Bone Marrow Parasite Burden among Patients with New World Kala-Azar Is Associated with Disease Severity

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Abstract. Kala-azar or visceral leishmaniasis, found mostly throughout the Indian Subcontinent, East Africa, and Brazil, kills 20,000–40,000 persons annually. The agents, *Leishmania donovani* and *Leishmania infantum*, are obligatory intracellular protozoa of mononuclear phagocytes found principally in the spleen and bone marrow. Protracted fever, anemia, wasting, hepatosplenomegaly, hemorrhages, and bacterial co-infections are typical features. One hundred and twenty-two (122) in-hospital patients were studied to verify if higher bone marrow parasite load estimated by quantitative polymerase chain reaction is associated with severe disease. The estimated median parasite load was 5.0 parasites/10⁶ human nucleated cells. It is much higher in deceased than among survivors (median 75.0 versus 4.2). Patients who lost more weight had a higher parasite burden, as well as patients with epistaxis, abdominal pain, edema, and jaundice. This study suggests that higher parasite load is influenced by wasting, which may lead to more severe disease.

INTRODUCTION

Kala-azar, or visceral leishmaniasis, is distributed worldwide in tropical and sub-tropical areas. The protozoa *Leishmania donovani* is the agent in the Indian Subcontinent and East Africa. In the rest of the world, the agent is *Leishmania infantum*, transmitted among domestic and wild canids and humans.¹ Non-complicated patients have just fever, anemia, and hepatosplenomegaly and the therapy is usually successful. However, even diagnosed patients have a mortality around 10%,^{2,3} killing 20,000–40,000 people annually worldwide.¹

Patients with the most severe disease may have hemorrhagic manifestations, bacterial infections, edema, dyspnea, hepatitis, diarrhea, vomiting, and renal failure.^{3–6} Serious anemia, neutropenia, and thrombocytopenia are laboratorial findings of patients with lethal outcome.⁷ Disseminated intravascular coagulation and thrombocytopenia are the main causes of bleeding.^{8,9} Among the patients with infections, both Gram-negative and Gram-positive bacteria have been found in patients with sepsis and other infections.¹⁰ The reason for the high proportion of bacterial infections is not known, because the well-known immunosuppression seen in kala-azar is specific to *Leishmania*.^{11,12} Other patients with severe disease show renal impairment,^{13,14} hepatitis,¹⁵ and interstitial pneumonitis.¹⁶

The amastigote forms of *L. infantum* and *L. donovani* are found inside neutrophils and mononuclear phagocytes, or freely, mostly in the spleen, which may harbor the largest parasite burden. Bone marrow also is highly parasitized. The liver and lymph nodes are also important sites of infection. Sporadically, parasites can be identified in the peripheral blood.^{17–20}

Regarding bone marrow, parasite load is usually heavier in hypercellular and in necrotic or nodular marrows, whereas the ones with granulomas and with fibrosis show lower intensity of parasitism.²¹ However, the modulation of this parasite burden has not been clearly described; nevertheless, patients

infected with human immunodeficiency virus (HIV) who relapse show more circulating parasites as seen by easy culturing or infectivity to sandflies,^{22,23} indicating that immunosuppression indeed increases parasite burden.

One important feature of kala-azar is the association of immunosuppression with systemic inflammation. Typically, lymphocytes are absent in the spleen and lymph nodes, whereas plasma cells and parasitized mononuclear phagocytes proliferate.¹⁸ These microscopic alterations reflect the profound cell-mediated immunosuppression with exaggeration of humoral immunity.^{24–26} Although a high level of sera or plasma interferon-gamma (INF- γ) and interleukin-10 (IL-10) are observed, low secretion of INF- γ and high production of IL-10 occur during *in vitro* stimulation, which suggests that high levels of circulating regulatory cytokines, such as IL-10, may blunt a specific Th1-type response.²⁷

Although *in situ* response to *Leishmania* fails, exaggerated plasma inflammatory cytokine response is observed,²⁸ showing that immunosuppression coexists with amplified and ineffective innate response, enabling both *Leishmania* proliferation and the development of symptoms. Moreover, the finding that patients with symptoms and signs of more severe disease have higher concentration of serum inflammatory cytokines²⁹ is suggestive that the causal pathway to death by kala-azar involves a dynamic process initiated by immunosuppression leading to a higher parasite burden and subsequently triggering progressive systemic inflammation. We then hypothesized that patients with a higher parasite burden would have a more severe disease. To test this idea, we designed a study to compare bone marrow parasite burden as measured by quantitative polymerase chain reaction (qPCR) in patients with severe kala-azar and to observe its association with the clinical and laboratorial abnormalities that are commoner in patients with lethal disease.

