect the host may have contributed to MIL relapse. First, this higher infectivity may have caused these parasites to (better) reach specific niches that may not be easily accessible to the drug and from which resurgence of visceral infection after the end of treatment may occur. Another effect might be that the higher parasite load caused by these more infectious parasites allows them to better manipulate the host system, as was observed earlier in the context of SSG resistance (12). Noteworthy, 11 out of the 12 strains from relapsing patients were isolated at the time of relapse. Hence, more research is required to understand whether the occurrence of more infectious parasites contributed to MIL relapse or if the MIL relapse generated parasites with a higher virulence. Therefore, future studies on several pairs of parasites (before treatment and at the time of relapse) are essential.

Interestingly, we demonstrated here that SSG-R strains were still present in Nepal, all of them in relapsed patients treated by MIL, even now that SSG has been abandoned in the region for several years. The sample size is too small to draw any conclusion on a possible heritage of SSG resistance in the outcome of MIL treatment, but this should definitively be monitored closely in the coming years. Metacyclogenesis assessment requires isolation of parasites, which may theoretically result in heterogeneous parasite populations in *in vitro* culture. However, since the *L. donovani* population in the Indian subcontinent is genetically homogenous (13), this likely has a limited impact on phenotypic studies such as the present study.

A higher infectivity of *L. donovani* being associated with both relapse after MIL treatment and SSG treatment failure (7, 8) significantly challenges our current view on visceral leishmaniasis treatment failure in the Indian subcontinent in general. It suggests that infectivity of the parasite, or related phenotypes, might be a more determinant factor for treatment failure in visceral leishmaniasis than drug susceptibility. Our work thus shows that it is highly recommended to also assess phenotypes other than drug susceptibility when characterizing parasites from patients with treatment failure. Our results also call attention to the risk of selecting more infective parasites during chemotherapy (14). Altogether, we advocate for an improved surveillance of VL treatment efficacy in the Indian subcontinent and other regions where VL is endemic.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00611-13/-/DCSupplemental.

File S1, XLSX file, 0.1 MB.
File S2, DOCX file, 0.1 MB.
File S3, TIF file, 0.5 MB.

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