

## Amastin Peptide-Binding Antibodies as Biomarkers of Active Human Visceral Leishmaniasis

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**Amastin surface proteins belong to a large family of developmentally regulated proteins comprising up to 45 members that have recently been discovered in the genus *Leishmania* and are highly similar to the amastin proteins in *Trypanosoma cruzi*. All members of the amastin gene family contain a highly conserved 11-amino-acid (aa) signature at the N terminus, which is unique to the amastin proteins and to the *Trypanosomatidae* family. Recent studies have demonstrated that this region is highly protective in a mouse model. The goal of the present study was to evaluate the potential of the 50-aa N-terminal domain of amastin proteins harboring the conserved 11-aa amastin signature peptide as a relevant immune biomarker of cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). We report here the amastin-binding total immunoglobulins (IgG) and/or IgG subclasses in the sera of patients at different stages of CL ( $n = 90$ ) and VL ( $n = 41$ ). In CL cases, there is no significant difference in seroreactivities between active, recovered, and nonhealed cases. However, the amastin peptide-reactive antibodies were present at high titers in 19 of 20 sera collected from patients with active VL compared to sera from patients recovered from VL and asymptomatic cases of VL. These data suggest that the amastin signature peptide could represent a relevant biomarker for the serodiagnosis of VL and, most importantly, that it could permit differentiation among the different stages of the disease.**

*Leishmania* protozoa are the causative agents of human leishmaniasis in nearly 90 countries, threatening over 350 million people, more than 12 million of whom are infected (1). Clinical manifestations of this disease range from self-healing cutaneous leishmaniasis (CL) to debilitating mucocutaneous leishmaniasis to life-threatening visceral leishmaniasis (VL). A measure of the seriousness of this parasitic disease is the two million new cases of human leishmaniasis that appear annually throughout the developing world (28). *Leishmania* organisms in the form of metacyclic promastigotes are transmitted to humans through the bite of an infected female sandfly. Within a short time, the promastigotes are taken by professional phagocytes (neutrophils, monocytes, and macrophages), as well as dendritic cells and fibroblasts. During this process, the promastigote loses its flagella and transforms into the non-flagellated amastigote form, in which it multiplies exclusively in the phagolysosomal compartment of macrophages (14).

At present, most diagnostic tools from PCR to antigen-based enzyme-linked immunosorbent assays (ELISAs) are not suitable for field conditions, and the diagnosis of VL still relies on bone marrow puncture (30). A better knowledge of *Leishmania* proteins should allow us to improve the diagnostic markers. The goal of the present study is to evaluate the serum reactivities of different stages of CL and VL cases to the conserved extracellular domain of newly identified surface antigens in *Leishmania* harboring the amastin signature sequence

(29). Amastins belong to a large family of surface proteins that have recently been discovered in the genus *Leishmania* (21) and show high similarity to the amastin proteins in *Trypanosoma cruzi*. The members of the amastin gene family, approximately 45 in all, are dispersed all through the genome of *Leishmania*. Amastin genes usually code for small proteins of about 200 amino acids (aa) (21). Amastins are unique to kinetoplastids and are expressed specifically in the amastigote stage of the parasite (21, 29). Amastin gene expression is up-regulated by a decrease in the pH (one of the properties of the intraphagolysosomal space). Due to its relative hydrophobic sequence and its localization in the membrane, it is believed that the amastin family plays a role in proton or ion traffic across the membrane to adjust the cytoplasmic pH. Nonetheless, the role of the amastin gene family in the virulence of *Leishmania* is still unclear (21). All members of the amastin gene family possess two predicted extracellular domains. The first domain, located between transmembrane helices 1 and 2, is 55 to 60 aa long and contains a highly conserved sequence of 11 aa at positions 52 to 62 that is present in all of the *Leishmania* and *Trypanosoma* amastin homologs and corresponds to the amastin signature sequence. This well-defined domain was not found in any other protein, which suggests that amastin surface proteins are probably unique to trypanosomatid protozoa. Although the putative function of the amastin signature sequence remains elusive, a recent report by Stober et al. showed that the N terminus of amastin proteins (aa 1 to 63) that harbors the 11-aa (CITLWGLKTDC) amastin signature sequence is highly immunogenic and induces protection in mice (27). In addition, Salotra et al. showed an upregulation of class III amastins (29) in post-kala-azar dermal leishmaniasis