MATERIAL AND METHODS

Patients. The study population was composed of 122 patients from an ongoing cohort study of hospitalized kala-azar patients in Teresina, Piauí, Brazil. From all 145 patients

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admitted from June to August of 2008 who had bone marrow samples, 23 were excluded because of low efficiency of qPCR (21 patients), or a lack of amplification of human or parasite DNA (two patients). All had the typical symptoms of fever, anemia, and hepatosplenomegaly plus positive serology by the indirect immunofluorescence test (Biomanguinhos, Rio de Janeiro, Brazil) or immunochromatographic test (Kala-azar Detect, Inbios, WA) or parasitological tests (direct bone marrow examination or bone marrow culture inoculated in NNN media with Schneider's insect culture media (supplemented with 2% filtered human urine) were included. A single physician examined patients on the first day of admission, when a sample of blood was taken. A standard clinical form, which included demographic information and clinical data, was fulfilled at the first examination. Complications, therapy, and the outcome of discharge or death were also registered. Only the first results of complete blood count and blood biochemistry were recorded. The ethical committee of the Federal University of Piauí approved the study. A written informed consent was obtained from each patient.

Bone marrow aspiration. The volume of 1–2 mL of bone marrow was aspirated from the sternum or ileum, when indicated.³⁰ Samples were distributed in culture media, smear preparation by slides apposition, and stored at -20°C . After staining with panoptic, slides were checked for at least 20 minutes before taken as negative at high magnification. Microscopy was used for diagnostic purposes only, not to estimate the amastigote burden.

Species identification. Isolates were tested with a modified indirect immunofluorescence technique using a fluorochrome conjugated with avidin/biotin accordingly as previously described³¹ with 23 leishmanial-specific monoclonal antibodies: B2, B5, B12, B11, B13, B18, B19, CO1, CO2, CO3, D13, L1, LA2, M2, N2, N3, V1, WA2, W1, W2, WH1, WIC, and T3.^{32,33}

Promastigotes of *L. infantum* isolated from a patient with kala-azar from Brazil were used to construct the standard curve. Initially, the DNA was extracted from 10^6 parasites and the DNA concentration and purity were measured in a NanoDrop 2000 Spectrophotometer (Wilmington, DE). Thereafter, duplicate serial dilutions of 10^4 , 10^3 , 10^2 , 10, and 1^{-1} were prepared. Samples were analyzed in triplicate. The number of parasites was calculated by the geometric mean of the repetitions for each sample.

DNA extraction. Bone marrow was routinely obtained from sternum or iliac crest puncture. Approximately 500 μL were stored, and then frozen at -80°C . The DNA was extracted with the QIAamp DNA mini kit (Valencia, CA), according to the manufacturer's instructions. The DNA was then eluted in 200 μL in buffer AE and stored at -20°C .

DNA quantification. The DNA quantification of *L. infantum* was carried out with qPCR by using the TaqMan technology. The reactions were carried out with primers and probes designed for mini-circle DNA (kDNA) of *L. infantum* (adapted from Rolão and others³⁴): 5'-GGC GTT CTG CAA AAT CGG AAA A-3' (sense); CCG ATT TTT GGC ATT TTT GGT CGA T-3' (antisense) and the probe FAM-TTT TGA ACG GGA TTT CTG - MGB-NFQ, for an amplified product of 72 base pairs (bp). The qPCR was carried out with a final volume of 20 μL , with 4 μL of sample DNA, 0.5 μL of Custom TaqMan Gene Expression Assays (Applied Biosystems - ref. 4331348), composed of primers and optimized

probes ($20\times$), 10 μL of TaqMan Gene Expression Master Mix (Applied Biosystems - ref. 4369016), and 5.5 μL of sterile ultra-pure water. The reactions occurred in 48-well microplates and were processed in the equipment StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) under the following conditions: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s, and 60°C for 1 min. Primers and probes and qPCR conditions for the human albumin gene were selected accordingly to previously published protocol.³⁵

Standard curve. Promastigotes of *L. infantum* isolated from a patient with kala-azar were used to construct the standard curve. The species of the isolate was confirmed by monoclonal antibodies. Initially, the DNA was extracted from 10^6 parasites and the DNA concentration and purity were measured in a NanoDrop 2000 Spectrophotometer. Thereafter, duplicate serial dilutions of 10^4 , 10^3 , 10^2 , 10, and 10^0 were prepared. Samples were analyzed in triplicate. The number of parasites was calculated by the geometric mean of the repetitions for each sample.

