Published in final edited form as:

Eur J Clin Microbiol Infect Dis. 2014 April ; 33(4): 639–649. doi:10.1007/s10096-013-1999-1.

Alteration of the serum biomarker profiles of visceral leishmaniasis during treatment

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Abstract

Until recently, chemotherapy for visceral leishmaniasis (VL; also known as kala-azar) was severely limited by factors such as high cost, route of administration, generation of side effects and potential for resistance. Although largely effective chemotherapies have become available with the introduction of new drugs and multi-drug regimens for VL, these could be further improved by the identification of biomarkers that are altered during effective treatment. The identification of such biomarkers in the circulation would also simplify efficacy trials. In this study we determined immunological signatures within the serum of ethnically and geographically-distinct VL patients (from Bangladesh and Brazil). Our results indicate that inflammatory and regulatory cytokines (IFN γ , TNF α , IL-10, IL-17), as well as levels of growth factors (FGF, VEGF), are elevated within the serum of VL patients from these sites. Examination of samples from Brazilian VL patients during and beyond standard treatment with meglumine antimoniate identified multiple parameters that revert to levels comparable to those of healthy endemic control individuals. The consolidation of these results provides a 'response to treatment' signature that could be used within efficacy trials to rapidly and simply determine successful interruption of VL.

Keywords

cytokines; chemokines; protozoa

Introduction

Many *Leishmania* parasites are pathogenic for humans and an estimated 2 million cases occur each year. Approximately 350 million people in *Leishmania*-endemic areas, although the geographic distribution of each *Leishmania* species affects the clinical manifestations and type of disease (1, 42). Visceral leishmaniasis (VL; also known as kala-azar) is caused by *L. donovani* in South Asia and Africa, while *L. infantum* causes VL in the Mediterranean, the Middle East, Latin America and parts of Asia (1, 42). VL is characterized by an infection of the reticuloendothelial system (RES; spleen, bone marrow and liver) and presents as a systematic inflammatory condition. If left untreated, VL is fatal within months in virtually

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Conflict of interest The authors declare that they have no conflict of interest.

all symptomatic patients and even when successfully treated, post-kala-azar dermal leishmaniasis (PKDL) can occur.

The pentavalent antimonials such as sodium stibogluconate (SSG) and meglumine antimoniate have been widely used to treat leishmaniasis for over 70 years, but resistance to these drugs is increasing (33, 37). Although previously hampered by factors such as high cost, toxicity or route of administration (25), various chemotherapies and additional drugs that are largely effective for the treatment of leishmaniasis are either now available or are being developed (26, 33, 35, 36, 39). Novel therapeutic strategies are also being developed for leishmaniasis to provide more cost-effective and tolerable treatment (38). Treatment strategies have typically investigated parasite burden as a measure of treatment efficacy, requiring invasive procedures such as bone marrow aspiration to collect pertinent samples. The further emergence of improved treatment regimen could be aided by the identification of additional biomarkers that are altered upon successful treatment (27).

The identification of relatively simple serological parameters would both simplify sample collection and expedite analyses of multiple samples. Particular biomarkers that are indicative of the severity of VL disease could be used to aid therapeutic prognosis and, if necessary, guide changes in treatment. Although circulating antibodies can and have been used to regulate entry into clinical trials, multiple lines of investigation indicate that anti-leishmanial antibodies can persist even after successful treatment with chemotherapy and are therefore not appropriate as surrogate indicators of cure (14, 15, 20, 21, 24, 28–30, 34). Other biomarkers indicative of the cellular immunological response to VL disease have been shown to reflect the severity of disease progression and could be useful as markers of recovery following therapy (8). With regard to the cellular immune response, active VL is characterized by a negative delayed type hypersensitivity (skin test) for parasite antigens and failure to produce Th1 cytokines and IL-10 is well documented as the predominant cytokine during active VL (4, 13, 17, 18). Conversely, the protective immunity of VL patients successfully cured by chemotherapy is associated with cell-mediated responses represented by production of Th1 cytokines like IFN γ and IL-2 upon antigen recall (3, 5).

