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Immune Responses of Leishmaniasis Patients to Heat Shock Proteins of *Leishmania* Species and Humans

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The course of human infection with *Leishmania braziliensis* is variable, ranging from self-healing infection to chronic disease. It is therefore a useful system in which to study immunoregulatory aspects of leishmaniasis, including the effects of parasite antigens on host responses. In the present study, we report on the cloning of, expression of, and comparative analyses of patient immune response to two different *L. braziliensis* genes homologous to the genes for the eukaryotic 83- and 70-kDa heat shock proteins. rLbhsp83 contains a potent T-cell epitope(s) which stimulated peripheral blood mononuclear cells (PBMC) from all *L. braziliensis*-infected individuals to proliferate and to produce interleukin-2 (IL-2) gamma interferon, and tumor necrosis factor alpha. The elicitation of IL-4 and IL-10 mRNAs was found to differ depending on the portion of the rLbhsp83 used to stimulate PBMC. rLbhsp83a, which represents the nearly full-length protein, stimulated IL-10 but not IL-4 mRNA. In contrast, a ~43-kDa protein representing the C-terminal region of Lbhsp83 stimulated the production of IL-4 but not IL-10 mRNA. rLbhsp70 stimulated PBMC proliferation from patients with mucosal disease but, unlike rLbhsp83, did not stimulate PBMC from self-healing individuals. PBMC from mucosal patients were not stimulated by rHuhsp70 to either proliferate or produce cytokines. This suggests that the hyperresponsiveness of mucosal patient PBMC to *Leishmania* heat shock proteins does not involve an autoimmune phenomenon resulting from cross-reactivity with self hsp70. In general, although the cytokine profile of patient PBMC in response to both of these *Leishmania* heat shock proteins represents a mixed Th1-Th2 pattern, the levels of gamma interferon and IL-2 were significantly higher than those of the Th2 cytokines IL-4 and IL-10. Patients with active mucosal and cutaneous disease but not self-healing individuals had significant anti-immunoglobulin G antibody titers to both rLbhsp83 and rLbhsp70 but not to the homologous rHuhsp70. It therefore appears that differential patient immune responses to *Leishmania* hsp83 and hsp70 may be of particular significance in the induction of protective immune responses as well as in the development of tissue damage in cases with particularly strong hypersensitive reactions.

Leishmanias are obligate intracellular protozoan parasites of macrophages that cause a spectrum of human leishmaniasis, including localized cutaneous (CL), diffuse cutaneous, mucosal (ML), and visceral (19). *Leishmania braziliensis* commonly causes CL. Patients with CL have strong delayed-type hypersensitivity and in vitro proliferative responses to leishmanial antigens (Ags) during both active and cured disease. Most patients with CL heal spontaneously (self-healing [7]). Patients with self-healing CL represent a potentially interesting group for comparative study because they may have developed immune responses capable of resolving their disease. However, in some infected individuals, chronic ML, characterized by severe and progressive destruction of the nasal, oral, and/or pharyngeal mucous membranes, arises (6, 13). In the case of active ML, the intradermal skin test and lymphocyte proliferative responses are exceptionally strong (4, 5). There appear to be a mixed cytokine profile associated with active CL or ML disease and a dominant Th1 response associated with healing. The determination of the cytokine profile in the peripheral blood mononuclear cells (PBMC) of patients with different clinical presentations as well as the identification of defined Ags that induce and elicit cell-mediated immune responses is important to our understanding of the nature of immune responsiveness during infection. Analysis of patient immune responses by defined *L. braziliensis* Ags is therefore necessary for identifying Ags for immunotherapy or immunoprophylactic applications as well as those that may be involved in pathological consequences. The present study was undertaken to clone, identify, and express *L. braziliensis* genes encoding Ags that elicit proliferative and cytokine responses in patient PBMC. Leukocyte responses and anti-immunoglobulin G antibody titers to recombinant *L. braziliensis* heat shock proteins 83 and 70 (rLbhsp83 and rLbhsp70) and the homologous rHuhsp70 from patients with different clinical forms of disease caused by *L. braziliensis* infection as well as in individuals that heal spontaneously were compared.