Data analysis. Parasite load was expressed as the number of amastigotes by 10^6 nucleated host cells. Comparison between the median of the parasite load according to the presence of outcomes, such as death, symptoms, and HIV status, was carried out using the Wilcoxon rank-sum test. The Spearman correlation coefficient was used for quantitative variables. A P value < 0.05 was generally used but results with a P value < 0.10 for some variables with a large difference of medians were also showed. All tests were carried out using the statistical package Stata 10 (College Station, TX).

RESULTS

Of the 122 patients, 78 were male (64%), 54 (44%) were < 2 years of age, and 19 (17%) were older than 40 years of age. Twenty of the 118 tested were infected with HIV (17%), with four deaths among them (24%). Ten patients died (8%), but only six of those without HIV infection (6%) (Table 1). The estimated mean of the bone marrow parasite load was 457.3 amastigotes per 10^6 human nucleated cells. The median was 5.1 with an interquartile range (IQR) of 0.5–79.8. The minimum value was 4×10^{-5} and the maximum was 2.2×10^4 per 10^6 nucleated cells. The HIV patients had a slightly more elevated median and mean parasite burden, but the difference was not statistically significant. Gender and age were not found to be associated with the estimated bone marrow parasite load.

A clear link between the bone marrow parasite load as measured both by qPCR and by microscopy was observed; patients with amastigotes identified during the bone marrow

TABLE 1
Characteristics of the study population*

Characteristic	Number (%)
Male gender	78/122 (63.9)
Age-group (years)	
0 to < 2	54 (44.3)
2 to < 18	24 (19.7)
18 to < 40	25 (20.5)
≥ 40	19 (16.6)
HIV-1 infection	20/118 (16.9)
Deaths	10/122 (8.2)

*HIV = human immunodeficiency virus.

FIGURE 1. Box plot of the bone marrow parasite load according to the result of the direct bone marrow examination. * Natural logarithm of the number of parasites per 10⁶ nucleated host cell.

smear examination had a much higher estimated parasite load (75.0 [IQR 0.8–274.7] versus 4.2 [IQR 0.3–68.1], $P < 0.001$) of those in whose bone marrow smear parasites were not found (Figure 1).

Nevertheless, patients who died had a much higher estimated bone marrow parasite load than those who survived (median 45.7 [IQR 7.6–180.4] versus 0.8 [IQR 0.1–3.7]), the difference was not statistically significant ($P = 0.09$), possibly caused by the low number of deaths in the data set (Figure 2).

The symptoms and signs that were statistically related to parasite load were weight loss, epistaxis, and abdominal pain. Patients or their relatives who thought they lost weight had a median parasite load more than one log higher than that of those that did not mention weight loss (19.8 [IQR 1.1–143.4] versus 1.5 [IQR 0.1–8.8], $P < 0.001$) (Figure 3). This association was confirmed by the positive correlation between the estimated weight losses with parasite load ($r = 0.29$, $P = 0.002$) (Figure 4). The influence of wasting on parasite burden was further illustrated by the correlation between the z-score of weight-for-age with parasite burden in children younger than 18 years of age ($r = 0.37$, $P = 0.002$) (Figure 4). Patients

FIGURE 2. Box plot of the bone marrow parasite load according to the outcome of the hospitalization. * Natural logarithm of the number of parasites per 10⁶ nucleated host cell.

FIGURE 3. Box plot of the bone marrow parasite load according to the claim of weight loss. * Natural logarithm of the number of parasites per 10⁶ nucleated host cell.

with epistaxis also had higher parasite burden than those without this type of hemorrhagic abnormality (68.1 [IQR 9.7–218.0] versus 3.6 [IQR 0.3–50.9], $P = 0.009$) (Figure 5). The median parasite load of patients with abdominal pain was 19.1 (IQR 1.0–107.0) versus 2.5 (IQR 0.3–29.4), $P = 0.02$.

Parasite load was much higher in patients with edema and jaundice, but the difference was not statistically significant. Patients with edema had a median parasite load of 9.1 (IQR 0.03–115.4), whereas those without edema had a value of 3.6 (IQR 0.3–74.1), $P = 0.06$. Parasite load was also higher in patients with jaundice 38.4 (IQR 4.0–87.9) as compared with 3.6 (0.4–79.0), $P = 0.06$, of patients without icterus. There was no correlation of parasite load with the symptoms of anemia, dyspnea, diarrhea, vomiting, spleen or liver size, bacterial infections, or other hemorrhagic manifestations. Furthermore, we found no correlation with any blood cell count.

