Serum soluble markers in the evaluation of treatment in human visceral leishmaniasis

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SUMMARY

Visceral leishmaniasis (VL) has a fatal course if not properly treated. Recovery from VL is linked to cellular immune response. Unresponsiveness to antimonial therapy reinforces the importance of determining parameters for treatment assessment. We analysed the pre- and post-treatment serum levels of soluble CD4 (sCD4), sCD8, sIL-2R, soluble intercellular adhesion molecule-1 (sICAM-1) and neopterin in groups of VL patients either responsive or not to standard antimonial therapy. Pretreatment serum levels of all markers except for sICAM-1 were significantly higher in VL patients than in healthy subjects from the same area ($P < 0.05$). sICAM-1 levels were similar in healthy controls and in VL patients refractory to antimonial therapy ($P = 0.25$), but significantly higher in patients responsive to treatment ($P = 0.02$). The comparison of pre- and post-treatment concentrations showed that all markers, except sCD4 and sICAM-1, presented a significant fall ($P < 0.05$) in patients responsive to antimonial therapy. However, only neopterin presented with levels compatible with those of healthy subjects at the end of treatment ($P = 0.30$). In refractory patients sICAM-1 presented with post-treatment levels significantly higher than the pretreatment determinations ($P = 0.03$), while sCD4 experienced a significant drop ($P = 0.01$). All markers displayed clearly distinct behaviour according to the patient's response to therapy. This makes all soluble molecules studied suitable for use as indicators of antimonial therapy response. Additionally the comparison of pretreatment levels of the markers between responders and refractory patients to antimonial therapy showed that serum concentrations of sIL-2R and sICAM-1 significantly differed among these two groups ($P = 0.02$ in each case), suggesting that they may be used in future as predictors of antimonial therapy response.

Keywords visceral leishmaniasis soluble markers antimonial

INTRODUCTION

American visceral leishmaniasis (VL) is a parasitic disease caused by the protozoan *Leishmania chagasi*. VL affects millions of people in tropical and subtropical regions of the globe, and has a fatal course if not treated [1,2]. Antimonial compounds, 10–20 mg/kg per day for 20 days, have been the therapy of choice for decades. Unfortunately, relapses and unresponsiveness to antimonial therapy have been widely reported, resulting in prolonged disease frequently accompanied by life-threatening bacterial infections that further darken the patient's outcome [3–6].

Currently, response to therapy in VL is assessed by clinical and parasitological parameters. Clinical and haematological improvement are highly influenced by the overall condition of

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the patient, such as nutritional status and coexistence of common causes of chronic blood loss. Splenomegaly may never completely subside in selected cases where portal hypertension evolved [7], and may also be caused by other conditions common in endemic areas of VL. Detection of parasites from splenic aspirates [8] has risks inherent to the procedure and needs experienced professionals to perform it in endemic areas, making its use non-viable for continuous therapeutic evaluation. Furthermore, persistence of VL organisms in treated patients has been reported [9].

In recent years, the concentrations of different soluble molecules in diverse body fluids have been employed as a safe, easy and fast means for the prediction and continuous assessment of therapeutic response in several disorders [9–15]. Such markers are intracellular or cell surface molecules shed into the circulation during the pathogenic process of the disease (e.g. cell activation, cell lysis), and reflect the progress of the underlying condition and the effectiveness of the treatment

Table 1. Levels of soluble markers of the immune system in the serum of visceral leishmaniasis (VL) patients before and after antimonial therapy

Patient no.‡	sCD4 (U/ml)		sCD8 (U/ml)		sIL-2R (U/ml)		sICAM-1 (ng/ml)		Neopterin (nmol/ml)	
	pre	post	pre	post	pre	post	pre	post	pre	post
1	22·26	6.58	1204	988	3404	4456	375	759	11·64	17·80
2	34·82	17·66	1132	582	>12500†	8087	444	800	43·51	5·28
3	26·66	16·62	268	662	4846	4362	62	844	29·15	8·57
4	31·26	18·30	2090	544	5385	1465	444	272	95·67	3·86
5	21·22	10·98	1156	1206	6216	5537	330	692	14·55	36·65
6	19·76	15·16	1352	1714	9334	10908	311	699	25·41	47·74
7	11·80	15·16	472	794	10132	3187	108	336	3·86	3·33
8	28·76	15·36	2934	658	>12500	1418	ND	ND	58·42	3·40
9	26·88	20·18	1802	2766	9864	>12500	832	826	31·36	33·84
10	ND*	ND	2162	2460	ND	ND	ND	ND	33·84	65·26
11	22·26	17·04	1800	698	12210	2356	390	413	8·88	4·89
12	10·76	15·78	1242	688	>12500	2385	1314	498	25·41	4·04
13	14·32	16·62	688	1730	>12500	>12500	616	673	ND	ND
14	25·62	15·78	1574	690	7306	2483	1092	246	16·89	3·08
15	17·46	12·02	3156	480	>12500	5669	505	310	43·51	3·08
16	34·40	24·16	1998	764	>12500	3688	896	641	52·65	5·89
17	24·58	20·18	2008	920	>12500	>12500	158	0·0	95·67	5·28
18	37·54	15·36	1176	2058	3891	>12500	ND	ND	21·07	25·41
19	15·58	33·98	1852	552	>12500	4286	1784	368	ND	ND
20	ND	ND	3304	926	ND	ND	ND	ND	83·40	5·14