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MATERIALS AND METHODS

Parasites. *L. braziliensis* (MHOM/BR/75/M2903), *Leishmania amazonensis* (IFLA/BR/67/PH8), *Leishmania chagasi* (MHOM/BR/82/BA-2,C1 and MHOM/BR/84/Jonas), *Leishmania donovani* (MHOM/Et/67/HU3), and *Leishmania infantum* (IPT-1) were used (3). Promastigotes were cultured in axenic media.

Library construction and isolation of genomic clones. A genomic expression library with sheared DNA from *L. braziliensis* (MHOM/BR/75/M2903) was constructed in bacteriophage ZAP II (Stratagene, La Jolla, Calif.). The expression library was screened with *Escherichia coli* preadsorbed serum from an *L. braziliensis*-infected individual with ML. Immunoreactive plaques were purified, and the pBSK(−) phagemid was excised by protocols suggested by the manufacturer. Nested deletions were performed with exonuclease III to generate overlapping deletions for single-stranded template preparations and sequencing as described elsewhere (12). Single-stranded templates were isolated following infection with VCSM13 helper phage as recommended by the manufacturer (Stratagene) and sequenced by the dideoxy chain terminator method (26) or by the *Taq* dye terminator system using the Applied Biosystems automated sequencer model 373A.

Expression and purification of rLbhsp83 and rLbhsp70 Ags. Recombinant

Ags of the genomic clones pLbhsp83a, pLbhsp83b, and pLbhsp70 were purified from 500 ml of isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cultures as described previously (30). pLbhsp70 lacks the first 131 residues as determined by sequence comparison with the homologous full-length clone of Lchsp70. The expression and purification of the full-length rLchsp70 were as described elsewhere (25). rHuhsp70 was a gift of Peter Murray, Whitehead Institute, Cambridge, Mass. All Ags used in our studies had negligible levels of endotoxin in a *Limulus* amebocyte assay.

Patients. Peripheral blood was obtained from individuals living in an area (Corte de Pedra, Bahia, Brazil) where *L. braziliensis* is endemic and where epidemiological, clinical, and immunological studies have been performed for over a decade (13, 31).

PBMC culture and proliferation assays. Peripheral blood from *Leishmania*-infected and uninfected individuals was collected, and PBMC were isolated from whole blood by density centrifugation through Ficoll (Winthrop Laboratories, New York, N.Y.). For in vitro proliferation assays, 2×10^5 to 4×10^5 cells per well were cultured in complete medium (RPMI 1640 supplemented with gentamicin, 2-mercaptoethanol, L-glutamine, and 10% screened pooled A+ human serum; Trimar, Hollywood, Calif.) in 96-well flat-bottom plates with or without 10 μg of the indicated Ags per ml or 5 μg of phytohemagglutinin per ml (Sigma Immunochemicals, St. Louis, Mo.) for 5 days. The cells were then pulsed with 1 μCi of [^{3}H]thymidine for the final 18 h of culture. Data are represented as mean counts per minute of triplicate cultures, and the stimulation index (SI) is defined as mean counts per minute of cultures with Ags/mean counts per minute of cultures without Ag. For determination of cytokine production 0.5 to 1 ml of PBMC was cultured at 1×10^6 to 2×10^6 cells per ml with or without the *Leishmania* Ags for 48 and 72 h. The supernatants and cells were harvested and analyzed for secreted cytokine or cytokine mRNAs as described below.

Cytokine enzyme-linked immunosorbent assay (ELISA) and PCR. Aliquots of the supernatants were assayed for gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), and IL-10 as described elsewhere (31). For cytokine mRNA PCR analysis, total RNA was isolated from PBMC and cDNA was synthesized by using poly(dT) (Pharmacia) and avian myeloblastosis virus reverse transcriptase. Following normalization to β-actin, diluted cDNA was amplified by PCR using *Taq* polymerase (Perkin-Elmer Cetus) with 0.2 μM concentrations of the respective 5′ and 3′ external primers in a reaction volume of 50 μl. The nucleotide sequences of the primer pairs and the PCR conditions used were as previously described (31). We verified that our PCR conditions were within the semiquantitative range by initially performing serial dilutions of the cDNAs and varying the number of cycles used for PCR. Plasmids containing the human sequences for IL-2, IFN-γ, IL-4, IL-10, and β-actin were digested, and the DNA inserts were purified after separation on 1% agarose gels. Radiolabelled ^{32}P probes were prepared by the random priming method (11). PCR products were analyzed by electrophoresis on 1.5% agarose gels, transferred to nylon membranes, and probed with the appropriate ^{32}P-labelled DNA insert.