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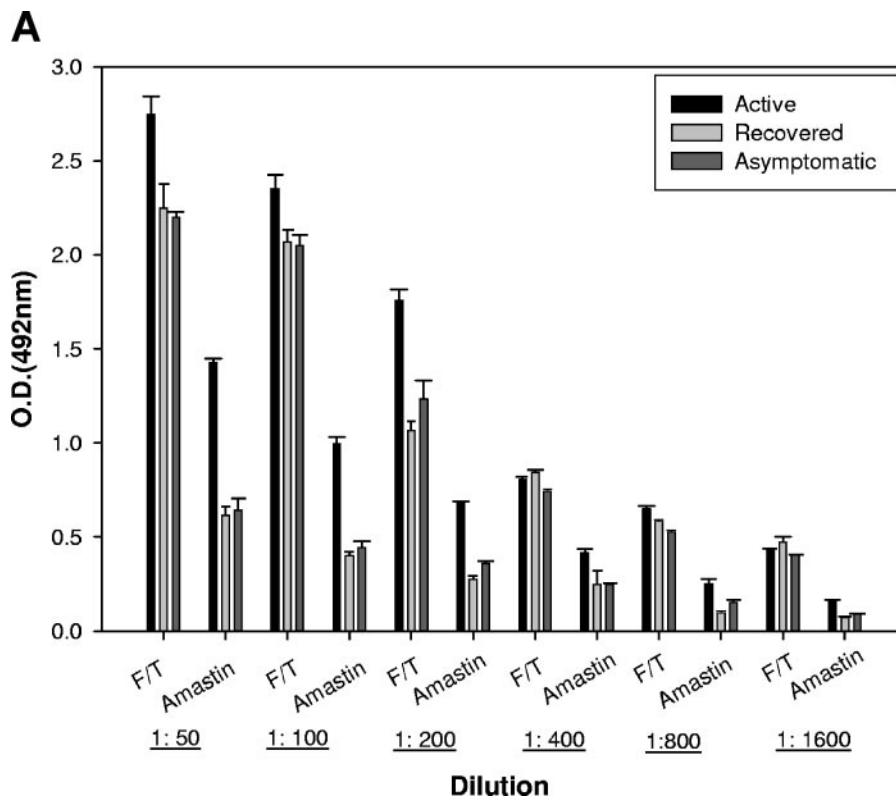


FIG. 2. Detection and concentration of amastin-binding antibodies in sera from patients with active VL. (A) Microtiter plates were coated with either 10 or 20  $\mu\text{g}$  of *L. infantum* lysates or the amastin signature peptide/ml, respectively. Six sera of active VL cases were pooled, and serial dilutions were made. Anti-human peroxidase-conjugated antibody at a 1:1,000 dilution was used. The results are given as the mean optical density (O.D.)  $\pm$  the SD at the indicated dilutions. (B and C) Western blotting analysis was performed by using 2  $\mu\text{g}$  of amastin signature peptide and 1  $\mu\text{g}$  of F/T lysates of *L. infantum*/lane and six sera samples (A to F) diluted to 1:100. Low-molecular-mass markers are indicated in kilodaltons on the left.

## RESULTS

**Sequence alignment of the N-terminal domain of four *L. major* amastin members and one *L. infantum* amastin member.** The alignment of the predicted amino acid sequence within the first conserved extracellular domain of several amastin homologs harboring the amastin signature sequence is shown in Fig. 1. Homologs within the *L. major* chromosomes 8 (LmjF08.0800; six identical copies) and 34 (LmjF34.0960 and LmjF34.0970) and the *L. infantum* amastin homolog LinJ34.0840 were compared. Sequence homology within the amastin signature region ranges from 48 to 100% among different *L. major* amastin homologs and is at 53% between the *L. major* (LmjF08.810) and *L. infantum* (LinJ35.840) homologs.