In this study, we assessed various inflammatory cytokines, chemokines and growth factors to generate an extensive immunological profile of VL patients infected with different *Leishmania* species and in different regions. We hypothesized that serum levels of some inflammatory mediators would rise, and concomitantly, some anti-inflammatory mediators would drop, during successful intervention, and tested this hypothesis by assessing the effect of standard meglumine antimoniate treatment on various circulating markers. Our results identify several markers that, when used in conjunction, could facilitate the evaluation of new VL treatments.

Materials and Methods

Study participants

A total of 76 newly diagnosed, previously untreated VL patients were recruited at either the Rajshahi Medical College Hospital, Rajshahi, Bangladesh and Hospital Universitário, Universidade Federal de Sergipe, Aracaju, Brazil). All VL patients were given complete clinical evaluations at the time of recruitment and were provided treatment. Healthy endemic controls (EC) were also recruited in each site. As further controls in Bangladesh, asymptomatic individuals were recruited by positive response in k39 rapid test. As further controls in Brazil, individuals with Chagas disease, tuberculosis and Dengue fever were recruited, as were individuals presenting with enlarged livers but who were negative by k39 rapid test (Table 1). Immune compromised individuals were excluded. Samples were also collected from a cohort of Brazilian VL patients at regular times throughout and after

treatment with meglumine antimoniate (20 mg/kg for 20 days). Blood was collected by venipuncture, sera prepared and stored at -20°C until assay. The study component in Bangladesh was approved by both the icddr and Rajshahi Medical College ethical review committees, and the study component in Brazil was approved by local and national ethics committee. All oversight committees ensured the study and data collection were conducted in accordance with the standards of the declaration of Helsinki. Participants were included after signing the appropriate informed consent forms.

Multiplex determination of biomarkers

Luminex assays were used to assess the serum content of the various biomarkers. Plexes were either custom developed upon request by Millipore (Billerica, MA; CCL2, CCL3, CCL4, CCL7, CCL22, CX3CL1, CXCL1, CXCL8, CXCL10, EGF, FGF-2, Flt3L, G-CSF, GM-CSF, IFN α 2, IFN γ , IL-1 α , IL-1 β , IL-2, IL-3 IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-1ra, sIL-2ra, sCD40L, TGF α , TNF α , TNF β , and VEGF; each analyte was detected across a range of 3.8–25,000 pg/ml) or purchased from Affymetrix (Fremont, CA; MPO, MMP-1, MMP-9, sICAM-1 and sVCAM-1 each analyte was detected across a range of 6.7–400,000 pg/ml). Assays were performed according to the manufacturer's instructions. Briefly, samples were diluted and incubated with anti-target coated magnetic beads. After washing, beads were incubated with detection antibodies, washed again, then incubated with streptavidin-PE. After final washes, PE incorporation onto bead was measured by reading on a Luminex 200 and concentration data were generated using MasterPlex® QT software (MiraiBio Group, South San Francisco, CA).

Parasite detection by PCR

Blood samples were shipped in Paxgene collection tubes containing 6.9mL Paxgene: 2.5mL blood. Prior to extraction, 200ng of a non-homologous linearized DNA segment was added as an internal standard (IS) to regulate extraction efficiency (10). Tubes were centrifuged, then pellets washed twice with nuclease free dH $_2$ O, and DNA extracted using the QIAamp DNA Mini Kit (Qiagen, Frederick, MD). Real time-PCR amplification of *Leishmania* DNA was conducted on a Roche LightCycler 480 (Roche, Pleasanton, CA) using ABI Taqman primers and probes targeting the conserved region of *Leishmania* 18S ribosomal RNA gene (ascension number X0773.1; 5'- CCGTTTCGGCTTTTGGTTGTTTAA; GCGATGGGAAAGCACTTGTC-3'; and reporter (FAM): CAGCTCCATAATCTCC) (32). Each sample was originally examined in duplicate, then samples that did not amplify, or had very late amplification, re-evaluated in triplicate. Samples were semi-quantified by assigning them a threshold cycle (Ct) when DNA was amplified, and were arbitrarily assigned a Ct of 55 cycles when no amplification occurred.