RESULTS

Cloning, characterization, and expression of *L. braziliensis* hsp83 and hsp70 Ags. An *L. braziliensis* genomic expression library was screened with sera from an ML patient (I) and plaques containing immunoreactive recombinant Ag were selected and assayed for their ability to stimulate PBMC from *Leishmania*-infected individuals. Several antigens were found to contain a potent T-cell epitope(s) as judged by their ability to stimulate patient PBMC to proliferate and secrete cytokine. Three of the Ags were identified as the *L. braziliensis* homologs of the evolutionarily conserved heat shock protein 83/90 and 70 (hsp83/90 and hsp70) family and were used in further studies.

In eukaryotes, the major hsps are grouped into three classes with molecular masses of 20 to 30, 68 to 73, and 82 to 90 kDa (8, 17, 22, 27). The last group is commonly referred to as hsp86 or hsp90 in higher eukaryotes (14, 15, 34) and hsp83 in the protozoans *Leishmania* spp., *Trypanosoma brucei*, and *Trypanosoma cruzi* (10, 21, 28, 29). The hsps in the 82- to 90-kDa class are cytosolic proteins which are abundant even in the absence of stress (14, 15, 34). Comparison of the cloned *L. braziliensis* heat shock Ags with members of the heat shock protein family from different species revealed extensive sequence homology with the hsp83 (hsp86/90) and hsp70 family.

Figure 1 shows alignment of the deduced primary structure of the *L. braziliensis* hsp83 Ags (Lbhsp83) with those of *L. amazonensis* (Lahsp83), *T. cruzi* (Tchsp83), and humans (Huhsp89a). The sequence of Lbhsp83a overlaps with those of Lahsp83 and Tchsp83 beginning at amino acid residue 48. On the basis of this homology, Lbhsp83a appears to lack the first 47 residues of the full-length 703 amino acid residues. Lbhsp83b contains the C-terminal 372 amino acid residues, and except for a single amino acid substitution (Leu to Ser at position 567), it is identical in its entire length of overlap (residues 332 to 703) to Lbhsp83a. Lbhsp83 has an overall homology of 94% (91% identity and 3% conservative substitution), 91% (84% identity and 7% conservative substitution), and 77% (61% identity and 16% conservative substitution) with *L. amazonensis* hsp83, *T. cruzi* hsp83, and human hsp89, respectively. Comparison of the Lbhsp83 sequence with the reported C-terminal amino acid sequences derived from the cloned *L. donovani* hsp83 gene (9) revealed 92% homology (88% identity and 4% conservative substitution [not shown]). Lbhsp70 has an overall homology of 97.4% (92.7% identity and 4.7% conservative substitution) with *L. chagasi* Lchsp70 and 72% with Huhsp70 (not shown). A comparison of Lbhsp70 with the hsp70 sequences of *L. chagasi*, *L. donovani*, *L. major*, and *T. cruzi* (9) revealed that most of the sequence divergence is restricted to the C-terminal region (not shown).

The 82- to 90-kDa hsps are phosphoproteins containing phosphoserine and phosphothreonine (2). Lbhsp83 contains three potential phosphorylation sites, RXS/T, at locations identical or similar (positions 132, 166, and 594) to those proposed for Lahsp83 (29). In addition, Lbhsp83 contains two regions of highly charged amino acid residues (positions 207 to 296 and 508 to 544), a conserved feature of the hsp83/90 family of proteins.

Figure 2 is a diagrammatic representation of the cloning, expression, and purification of the two *L. braziliensis* hsp83 Ag genes: those for hsp83a (residues 48 to 703) and the 43-kDa C-terminal portion, hsp83b (residues 331 to 703). Lbhsp70 and Lchsp70 were similarly expressed and purified (not shown). Recombinant Lbhsp83a and Lbhsp70 were used to immunize rabbits for the production of monospecific antisera. Immunoblots of *L. braziliensis* lysates with the respective rabbit antisera revealed protein species of sizes expected for hsp83 and hsp70 (not shown). The same immune sera did not react with control *E. coli* lysate, indicating that the purified recombinant Ags were devoid of contaminating *E. coli* Ag (not shown). The purified Ags were subsequently tested for their effectiveness in stimulating T cells from *L. braziliensis*-infected individuals as well as uninfected controls.