**Detection of amastin-binding antibodies in sera from patients with active VL.** To evaluate the antigenicity of the amastin signature peptide, a mixture of 10 active sera isolated from patients with VL was analyzed by ELISA using a highly pure synthetic peptide harboring the amastin signature sequence and crude lysates (F/T antigen) from *L. infantum* promastigotes. A titration curve was determined, and the optimal concentrations were found to be 10  $\mu\text{g}/\text{ml}$  for F/T antigens and 20  $\mu\text{g}/\text{ml}$  for the amastin signature peptide. A strong reactivity of pooled sera from six patients with VL was observed toward promastigote crude antigen preparation, with no significant differences between different dilutions and between the three

groups of active, asymptomatic, and recovered VL (Fig. 2A). In contrast, the sera from active patients with VL demonstrate a strong antibody response against the amastin signature peptide, which is significantly different from that observed with asymptomatic and recovered VL groups (Fig. 2A). The reactivity against the crude antigen preparation from *L. infantum* promastigotes (F/T antigen) and the amastin signature was also confirmed by Western blotting using sera from six patients with active VL (A to F) (Fig. 2B and C). As we have previously reported, there are several antigens in the F/T lysate that were strongly revealed by sera from active VL cases (20). These bands are mainly p14 and p18 and proteins with molecular masses of 60 to 62 kDa. As shown in Fig. 2C, all sera were also reactive with the amastin signature peptide (the upper part of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel does not contain any band).

**The amastin-reactive antibodies are present at higher titers in sera collected from patients with active VL and are potentially relevant immune signatures for detecting VL.** In order to determine whether the amastin signature synthetic peptide could be considered a valuable tool for the serodiagnosis of CL and/or VL, we analyzed the reactivity of 90 CL and 40 VL sera against this peptide. According to the clinical features of each donor, sera were separated into three groups for both CL and VL patients. For the CL patients, the first group was composed of sera from 30 individuals with recent active lesion without