Statistical analyses

Exploratory data analysis, including box-plot and scatter plot, medians and interquartile range (IQR) were used to analyze the analyte levels among different study groups. Statistical significance was assessed by Kruskal-Wallis one way ANOVA followed by Dunnett's Multiple Comparison Test (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego CA). Results were considered statistical significant when p -values < 0.05 were obtained.

Results

Th1/2 cytokine profile of VL patients

The Th1 associated cytokines IL-12 and IFN γ are well known to be associated with clearance of *Leishmania* parasites and clinical cure, while Th2 cytokines inhibit the

protective response and can exacerbate disease (2–5, 13, 17, 18). Of the analytes measured, the Th1-associated cytokines IFN γ , IL-12p40 and IL-12p70 gave the clearest discrimination of patient from control samples (Figure 1 and Supplemental Figure 1). While low to undetectable levels were observed in all control groups in both Bangladesh and Brazil, cytokine levels above 50pg/ml were found in the serum of 60% and 67% (IFN γ), 63% and 75% (IL-12p40), 37% and 38% (IL-12p70) of Bangladeshi and Brazilian VL patients, respectively.

Somewhat paradoxically, Th2 cytokines were also found to be circulating in the sera of minor subsets of VL patients but not controls (Figure 1 and Supplemental Figure 1).). Most notably IL-4 was detected at over 50 pg/ml in the serum of 17% Bangladeshi VL patients and 29% Brazilian VL patients, while IL-13 was above this threshold in 17% and 21% of these VL patients, respectively.

Analysis of regulatory cytokines also revealed significant differences between VL patients and controls (Figure 1 and Supplemental Figure 1). IL-10 was detected at median levels of 159 and 353 pg/ml in VL patients, such that 83% and 92% of Bangladeshi and Brazilian patients had circulating serum levels greater than 50 pg/ml. Serum IL-17 was detected above 50 pg/ml in 53% and 50% of these VL patients, respectively.

Inflammatory cytokine profile of VL patients

The most striking difference in pro-inflammatory cytokine levels in the Brazilian VL population could be observed for TNF α , where 83% VL patients (20 of 24) had circulating levels above 50 pg/ml, in contrast with only a single TB patient that fit this criterion within the control groups (Figure 2). VL patients presented with elevated circulating IFN α 2 levels in comparison with controls (Figure 2). In Bangladesh circulating IFN α 2 levels were greater than 75 pg/ml in 50% VL patients and in Brazil 67% VL patients were above this threshold. In contrast, all controls groups had fewer than 8% recruits presenting with IFN α 2 above 75 pg/ml.

Analysis of samples from Bangladesh indicated that IL-1 α was above 50 pg/ml in the serum of a subset of VL patients (50%, 15 of 30) and asymptomatic individuals (35%, 6 of 17) (Figure 2). A subset of healthy Bangladeshi controls (4 of 10, 40%), however, also fit this criterion. This general observation held true among the samples from Brazil, with IL-1 α levels above 50 pg/ml in 50% VL patients (12 of 24). Similar patterns, albeit at lower levels and reduced overall proportion, were observed for circulating IL-6 in both Bangladeshi and Brazilian groups (Supplemental Figure 1). IL-1 β and TNF β were virtually undetectable, while TGF α was found constitutively in all groups, such that these markers could not discriminate any groups (Supplemental Figure 1).