rLbhsp83 is a potent stimulator of PBMC from *L. braziliensis*-infected individuals. Human T-cell responses to both Lbhsp83 Ags and *L. braziliensis* lysates were assessed with PBMC from patients with ML, CL, and self-healing CL. Cells from all ML patients proliferated strongly in response to rLbhsp83, with SIs ranging from 19 to 558 and 20 to 1,634 in response to parasite lysate (Table 1). Proliferation of PBMC from CL patients was variable and except for levels in two patients (IV and VII), levels were significantly lower than those of ML patients. By comparison, the proliferative responses of individuals with self-healing CL to rLbhsp83 were similar to those of individuals with ML. However, the responses of all six self-healing individuals to rLbhsp83a were consistently higher than those to rLbhsp83b (Table 1). This suggests that PBMC from self-healing CL patients preferentially recognize a T-cell epitope(s) located within the amino portion of Lbhsp83. In the case of two self-healing individuals (II and IV), the proliferative responses to rLbhsp83 were higher than those to parasite lysate. PBMC from uninfected individuals were only marginally stimulated by *Leishmania* Ag.

Patient PBMC responses to rhsp70. The proliferative re-

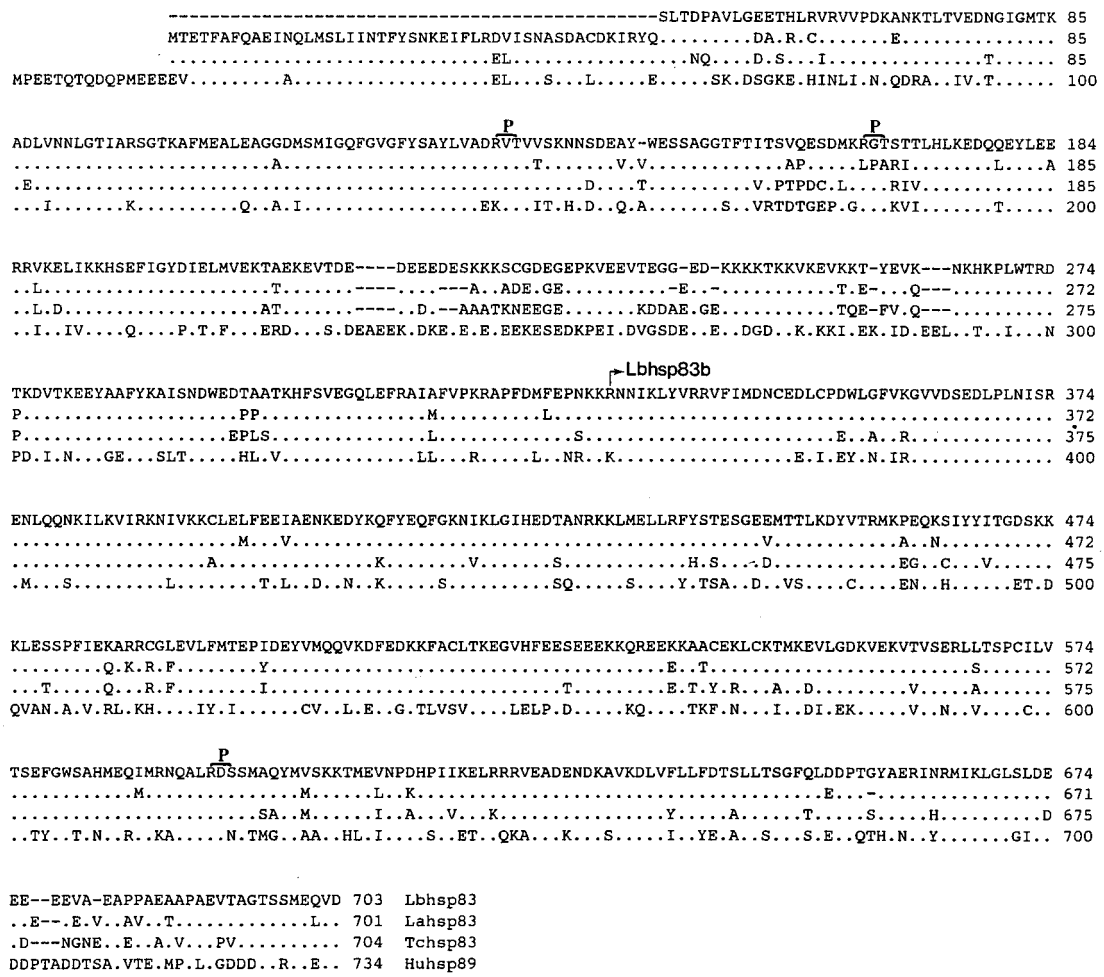

FIG. 1. Comparison of the predicted amino acid sequences of *L. braziliensis* hsp83 (Lbhsp83) and the homologous proteins from *L. amazonensis* (Lahsp83), *T. cruzi* (Tchsp83), and humans (Huhsp89a). Dots represent amino acids that are identical to Lbhsp83, and gaps (dashes) are introduced to maximize homology. The amino terminus representing the first residue of rLbhsp83b is indicated. The three potential phosphorylation sites within Lbhsp83 are identified with the letter P.

sponses and cytokine secretion profile of PBMC from the three patient groups stimulated with rLbhsp83 were compared with the responses elicited by rLbhsp70. rLbhsp70 stimulated PBMC from seven of nine ML patients tested, with SIs from 8 to 569 ($\bar{x}$ = 99) (Fig. 3). PBMC from the other two ML patients were only marginally stimulated (SIs = 2 and 3). The proliferative responses of PBMC from most CL patients in response to rLbhsp70 were comparatively lower (average SI = 4.2). In general, the responses