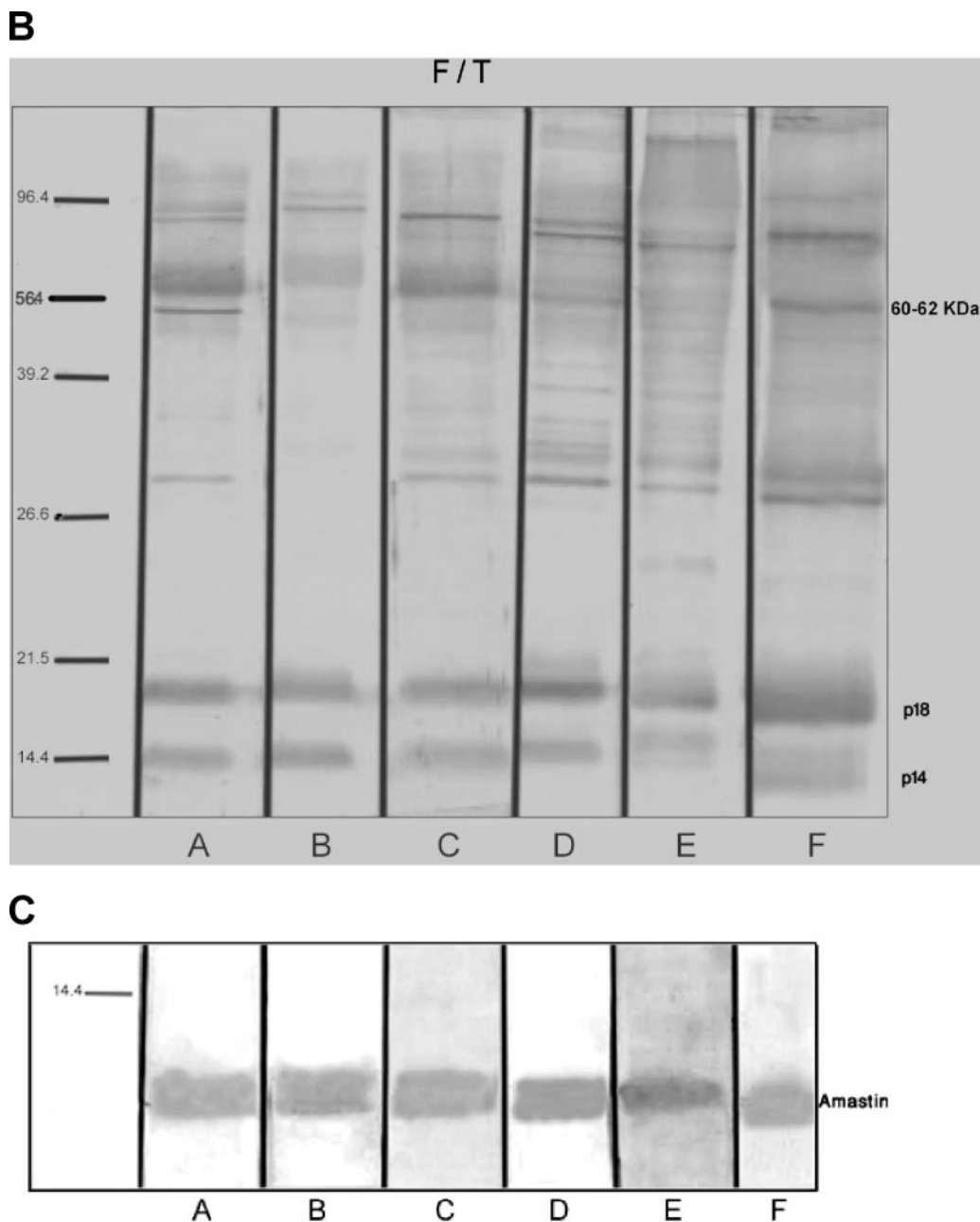


FIG. 2—Continued.

any treatment (all LST negative). The second group consisted of nonhealed cases and was composed of sera from 30 individuals who had had the lesion for more than 3 years (90% LST negative). The third group included 30 individuals who had recovered completely from disease, and all had positive LSTs. We used 10 normal sera that have been collected from individuals who live where the disease is not endemic and the LST was negative. Figure 3 shows the mean reactivity values for each group of CL sera against the amastin signature peptide. The CL active sera reacted with a mean absorbance value of 0.55 (SD = 0.24), nonhealed sera reacted with a mean absorbance value of 0.49 (SD = 0.24), and recovered sera reacted with a mean absorbance value of 0.52 (SD = 0.12). We found

that 20% of sera from active CL patients, 8% of sera from nonhealed individuals, and 16% of recovered sera were higher than the cutoff value (mean of normal serum reactivity + 5 SD), which is 0.76. The difference between all three CL groups and normal individuals is highly significant ( $P < 0.05$ ). In contrast, the serum reactivities between these three groups are not significant ( $P > 0.05$ ). Therefore, in CL cases, it is not possible to differentiate between disease stages using the amastin signature peptide. The serum reactivity toward the *L. major* F/T antigen preparation in all three groups is significantly higher than in normal cases, and it is also higher than the response against the amastin signature peptide (Fig. 3). Thus, in the case of CL patients, we were not able to differentiate



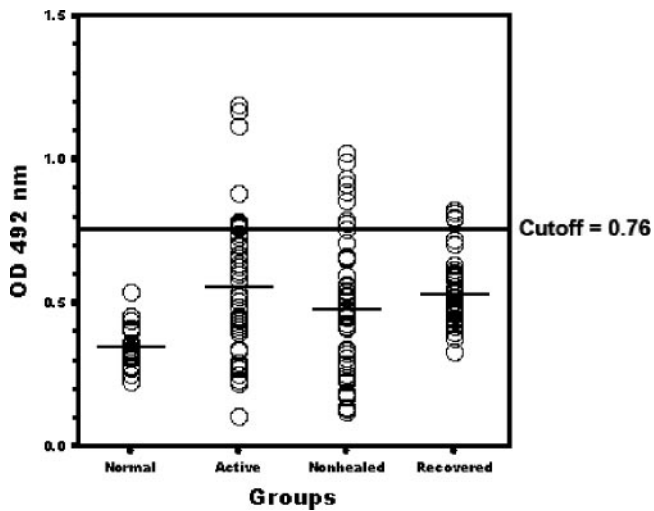


FIG. 3. Reactivity of sera from patients with CL and healthy controls with amastin signature peptide. An ELISA was performed with sera diluted 1:50 from all patients at different stages. The horizontal line represents cutoff values that were calculated by adding 5 SD values to the mean optical density (OD) of normal sera.

between the different stages of the disease using either the crude antigen preparation or the amastin peptide.