Growth factor profile of VL patients

A number of growth factors were affected by *Leishmania* infection (Figure 3 and Supplemental Figure 2). Not surprisingly, the T cell growth factors IL-2 and IL-15 were found to be circulating in the blood of VL patients but were absent in controls (Figure 3). The hematopoietic growth factor, Flt3L, was similarly found to be elevated in the sera of VL patients, as were vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF2) (Figure 3 and Supplemental Figure 2). GM-CSF was increased in the sera of Brazilian VL patients versus the negligible levels in the sera of Brazilian controls, although it was present in the sera of all Bangladeshi volunteers such that a difference of VL patient from controls could not be discerned in that population. IL-3, EGF and G-CSF (Supplemental Figures 1 and 2) were not significantly different between VL and EC in either country.

Chemokine profile of VL patients

We also examined a number of chemokines to determine if they could distinguish VL patients from controls (Supplemental Figure 3). The sole chemokine analyzed that demonstrated significant differences between VL patients and EC was CXCL10, although this difference was only noted in the Brazilian population.

Various biomarkers are reduced during treatment

Taken together, our data indicate a variety of serum biomarkers found at levels in VL patients distinct from those observed during other diseases and in healthy individuals. We hypothesized that many of these biomarkers would be altered during the course of anti-leishmanial treatment. To identify which markers were affected, and how rapidly, sera were collected at regular intervals from a cohort of patients undergoing chemotherapy with meglumine antimoniate. Several cytokines were rapidly reduced following the initiation of treatment (Figure 4 and Supplemental Figure 4). Of all the analytes examined, IL-10 was the most rapidly reduced, falling to 20% of time of diagnosis levels by 2–3 weeks of treatment and continued to decline to undetectable levels 6 months after treatment (Figure 4B). TNF and IFN γ levels were also notably diminished during treatment, with both of these inflammatory cytokines reduced by approximately 50% by 2–3 weeks of treatment (p -values < 0.05 versus time of diagnosis). IL-1 α , sIL-2Ra and FGF2 followed a similar decline during treatment (Supplemental Figure 4).

Some biomarkers increase or are not altered during treatment

In contrast to those markers that declined upon treatment, a handful of markers were unaffected and some actually increased (Figure 5 and Supplemental Figure 4). The most obviously increased was sCD40L, although MPO and MMP9 were also significantly increased at various times after treatment (Figure 5). sCD40L and MMP9 were significantly increased as early as two weeks after the initiation of treatment, whereas MPO required 6 months to demonstrate statistically significant elevations.

Correlation of biomarkers with circulating *Leishmania* DNA

DNA was extracted from several blood samples in order to semi-quantify circulating parasite DNA. As expected, and consistent with data generated from samples obtained during therapy, parasite DNA levels were highest in the samples that demonstrated the highest concentrations of IL-10, sIL-2Ra, TNF, IL-6 and IFN γ , and the lowest concentrations of MMP9 (Figure 6). As anticipated, circulating *Leishmania* DNA levels were reduced by treatment (Figure 7). Taken together, our data identify several circulating biomarkers whose concentrations appear linked to magnitude of *Leishmania* infection.

Discussion

Novel therapeutic strategies are being developed for VL to provide more cost-effective and tolerable treatment. These strategies have typically investigated parasite burden as a measure of treatment efficacy, requiring invasive procedures such as bone marrow aspiration to collect pertinent samples. The use of relatively easy to obtain serological parameters could simplify sample collection, facilitate expeditious analyses of multiple samples and accelerate trials of new treatments (27). By analyzing the levels of various chemokines and cytokines in the serum at the time of initial diagnosis, we established an immune profile that distinguished VL patients from healthy individuals and those with other diseases. Furthermore, we identified markers whose concentrations correlated with circulating *Leishmania* DNA (which is likely reflective of parasite burden) and demonstrated that these are changed by treatment.