of PBMC from ML patients to stimulation with rLbhsp70 were comparable to those observed with rLbhsp83, while PBMC from CL individuals were only marginally stimulated by rLbhsp70. A similar comparison of PBMC from all six self-healing CL patients revealed that while their proliferative responses to rLbhsp83 were comparable to those of ML patients (above), self-healing CL PBMC either did not proliferate or showed a significantly lower proliferative response to rLbhsp70 (Fig. 3). A similar finding was observed when the same group of patients' PBMC were stimulated with the full-length Lchsp70 homolog (not shown), suggesting the conservation of a T-cell epitope(s) between *L. braziliensis* and *L. chagasi*.

To determine whether cross-reactive epitopes between *Leishmania* hsp and the human homolog could account for the hyperresponsiveness observed with PBMC from ML patients, we analyzed the responses of PBMC from ML patients to the cloned rHuhsp70. In general, the proliferative responses of ML patient PBMC to rHuhsp70 were comparable to those of uninfected individuals (29a) despite the fact that *Leishmania* and human hsp70s share ~72% homology. Therefore, the present study has revealed differences in patient immune responses to two different members of the *Leishmania* hsp family. In addition, the hyperproliferative response observed in ML patients does not appear to be the result of an autoreactive response to self hsp70.

PCR analysis of cytokine mRNA following Ag stimulation of patient PBMC. A more detailed analysis of cytokine patterns of PBMC from ML patients was performed by reverse transcriptase PCR. Cytokine mRNAs were evaluated in cells prior to culturing (Fig. 4, lanes O) or following culturing in the absence (lanes −) or presence of the indicated Ag for 48 and 72 h. Figure 4A shows the results for five of the six ML patients whose PBMC were analyzed. In about half of the ML patients, noncultured (resting) PBMC had detectable levels of mRNA for IFN-γ, IL-2, and IL-4 but not IL-10. CL patient PBMC, however, had IL-10 mRNA in the resting state in addition to mRNAs for the other cytokines tested (Fig. 4B). Following in vitro culture without Ag, the levels of mRNA for IFN-γ, IL-2, and IL-4 in resting cells from ML patients decreased to back-