* ND, Not determined due to insufficient material.
† > 12 500 level was above the detection limit of the test.
‡ Patients 1–10 were refractory to antimonials; patients 11–20 were responsive to antimonials.

without necessarily playing an active part in the pathogenic process. Unlike splenic aspirates, the collection of samples for analyses offers minimal risk, and the laboratory techniques, usually ELISAs and radio immunoassays, are fast and require a minimum of training to be reliably performed.

The existence of alternative therapeutic regimens for the treatment of VL cases prompts for the detection of parameters capable of assessing and predicting the ongoing therapeutic response to antimonials in these patients. In the present study, we analysed the serum levels of soluble markers of the immune

Table 2. Levels of statistical significance of the comparisons between serum concentrations of soluble markers in visceral leishmaniasis (VL) patients (before and after antimonial therapy) compared with values from healthy subjects from the same endemic area

Markers	Responders		Refractory	
	Before	After	Before	After
sCD4	<0·01*	<0·01	<0·01	<0·01
sCD8	<0·01	0·01	0·02	<0·01
sIL-2R	<0·01	<0·01	<0·01	<0·01
sICAM-1	0·02	0·05	0·25	<0·01
Neopterin	0·01	0·30	0·01	0·03

*P values obtained from Wilcoxon's rank test, between indicated patient group and normal controls.

system thought to be directly or indirectly involved in the pathophysiology of VL. Soluble IL-2 receptor (sIL-2R), sCD4, sCD8 and soluble intercellular adhesion molecule-1 (sICAM-1) and neopterin were evaluated in VL patients, before and after antimonial therapy. sIL-2R has been demonstrated to be a major mediator of the non-specific immunosuppression observed in VL patients [16]. Neopterin is produced by activated phagocytes and its levels have been employed as a marker of phagocyte activation, disease progression and therapeutic response in several conditions [11]. sCD4 and sCD8 have been evaluated due to the potential role of suppressor T cells in the Leishmania antigen-specific immunosuppression observed during VL [17], and because of reports showing that a relative increase in $CD8^+$ and decrease in $CD4^+$ T cell subpopulations occur during active disease [17–19]. ICAM-1 is an adhesion molecule present on the surface of endothelial cells and fibroblasts [20], whose expression is induced by proinflammatory lymphokines such as interferon-gamma (IFN-γ) and IL-1 [21,22]. The observations that mononuclear cells of VL patients are unable to secrete IFN-γ upon stimulation with leishmanial antigen [23], and human peripheral blood monocytes infected in vitro with L. donovani do not secrete IL-1 after challenge with leishmania [24], led us to investigate whether the levels of sICAM-1 in the serum of these patients could give insight into evaluating the outcome of treatment.