It has previously been reported in various settings that VL patients exhibit mixed Th1/ Th2 responses and present with elevated levels of circulating IFN γ , TNF α , IL-1, IL-6, and IL-10, and our results corroborated these findings (6–8, 16, 33, 40). We also noted that IL-17 was elevated in subsets of VL patients in both regions, further demonstrating the complexity of the response which involves inflammatory and immune regulatory molecules. In both of our study sites, IL-10 was found at elevated levels in the circulation of the greatest proportion of VL patients. It is noteworthy that IL-10 has also recently been shown that high IL-10 levels correlate with high parasite burden in Indian VL patients (41). Again, our results are in agreement with this observation, although in addition we found that IL-2Ra, IL-6 and IFN γ levels also correlated with circulating *Leishmania* DNA among Brazilian VL patients. Of note, we observed differences between the naturally circulating levels of several markers, including IL-6, between Bangladeshi and Brazilian subjects. The reasons for these differences are unclear, but it is possible that different genetic makeups of the hosts and pathogens, or environmental stimuli, could contribute to this discrepancy.

Identifying and differentiating biomarkers that are altered as a result of infection, active disease or secondary symptoms is difficult. IL-10 and sIL-2Ra were readily measured in the sera of non-VL patients with enlarged livers while circulating IFN γ and FGF2 were not. These data therefore suggest that IL-10 and sIL-2Ra might be more closely associated with symptoms than *Leishmania* infection. Hepatocytes can produce IL-10 that can limit naive CD4 T cell activation and IL-10 can limit fibrosis (19, 31). Thus, although clearly linked with controlling the anti-*Leishmania* immune response, the primary role of IL-10 and the reason for its high circulating levels may be attempts at preserving liver function. It has previously been reported that sIL-2Ra declines rapidly during 30 day treatment with SSG, and patients with the highest sIL-2Ra levels may be predisposed to develop PKDL (12). Our data support the use of declining sIL-2Ra levels as a measure of improving liver condition during VL therapy, but the current study was not sufficiently powered to draw links with PKDL incidence.

We recently reported that Brazilian VL patients present with low sCD40L levels in their circulation in contrast with the high levels observed in endemic controls (9). In Bangladesh, sCD40L levels were indistinguishable between VL patients and endemic controls although, interestingly, asymptomatic individuals had elevated plasma concentrations. This may reflect differing *Leishmania* infectivity rates or may be influenced by the different pathogen compositions of these regions. Levels of sCD40L and MMP9 both demonstrated a continual rise throughout treatment of Brazilian patients. These alterations are consistent with the reported observation that sCD40L can trigger increases in MMP9 production but are somewhat in conflict with a report that *in vitro* infection of human macrophages with *L. braziliensis* increases secretion and activation of MMP9 (22). It is known that neutrophils can enhance the release of sCD40L, which is produced by platelets. It is therefore noteworthy that neutrophil depletion can lead to an increase in *L. donovani* parasite growth following experimental infection (23), and that perturbations in other neutrophil functions have previously been noted in VL patients (11). How the levels of IL-17, which contributes to neutrophil development, are impacted remains unclear. Regardless, these data suggest that the reduced levels of sCD40L during active *L. donovani* infection may be a function of parasite-induced perturbation of neutrophils. This hypothesis remains to be proven.

Among the greatest challenges for development of new intervention schemes for VL is expedient evaluation of candidate strategies, particularly when long periods of time are required to generate data relating to clinical outcome. Determining the immunological profiles that are associated with ongoing or cured VL is valuable for evaluating chemotherapeutic or immunological strategies to treat or prevent VL. Our results provide

further evidence of an immune signature associated with VL and identify parameters that change upon successful treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was funded by grants from the Bill and Melinda Gates Foundation (631 and 39129 to SR and GCE Round 2 Award 53053 to DM), Oficina de Prioridades de Pesquisa para o Sistema Único de Saúde, Fundação de Apoio à Pesquisa e à Inovação Tecnológica do Estado de Sergipe and Brazilian Research National Council (CNPq) (to RA). The authors thank all patients and donors for their participation.