DISCUSSION

The estimated bone marrow parasite measured by using the qPCR protocol was endorsed by the microscopic examination. Indeed, the parasite load was almost 20 times higher among patients with a positive microscopy than among those with a negative result. Mary and others³⁵ also found a strong concordance between their qPCR protocols with microscopy in France. Furthermore, the median of parasites was similar to that depicted by the same authors for bone marrow positive samples.

The higher estimated parasite load in the deceased patients, and more clearly, among the patients with more severe disease, with wasting, epistaxis, edema, and jaundice, suggests that the higher the number of parasites present in the bone marrow the more severe kala-azar is. Higher parasite load can be considered as a contributing cause of death because bone marrow aspiration was taken at admission, preceding the lethal outcome.

Wasting has been shown to be a risk factor for the death of patients with Sudanese kala-azar.^{2,6} In fact, malnutrition is a powerful cause of immunosuppression.³⁶ Therefore, wasting may have played a role in the relationship between parasite load and disease severity by both increasing mortality by

FIGURE 4. Correlation and regression lines of the bone marrow parasite load with the estimated weight loss and with the z-score of weight-for-age of children < 18 years old.

allowing opportunistic bacterial infections, also common in visceral leishmaniasis, and by tolerating parasite replication. Interestingly, parasite burden was not associated with HIV status. Although parasitemia seems to be higher in HIV patients³⁷ bone marrow was not affected, likely because of its peculiar characteristic as a primary lymphoid tissue.

Symptoms and signs of severe kala-azar are a consequence of systemic inflammation as shown previously.^{29,38} However, in this study, symptoms, signs, and laboratory tests of severe disease such as hemorrhagic manifestations, edema, jaundice, bacterial infections, and blood cell count were not as strongly associated with parasite burden as wasting was. Epistaxis was the sole exception, possibly a result of the symptom's lack of specificity; in addition to edema and jaundice, it has been identified to be a strong risk factor for death in patients with visceral leishmaniasis.^{3,39} Altogether, these observations suggest that disease-induced malnutrition is the main factor contributing to parasite replication, which secondarily may lead to systemic inflammation, depending upon virulence factors and genetically driven host response^{40–43}. Therefore, the present data suggest that the association of higher parasitemia

with malnutrition and with death may be caused by a nutrition-dependent immunosuppression, leading both to higher parasite burden and to disease severity. However, in opposition to our findings, a previous study²¹ found higher bone marrow parasite burden in patients with better prognosis and higher mortality in those with a low parasite load observed by microscopy. One possible explanation for the distinct findings is that our measurement of parasite load was not absolute, but calculated relative to bone marrow cell count.

Previous work by Verma and others⁴⁴ on parasitemia in Indian post-kala-azar dermal leishmaniasis (PKDL) showed a strong correlation of parasitemia of *L. donovani* with serum levels of IL-10, suggesting that IL-10 leads to higher parasitemia. This observation is in line with previous data showing the role of this cytokine on the control of *L. donovani* in or by the spleen.^{45–47} Our unpublished data with *L. infantum* also shows the same importance of IL-10. Now, the present data suggest that, besides the spleen, bone marrow plays a central function on the pathogenesis of *L. infantum*. Indeed, bone marrow is not just a hematopoietic tissue; its role as a site of primary immune response or memory supplants that of the secondary lymphoid tissues, like the spleen. Moreover, bone marrow is possibly the main site of immune regulation, where a large population of CD4 + CD25 + regulatory cells is present.⁴⁸ Therefore, our data, together with the recent literature, allows the speculation that malnutrition-induced immunodeficiency, jointly with IL-10 from bone marrow or spleen, promotes disease development by favoring parasite replication, which leads to exaggerated systemic inflammation, finally followed by more severe disease and death. One may wonder if inducing wasting is an adaptive characteristic of viscerotropic *Leishmania* to increase transmission.

In conclusion, we found a strong correlation between of kala-azar patients and bone marrow parasite load. Furthermore, patients who died, and those with a dramatic clinical course with epistaxis, edema, and jaundice had an important higher parasite load. These findings suggest that malnutrition may lead to immunosuppression allowing parasite replication. Together with *Leishmania* virulence factors, this process might initiate a vicious cycle in which a high secretion of regulatory IL-10 allows progressive parasite replication,

FIGURE 5. Box plot of the bone marrow parasite load according to the occurrence of epistaxis. * Natural logarithm of the number of parasites per 10^6 nucleated host cell.

which might lead to excessive inflammatory cytokines, systemic inflammation, and mortality.

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