The population analysed in this study comprised antimonial responsive and refractory VL patients. The concentrations of each of the above mentioned serum soluble molecules were determined before and after antimony therapy.
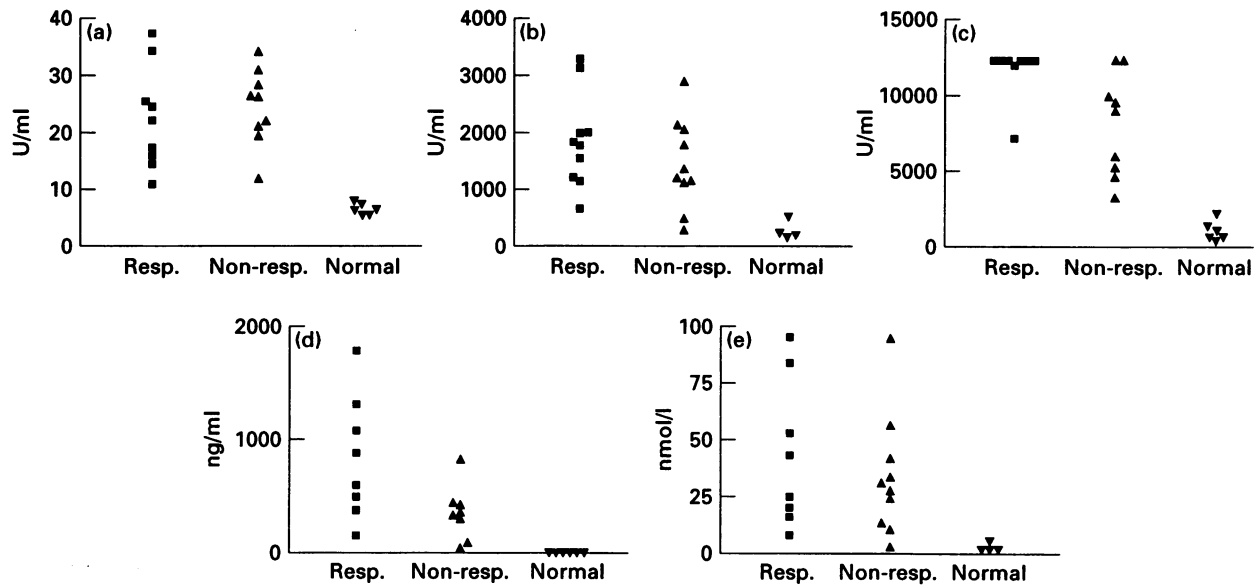
Fig. 1. Levels of soluble (s) CD4 (a), sCD8 (b), sIL-2R (c), soluble intercellular adhesion molecule-1 (sICAM-1) (d) and neopterin (e) in visceral leishmaniasis patients who were later proved to be responsive (Resp.) or refractory (Non-resp.) to antimonial therapy compared with levels of control subjects (Normal) from the same area. Results are means of duplicate determinations of each sample and each point represents a single patient.

PATIENTS AND METHODS

Patients and groups

All patients enrolled in the present study came from endemic areas of VL in the state of Bahia, north-eastern Brazil. Clinical diagnosis of VL was confirmed by at least one laboratory test (serology or parasite cultivation). All patients received antimonial therapy. After treatment, each patient was re-evaluated according to the same clinical and laboratory criteria and classified into one of two groups: (i) responsive to antimonial therapy; and (ii) refractory to antimonial therapy. Clinical information regarding study patients has been published elsewhere [25,26].

Sample collection and storage

Blood samples were collected from each patient before treatment and 30 days post-treatment at the moment of re-evaluation. Serum was collected, aliquoted and frozen at −20°C until tests were performed. Repeated freezing and thawing was avoided by dividing the tests into two batteries, in between which the samples were maintained at 4°C.

Detection of soluble receptors and neopterin in serum samples

Levels of sCD4, sCD8, sIL-2R and sICAM-1 in the serum samples were determined by ELISA (T Cell Diagnostics, Inc., Cambridge, MA). Serum levels of neopterin were determined by radioimmunoassay (IMMUtest Neopterin; Henning, Berlin, Germany). All tests were conducted as described by the manufacturers. Serum levels of the markers were calculated by interpolating the optical density readings of test samples in standard curves calculated from samples of known concentrations supplied with the kits and assayed in parallel.

Statistical analysis

All statistical analyses performed employed non-parametric tests. Signed Wilcoxon rank test was used in comparisons

among individuals of the same group, while Wilcoxon rank test was used in comparisons between different groups. $P < 0.05$ was considered significant. The range of normal values for each soluble marker was considered to be that within the limit of 1·96 s.d. of the average concentrations observed in samples of healthy individuals of the same region assayed in parallel.

RESULTS

Pretreatment serum levels of soluble markers

The serum levels of sCD4, sCD8, sIL-2R, sICAM-1 and neopterin in VL patients responsive and refractory to antimonial therapy (as well as normal controls) were determined by ELISA or radioimmunoassay immediately before and after treatment (Table 1). The results of the pretreatment determinations, those of normal controls and the number of samples tested in each group are displayed in Fig. 1. Levels observed in pretreatment samples of VL patients were higher than those in normal controls for all markers (Fig. 1 and Table 2).