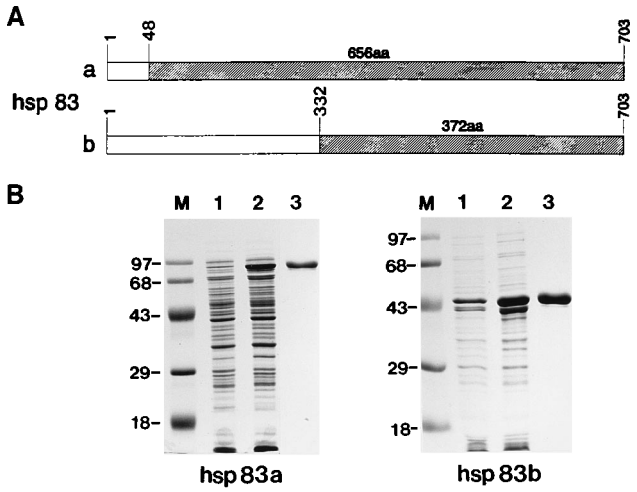

FIG. 2. Cloning and expression of Lbhsp83 Ag genes. (A) Diagrammatic representation of the cloned and expressed portions of two different portions (a and b) of the Lbhsp83 Ag. Numbers indicate the positions of amino acid residues relative to the amino terminus. Shaded areas indicate the expressed portions of the recombinant Ags. (B) Expression and purification of recombinant Lbhsp83 Ags. Coomassie blue-stained sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis of molecular weight markers (lanes M), *E. coli* lysates from uninduced (lanes 1) and induced (lanes 2) cultures, and the purified recombinant Ags (lanes 3). The recombinant Ags have ~4 kDa of LacZ plasmid fusion sequences at their amino termini.

ground levels while IL-10 mRNA levels increased. In contrast, PBMC of most CL patients had stable or increased IL-10 mRNA, while the mRNAs for IL-2, IFN-γ, and IL-4 were reduced to barely detectable levels in the absence of Ag stimulation.

In PBMC of three ML patients, stimulation with lysate resulted in increased expression of mRNA for IFN-γ, IL-2, and IL-4 but not IL-10. By comparison, both rLbhsp83 Ags elicited the production of mRNA for IFN-γ and IL-2 from all ML patient PBMC tested. In contrast, profiles of mRNA for IL-10 and IL-4 differed for the two hsp83 Ags. Lbhsp83a stimulated the production of IL-10 but not IL-4 mRNA (patients I, II, and III), while Lbhsp83b stimulated the production of IL-4 but not IL-10 mRNA in all six patients.

All CL patients tested responded to both rLbhsp83 Ags as well as to the parasite lysate by upregulating the synthesis of mRNAs for IL-2 and IFN-γ, and in two of four patients (I and IV), the level of IL-4 mRNA also increased, indicating stimulation of both Th1 and Th2 cytokines. Interestingly and as in the case of ML patient uncultured PBMC which did not have detectable levels of IL-10 mRNA, Lbhsp83a and not Lbhsp83b stimulated PBMC from one CL patient (IV) to synthesize IL-10 mRNA. However, in the other three patients (I, II, and III) with resting levels of IL-10 mRNA, both rLbhsp83 Ags as well as the parasite lysate downregulated the expression of IL-10 mRNA. Reverse transcriptase PCR analysis of mRNA from patient PBMC following stimulation with rLbhsp70 revealed a mixed Th1-Th2-type cytokine profile but with most ML patients showing a stronger bias for IFN-γ and IL-2 (not shown). While PBMC from ML patients responded to rLbhsp83 and rLbhsp70 Ags with a mixed Th1-Th2 pattern, the cytokine profiles for both the mRNA and secreted products were predominantly of the Th1 type. PCR products for IL-2 and IFN-γ were readily detected on ethidium bromide-stained gels, whereas IL-4 and IL-10 were detected only by radioactive probing of the resolved PCR products.

Levels of secreted cytokines produced in the supernatant in response to Lbhsp83 and Lbhsp70. PBMC supernatants were also assayed for the presence of secreted IFN-γ, TNF-α, IL-4, and IL-10. Cells from all ML and self-healing CL patients (seven and six patients, respectively) and from four of seven CL patients were analyzed for secreted IFN-γ following stimulation with both rLbhsp83 Ags and parasite lysate (Fig. 5A). In general, rLbhsp83a stimulated patient PBMC to secrete higher levels of IFN-γ than did rLbhsp83b (0.2 to 36 and 0.13 to 28 ng/ml, respectively). The presence of secreted IFN-γ correlated well with the corresponding mRNA detected by PCR.

Six of the seven ML patients whose PBMC proliferated in response to rLbhsp70 also produced IFN-γ with values ranging from 0.2 to 35 ng/ml (Fig. 5A). PBMC from the seventh patient, which did not secrete IFN-γ, had the lowest SI. Little or no IFN-γ was detected in the supernatants of PBMC from all other ML and CL patients, in agreement with the marginal proliferative responses of those PBMC. Similarly, PBMC from self-healing CL patients secreted little or no IFN-γ in response to stimulation with rLbhsp70. This same patient group was stimulated by rLbhsp83 to secrete IFN-γ with values ranging from 0.4 to 30 ng/ml (Fig. 5A).