In the case of individuals with VL, the first group consisted of 21 clinically confirmed active cases prior to any treatment (all LST negative). The second group consisted of 10 individuals with no symptoms of VL but with a positive LST, and the third group was composed of 10 individuals who had recovered from VL by treatment and had positive LSTs. In Fig. 4, the specific total IgG reactivities against the amastin signature peptide are shown. Clearly, the active VL sera reacted with a mean absorbance value of 1.71, much higher than the cutoff value at 0.74. Both asymptomatic and recovered VL sera showed much lower mean absorbance values that are closer to

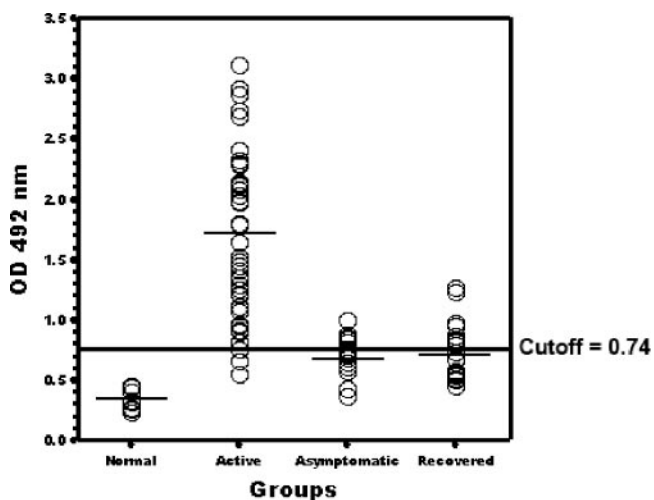


FIG. 4. Reactivity of sera (total IgG) from patients with VL and healthy controls with amastin signature peptide. An ELISA was performed with sera diluted 1:50 from all patients at different stages. The horizontal line represents cutoff values that were calculated by adding 5 SD values to the mean optical density (OD) of normal sera.

TABLE 1. Percentage of serum reactivities toward amastin signature peptide in normal and VL cases

VL status (no. of cases)	F/T (poly Ab; IgM, IgG, and IgA)		Amastin signature (poly Ab; IgM, IgG, and IgA)	
	Mean absorbance $\pm$ SD	% Reactivity	Mean absorbance $\pm$ SD	% Reactivity
Active (21)	2.99 $\pm$ 0.15	100.0	1.71 $\pm$ 0.69	95.2
Asymptomatic (10)	2.18 $\pm$ 0.81	90.0	0.69 $\pm$ 0.15	25.0
Recovered (10)	2.15 $\pm$ 0.77	90.0	0.73 $\pm$ 0.23	30.0

the cutoff value. Of 21 sera collected from patients with active VL, 95.2% reacted with the amastin signature synthetic peptide (mean absorbance  $\pm$  SD = 1.71  $\pm$  0.63), whereas 100% were reactive with F/T antigen of *L. infantum* (mean absorbance  $\pm$  SD = 2.9  $\pm$  0.15) (Table 1). The difference between mean absorbance values of the active VL group and normal cases is highly significant ( $P < 0.05$ ). The mean absorbance values against the amastin signature peptide for the asymptomatic and recovered individuals are at 0.71 (SD = 0.24) and 0.74 (SD = 0.56), respectively, and there is no significant difference between these two groups (asymptomatic and recovered cases). In contrast, there is a significant difference between active VL and these two groups. Similar results were obtained with the *L. infantum* amastin signature peptide (data not shown). Overall, these data suggest that the amastin signature peptide represents a potentially relevant immune signature for detecting active VL, and it could distinguish between active and other stages of visceral leishmaniasis.