Serum concentrations of sCD4, sCD8, sIL-2R, sICAM-1 and neopterin determined before antimonial therapy were compared between responders and refractory VL patients, and the results are listed in Fig. 1 and Table 3. Serum concentrations of sCD4, sCD8 and neopterin did not vary significantly between responders and refractory patients. Pretreatment levels of sIL-2R and sICAM-1 were higher in responders than in refractory patients ($P = 0.02$, for both markers). These distinct pre-therapeutic behaviours of sICAM-1 and sIL-2R among VL patients indicate that these markers may prove useful as variables with predictive value on antimonial therapy outcome.

Post-treatment serum levels of soluble markers

Post-treatment serum levels of sCD4, sCD8, sIL-2R and

Table 3. Levels of statistical significance of the comparisons between serum concentrations of soluble markers in visceral leishmaniasis (VL) patients responsive and refractory to antimonial therapy

Marker	Pre × pre*	Responders†	Refractory†
sCD4	0·47	0·21	0·01
sCD8	0·26	0·03	0·96
sIL-2R	0·02	0·03	0·21
sICAM-1	0·02	0·06	0·03
Neopterin	0·62	0·02	0·65

* Pretreatment samples from responder patients compared with those from refractory patients by Willcoxon's rank test.

† Comparisons of pre- with post-treatment samples from the same groups by signed Willcoxon's rank test.

sICAM-1 in VL patients were significantly higher than those observed in healthy subjects (Table 2). VL patients responsive to therapy presented with elevated serum levels of neopterin before treatment ($P = 0·01$) and levels compatible with those of healthy subjects after therapy ($P = 0·3$). Determinations of neopterin in refractory patients were significantly higher than in the control group both before and after therapy (Table 2).

Progression of soluble marker levels in serum of VL patients

The results observed before and after treatment within each group were compared (Fig. 2), and the levels of statistical significance are displayed in Table 3. This was carried out in an attempt to assess the evolution in serum levels of the studied markers according to the response to antimonial therapy. There was no patient in whom all markers displayed the same evolution (i.e. either raised or dropped), indicating a heterogeneity in the behaviours of these markers. Analysis of Table 3 and Fig. 2 reveals that successful antimonial therapy resulted in

a significant drop in the serum levels of sCD8, sIL-2R and neopterin among responders ($P < 0·05$). On the other hand, refractory patients presented a significant drop in sCD4 ($P = 0·01$) and an increase in sICAM-1 ($P = 0·03$). sCD4 and sICAM-1 did not show significant differences between pre- and post-treatment serum levels in responders. sCD8, sIL-2R and neopterin did not change in refractory patients.

DISCUSSION

The serum levels of several soluble molecules of the immune system were analysed in VL patients responsive or refractory to antimonial therapy. Overall, data for each time point showed a high degree of dispersion in both groups of VL patients. This was not observed among control subjects, suggesting the variation may be due to the underlying condition: (i) the participants enrolled in the study at different stages of disease; and (ii) individual variability of the immune response of patients. The concept of variable clinical courses in VL is further supported by the observations that not all infected individuals develop full blown VL, and that incubation periods may vary widely from a few weeks to years. Non-parametric tests based on median rather than mean concentrations of serum markers were used in order to alleviate the effects of this dispersion in the analysis of data. Paired versions of these tests were used when applicable to decrease even further any bias caused by the dispersion of the data.

Serum levels of all studied markers displayed an outcome-specific evolution, making them suitable for the evaluation of the ongoing treatment. We particularly think that the frequent monitoring of markers that displayed opposite behaviours, like sCD4 and sCD8, would be an easy, objective and safe way of continuously evaluating potential therapeutic failure or success in VL patients undergoing antimonial therapy.