PBMC from four of five ML patients (I, II, V, and VII) had supernatant TNF-α levels (0.8 to 2.2 ng/ml) higher than those detected in cultures of PBMC from uninfected controls following stimulation with parasite lysate (Fig. 5B). Similarly, the same PBMC were stimulated by rLbhsp83 to produce levels of TNF-α in supernatant ranging from 0.61 to 2.9 ng/ml. Compared with those of uninfected controls, PBMC from three (I, V, and VI), five (I, II, IV, V, and VI), and two (II and V) of six individuals analyzed produced higher levels of TNF-α in response to parasite lysate, rLbhsp83a, and rLbhsp83b, respectively. The levels of TNF-α produced by PBMC from CL patients in response to parasite lysate were comparable to those produced by uninfected controls. However, rLbhsp83 stimulated TNF-α production in the PBMC of two of these patients. rLbhsp83a stimulated higher levels of TNF-α production than did rLbhsp83b. In the absence of Ag stimulation, only PBMC from ML patients (five of six) produced detectable levels of supernatant TNF-α (60 to 190 pg/ml).

rLbhsp70 stimulated PBMC from only three ML patients and one CL patient but not from any of the self-healing CL patients to secrete TNF-α (Fig. 5B). No significant levels of IL-4 or IL-10 were detected in any of the patient PBMC following stimulation with rLbhsp70. Therefore, the hyperresponsiveness of PBMC from ML patients to rLbhsp70 is associated with dominant production of IFN-γ. Stimulation of ML patient PBMC with rHuhsp70 did not result in the production of IFN-γ or TNF-α.

In agreement with the IL-10 mRNA, IL-10 was detected by ELISA in the Ag-stimulated PBMC culture supernatants from ML and CL patients. The levels (49 to 190 pg) were significantly higher (up to 10-fold) following stimulation with rLbhsp83a compared with those after parallel stimulation of the same cells with rLbhsp83b (Fig. 6). Parasite lysate also stimulated PBMC from some of the patients to produce IL-10. Although rLbhsp83 stimulated PBMC from uninfected individuals to produce IL-10, with one exception, the levels were lower than those observed with patient PBMC. IL-4 was not detected in any of the supernatants analyzed. Therefore, the level of any secreted IL-4 is below the detection limit of the ELISA employed (50 pg/ml). Taken together, the results demonstrate that a predominant Th1-type cytokine profile is associated with PBMC from *L. braziliensis*-infected individuals following stimulation with rLbhsp83 Ags.

TABLE 1. In vitro proliferation of PBMC from *L. braziliensis*-infected individuals in response to rLbhsp83 Ags

Group and patient	Lysate[a]	SI	Lbhsp83a[a]	SI	Lbhsp83b[a]	SI
ML						
I	41.3 (1.3)	294	32.5 (6.6)	221	46.7 (1.4)	318
II	44.2 (0.5)	104	20.0 (3.7)	47	36.7 (0.76)	86
III	27.4 (1.5)	150	8.1 (1.7)	44	9.9 (0.32)	54
IV	52.7 (3.3)	138	54.1 (6.2)	142	32.0 (1.3)	84
V	140.6 (7.6)	308	151.8 (5.7)	333	150.4 (7.9)	331
VI	15.8 (1.8)	20	21.3 (4.4)	28	14.4 (1.3)	19
VII	300.1 (9.4)	1,634	102.1 (7.6)	558	41.7 (4.9)	228
CL						
I	0.26 (0.0)	1.5	0.57 (0.3)	3.3	0.43 (0.17)	3.3
II	55.63 (8.6)	218	0.42 (0.0)	1.6	0.8 (0.14)	3.2
III	0.39 (0.5)	4.0	3.41 (0.5)	9	2.6 (0.9)	6.6
IV	19.14 (1.3)	87	7.17 (0.6)	32	5.9 (0.9)	27
V	0.32 (0.2)	3.0	1.47 (0.5)	14	0.3 (0.1)	3.0
VI	0.77 (0.1)	4.7	1.44 (0.2)	9	1.3 (0.6)	8.0
VII	4.01 (1.0)	2.0	60.3 (8.5)	15	66.7 (3.9)	16.6
Self-healing CL						
I	19.7 (4.4)	94	61.3 (4.6)	293	5.0 (2.0)	24
II	0.6 (0.1)	6.5	7.0 (2.0)	79	1.2 (0.8)	13
III	59.6 (7.1)	519	49.4 (3.1)	429	21.4 (3.7)	186
IV	0.2 (0.1)	1.6	13.1 (1.7)	108	0.6 (0.1)	5
V	27.1 (2.0)	225	6.3 (2.6)	52	3.0 (1.5)	25
VI	130.3 (14)	340	28.2 (2.9)	74	7.7 (3.8)	20
Control (uninfected)						
I	0.19 (0.0)	1.4	0.18 (0.0)	1.3	0.40 (0.16)	2.8
II	0.31 (0.1)	1.7	0.19 (0.0)	1.0	0.27 (0.0)	1.5
III	0.44 (0.2)	4.1	0.48 (0.1)	5.0	0.51 (0.2)	5.2
IV	0.40 (0.1)	3.2	0.52 (0.2)	5.1	0.50 (0.1)	5.0