**Profile of total IgG and IgG subclasses against the amastin signature peptide in VL patients.** In the present study, we attempted to define whether the total IgG and/or IgG subclasses against the amastin signature peptide were also able to differentiate between disease stages of VL. We therefore tested the IgG subclasses (IgG1, IgG2, IgG3, and IgG4) against the amastin signature in different VL groups. Our data showed no significant difference (one-way analysis of variance) in serum reactivities between the different VL disease stages toward each IgG subclass (Table 2). In contrast, significant differences were observed between total IgG in different VL stages. The patients with active stage showed significantly higher reactivity toward amastin signature peptide than asymptomatic and recovered VL individuals. It has been shown that classes and subclasses of antibody response are sometimes instrumental because each isotype has a distinct biological function (10). Hence, it is important to determine the level of each subclass in response to the amastin signature peptide. It is important to note that the level of IgG3 is significantly higher than for the other subclasses (IgG3  $\gg$  IgG2 > IgG1 > IgG4) in all three VL groups (active, asymptomatic, and recovered cases). The mean absorbance value of each IgG subclass in all three VL groups is significantly higher than for normal cases ( $P < 0.05$ ).

## DISCUSSION

The diagnosis of VL is still based on parasite detection in aspirates from lymph node, bone marrow, and/or spleen. This parasitological method is not sensitive and involves an invasive

TABLE 2. Total IgG and IgG subclasses against amastin signature peptide in the sera isolated from VL patients and normal healthy individuals

Subject type (no. of cases)	Mean absorbance $\pm$ SD				
	Total IgG	IgG1	IgG2	IgG3	IgG4
Normal (10)	0.22 $\pm$ 0.07	0.12 $\pm$ 0.018	0.22 $\pm$ 0.15	0.28 $\pm$ 0.04	0.23 $\pm$ 0.03
Active (21)	1.71 $\pm$ 0.63	0.61 $\pm$ 0.24	0.69 $\pm$ 0.21	1.31 $\pm$ 0.52	0.63 $\pm$ 0.23
Asymptomatic (10)	0.71 $\pm$ 0.24	0.45 $\pm$ 0.14	0.15 $\pm$ 0.13	1.54 $\pm$ 0.29	0.46 $\pm$ 0.13
Recovered (10)	0.74 $\pm$ 0.56	0.56 $\pm$ 0.23	0.69 $\pm$ 0.09	1.39 $\pm$ 0.23	0.48 $\pm$ 0.07

procedure. Some VL patients are very anemic, have coagulation disorders, and are not fit for these invasive aspirations. In addition, the sensitivity of the parasitological method on aspirates of bone marrow and lymph node is also much lower, and up to 50% of cases of visceral leishmaniasis are expected to remain untreated, if the treatment is conditional on diagnosis confirmation (4). Among the various serological tests that have been described, the complement fixation test, the indirect immunofluorescence test, ELISA, and the direct agglutination test are the most frequently used. Each of these assays has its advantages and disadvantages. The antigen used in these assays is usually derived from *Leishmania* promastigotes cultured in vitro (8). In recent years, the direct agglutination test has been the preferred technique in many studies because the test is easier to use under field conditions and does not require any equipment; variation in antigen quantity among batches has, however, been a substantial limitation to its use (6). The rapid development of molecular biology techniques in the last decade has opened the way for the use of highly purified recombinant specific antigens. Synthetic peptides have proven to be valuable tools in the diagnosis of a variety of diseases (16).

In the present study, we used a highly pure 52-aa synthetic peptide corresponding to the first extracellular domain of *Leishmania* amastin surface proteins. This peptide contains a highly conserved sequence of 11 aa, which is present in all of the *Leishmania* and *Trypanosoma* amastin homologs and represents the amastin signature sequence (21). This well-defined domain was not found in any other protein in the databases, which suggests that the family of amastin surface proteins is probably unique to trypanosomatid protozoa. Since the expression of amastins is specific to the amastigote stage and this stage is associated with pathology in humans, we were interested in examining the antibody response against the highly pure amastin signature peptide. We showed that the amastin antibody response was specific for the active stage of VL patients. Using serial dilutions in the ELISA, we showed that the seroreactivity toward amastin peptide is similar to that obtained with the crude promastigote lysates (F/T antigen). By comparing different stages of VL, we showed that the amastin-reactive antibodies are present at high titers in 20 of 21 sera collected from patients with active VL (95% reactivity) and thus could be used as a stage-specific disease marker. This is an important finding since there are not too many antigens which could show stage specificity for VL. During the last few years, many purified and recombinant antigens, such as soluble leishmanial antigen (13), gp63 (25), H2A (26), KMP11 (18), rPSA (3), rCPB (20), rK39 (2), and A2 (5), have been used in serological tests. It has been reported that, during the acute phase of the VL disease, the host may produce specific antibodies, including the A2 and K39 antigens (15). Therefore, amastin is