The early normalization of serum levels of neopterin in responders gives it a potential role as indicator of treatment
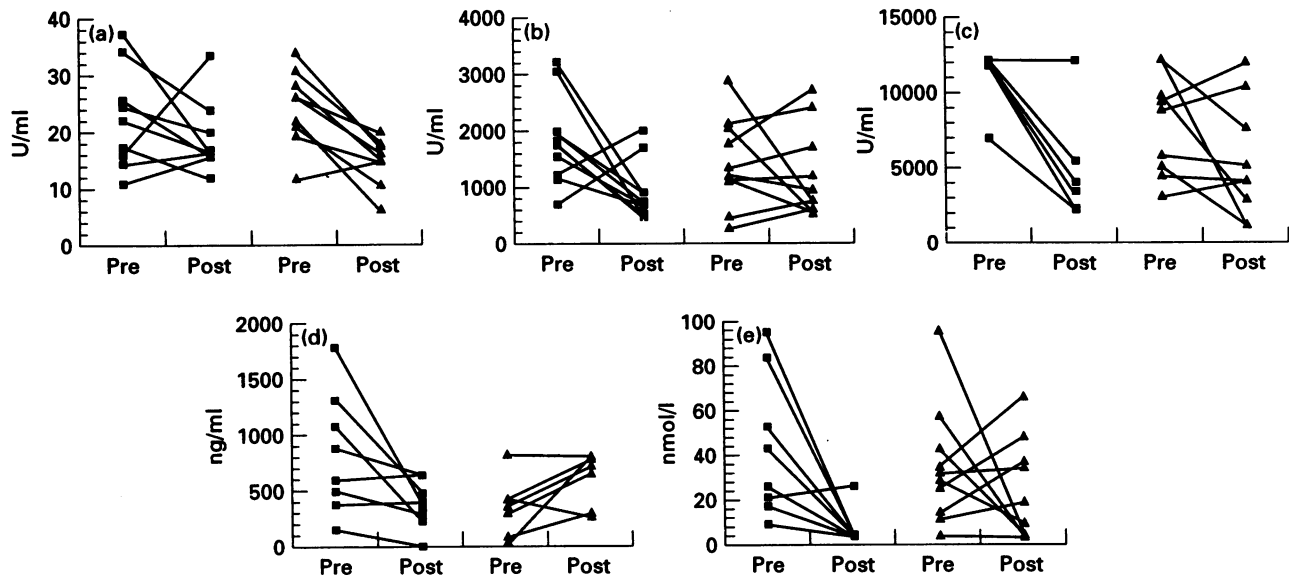
Fig. 2. Pre- and post-treatment serum levels of soluble (s) CD4 (a), sCD8 (b), sIL-2R (c), soluble intercellular adhesion molecule-1 (sICAM-1) (d) and neopterin (e) in visceral leishmaniasis patients who responded (■) or were refractory (▲) to antimonial treatment. Pre- (left) and post-treatment (right) levels for each patient are linked by lines.

success. In analogy to observations in malaria, we believe that the normalization in serum levels of neopterin detected in responsive patients at the end of the treatment course reflects the clearance of parasites from their phagocytic reservoir in the host.

The significant differences observed in pretreatment levels of sIL-2R and sICAM-1 among responder and refractory patients suggest the feasibility of identifying individuals who would most probably benefit from treatment. Larger samples will be needed to trace precisely the threshold between serum concentrations of sIL-2R predictive of therapeutic success or failure, since the concentrations of this marker were significantly elevated in both groups of patients in comparison with normal controls. A possible explanation of the observed association of high pretreatment levels of sIL-2R and sICAM-1 and favourable treatment outcome, would be that these soluble molecules are early indicators of T cell response and consequent IFN-γ production in VL. Activated T cells release sIL-2R in the circulation [27] and produce IFN-γ, the major activator of leishmanicidal activity of phagocytes. IFN-γ is able to up-regulate ICAM-1 expression [21,22]. So the observations that sIL-2R and sICAM-1 are more elevated in the serum of VL patients that successfully responded than in those that did not respond to antimonial therapy may indicate that the former individuals had a more favourable background to fight the infection, one which was synergized by the treatment. This line of thinking helps resolve the apparent paradox that sIL-2R, the non-specific immunosuppressor observed in the VL patients' serum, and sICAM-1, shed into the circulation during the course of tissue-destructive processes, are not indicative of poorer outcome.

Drops in the levels of tumour necrosis factor-alpha (TNF-α) [26,28] and of IFN-γ and IL-6 [28] have been demonstrated after therapy in VL. To date only sIL-2R has been evaluated in its ability to predict or monitor treatment outcome in VL. Barral-Netto *et al.* [16] and Vitale *et al.* [9] showed that the high pretreatment serum levels of this soluble receptor had dropped significantly at the end of successful antimonial therapy, and concluded that sIL-2R could be employed as a marker of disease activity. In the present study we confirmed these observations and extended this analysis to refractory patients. We found that levels of sIL-2R drop during treatment in both groups of patients, although much less markedly in non-responders. Thus, we think that sIL-2R has the potential to be used as a marker of therapeutic response and for the prediction of treatment outcome. However, as discussed above for the use of sIL-2R levels for prediction of treatment outcome, larger samples must be evaluated for better defining the dropping ratios that would be indicative of good therapeutic response.

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