Abbreviations

CL	cutaneous leishmaniasis
DAT	direct agglutination test
IFN	interferon
IL	interleukin
LST	leishmanin skin test
MST	Montenegro skin test
PKDL	post kala-azar dermal leishmaniasis
RES	reticuloendothelial system
VL	visceral leishmaniasis

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Figure 1.
Differential presence of immune modulatory cytokines in VL patients at time of diagnosis. Plasma from clinically diagnosed VL patients and controls were analyzed by luminex assay. # and ## = p -value < 0.05 and 0.01, respectively, versus EC from the same region (Bangladesh or Brazil).

Figure 2.

Differential presence of innate cytokines in VL patients at time of diagnosis. Plasma from clinically diagnosed VL patients and controls were analyzed by luminex assay. # and ## = p -value < 0.05 and 0.01, respectively, versus EC from the same region (Bangladesh or Brazil)

Figure 3.

Differential presence of growth factors in VL patients at time of diagnosis. Plasma from clinically diagnosed VL patients and controls were analyzed by luminex assay. # and ## = p -value < 0.05 and 0.01, respectively, versus EC from the same region (Bangladesh or Brazil)

Figure 4.

Reductions in serum cytokines during anti-leishmanial treatment. Plasma were collected from clinically diagnosed VL patients in Brazil at the time of diagnosis and at various times during or after treatment (20 mg meglumine antimoniate /kg for 20 days). In A, total levels are plotted on the y-axis and time relative to the initiation of treatment is indicated on the x-axis. In B, biomarker levels at each time point were normalized versus the value obtained from the same patient's intake sample (time of diagnosis or within the first week after the initiation of treatment). Time relative to the initiation of treatment is indicated on the x-axis: day 0–7, n = 46; day 14–21, n = 40; 1 month, n = 35; 1.5 month, n = 31; 2 month = 30; 6 month = 7; and 12 month, n = 3. * = *p*-value < 0.05 versus time of diagnosis.

Figure 5.

Biomarkers that rise during anti-leishmanial treatment. Plasma were collected from clinically diagnosed VL patients in Brazil at the time of diagnosis and at various times during or after treatment (20 mg meglumine antimoniate /kg for 20 days). In A, total levels are plotted on the y-axis and time relative to the initiation of treatment is indicated on the x-axis. In B, biomarker levels were also normalized versus the mean value obtained from samples obtained at the time of diagnosis or within the first week after the initiation of treatment. Time relative to the initiation of treatment is indicated on the x-axis: day 0–7, n = 46; day 14–21, n = 40; 1 month, n = 35; 1.5 month, n = 31; 2 month = 30; 6 month = 7; and 12 month, n = 3. * = p -value < 0.05 versus time of diagnosis.

Figure 6.

The relationship of circulating *Leishmania* DNA levels with biomarker concentration. Circulating parasite DNA levels were established within samples by real-time PCR and these are plotted versus relative levels of the indicated biomarkers within matching plasma. An arbitrary threshold of 55 was assigned for PCR negative samples, with the solid line representing the best fit linear regression and the dashed lines representing the 95% confidence intervals.

Figure 7.

Circulating *Leishmania* DNA levels relative to time of treatment. Circulating parasite DNA levels were established within samples by real-time PCR, and these are plotted against time from the initiation of treatment. An arbitrary threshold of 55 was assigned for PCR negative samples, with the solid line representing the best fit linear regression and the dashed lines representing the 95% confidence intervals.

Table 1

Summary of study participants.

Site	Sample categorization (total number)	Sex (M/F)	Mean age (range, years)
Dhaka, Bangladesh	VL (30)	20/10	23.0 (4–55)
	asy + (17)	10/7	25.0 (6–55)
	EC (10)	5/5	25.8 (9–55)
Sergipe, Brazil	VL (46) ^I	27/19	11.3 (2–44)
	enlarged liver (9)	-	-
	Chagas (16)	-	-
	TB (16)	-	-
	Dengue (16)	-	-
	EC (63)	-	-

^I a subset of 24 samples were used for the generation of time of diagnosis data (day 0) while all samples (days 0–7) were used for the generation of effect of treatment data.