another antigen that acts similarly, and it would be possible to make a diagnostic kit consisting of these three antigens. Although the use of peptide or recombinant protein mixtures requires a rigorous standardization and close monitoring of the solid phase during the antigen adsorption step in order to warrant reproducible performance, the fusion of different antigenic peptides into a single stable molecule represents an interesting alternative that permits a uniform adsorption without the loss of the individual peptides.

In the present study, we also assessed the level of IgG subclasses toward amastin signature peptide and showed that IgG3 has the highest absorbance in all three stages. Although in our study there are no significant differences between the IgG3 level at different stages of VL patients, there are two other studies from Sudan and Brazil in which the researchers monitored each patient before and after treatment (7, 19). Elasad et al. determined IgG subclasses longitudinally in 28 VL patients before treatment and after 1 month of treatment with Pentostam. Those authors noted a significant decrease in antibody levels for subclasses IgG1 and IgG3 after treatment. This may be due to immune regulation at the helper cell level, and treatment might have lowered the parasite load and allowed the immune response to switch from a Th2 to Th1 cytokine response (7). In a study from Brazil, Pedras et al. showed that IgG subclass antibody detection constitutes a valuable alternative to increase the efficiency of the serological diagnosis of mucosal leishmaniasis. Those authors found that the anti-*Leishmania braziliensis* IgG3 is the first antibody that decreases in serum 90 days after treatment (19). IgG3 is one of the key components of Fc $\gamma$ R-mediated effector response (antibody-dependent cell-mediated cytotoxicity, immune phagocytosis) and can neutralize pathogens at their entry into the body (17). Fc $\gamma$ Rs are largely expressed by many cells, such as neutrophils, monocytes, macrophages, and NK cells (11). It has been shown that in malaria cases, IgG3, which represents a small percentage of total IgGs and has a significantly shorter half-life than other IgG subclasses, can be more efficient than IgG1 in clearing malaria infections, and it is more protective (9). Furthermore, it has recently been shown that IgG3 is more potent than other subclasses in neutralizing human immunodeficiency virus type 1 (24). Therefore, it is highly crucial to have the most appropriate secondary antibody response in infected individuals, since this type of response has the best chance of clearing the infection and/or controlling disease (10). This could be fundamental for vaccine strategies, and for this reason we consider amastin to be a good vaccine candidate against leishmaniasis, as has recently been suggested by the work of Stober et al. with mice (27).

In CL cases, the percentage of seroreactivity against the amastin signature region in active stage is only 20%, and it is

not as strong as for VL active individuals. This may be due to pathophysiological differences that are present in these two different forms of leishmaniasis (15). The level of amastin antibody production is much higher in VL than in cutaneous forms of the disease. This could also be explained by differences in the expression of these amastin homologs between *L. major* and *L. infantum*. Our recent data suggest that there are important differences, at least at the mRNA expression levels, between several amastin homologs of *L. major* and *L. infantum* (21; unpublished data).

Overall, we show here for the first time that the amastin conserved signature sequence at the N-terminal extracellular domain is highly immunogenic in both CL and VL. Due to the unique feature of amastins, which are present only in trypanosomatids, the possibilities for a cross-reactivity with other common diseases, such as malaria and tuberculosis, in areas where *Leishmania* is endemic are almost nonexistent. The amastin signature sequence is an interesting target for further investigation of VL caused by *L. donovani*, especially the rare cases of PKDL. There is a recent report using microarrays showing that, among PKDL cases, there is specific upregulation of class III amastins (12). At present, we are focusing on *L. donovani*-infected individuals to further investigate the potential of amastin peptides as sensitive and stage-specific diagnostic tools against this fatal disease.

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