[a] Mean [^{3}H]thymidine incorporation [10^3 cpm (standard deviation)].

Serological responses to hsp83 and hsp70 in *L. braziliensis* patients. To determine the correlation between the observed T-cell responses and antibody production to Lbhsp83 and Lbhsp70, we compared the antibody (immunoglobulin G) reactivities to Lbhsp83 in sera from the three patient groups (Fig. 7). The ELISA reactivities of ML patient sera with rLbhsp83a were comparable to those observed with parasite lysate, and in general, there was a direct correlation between ML patient anti-Lbhsp83 antibody titer and T-cell proliferation. Of 23 serum samples from ML patients analyzed, 22 were positive (~96%) with absorbance values of 0.20 to >3.0. Eleven of the ML patient serum samples had optical density values that were >1. In general, CL patients had significantly lower anti-Lbhsp83 antibody titers ($\bar{x}$ = 0.74; standard error of the mean [SEM] = 0.1) compared with those of ML patients. Therefore, ML and CL patient anti-rhsp83 antibody titers correlated with their respective T-cell proliferative responses. Anti-rLbhsp83 antibody titers were significantly higher in patients with ML ($\bar{x}$

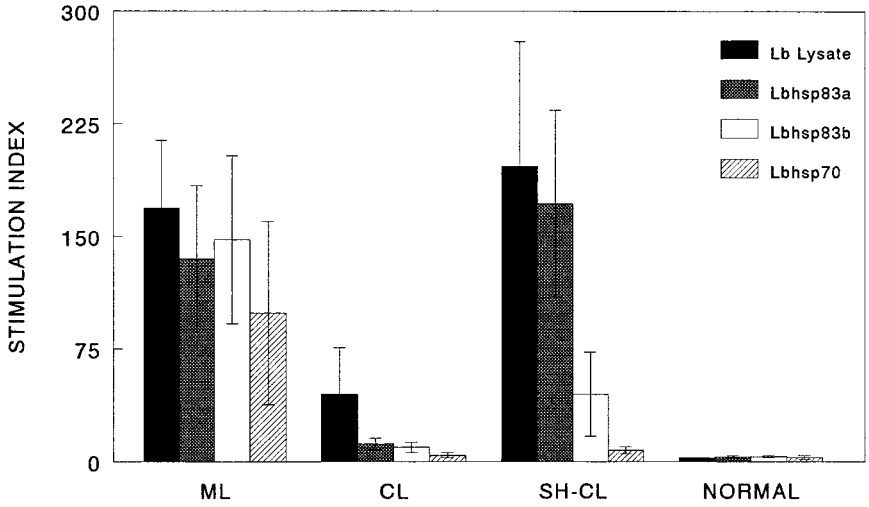

FIG. 3. Comparison of the proliferative responses (stimulation index) of PBMC from *L. braziliensis*-infected individuals with different clinical presentations (SH-CL, self-healing CL) and from uninfected controls (normal) following stimulation with rLbhsp70 and rLbhsp83. The SI represents mean counts per minute of cultures with Ag/mean counts per minute of cultures without Ag stimulation. Mean values ± SEMs are indicated for each patient group.

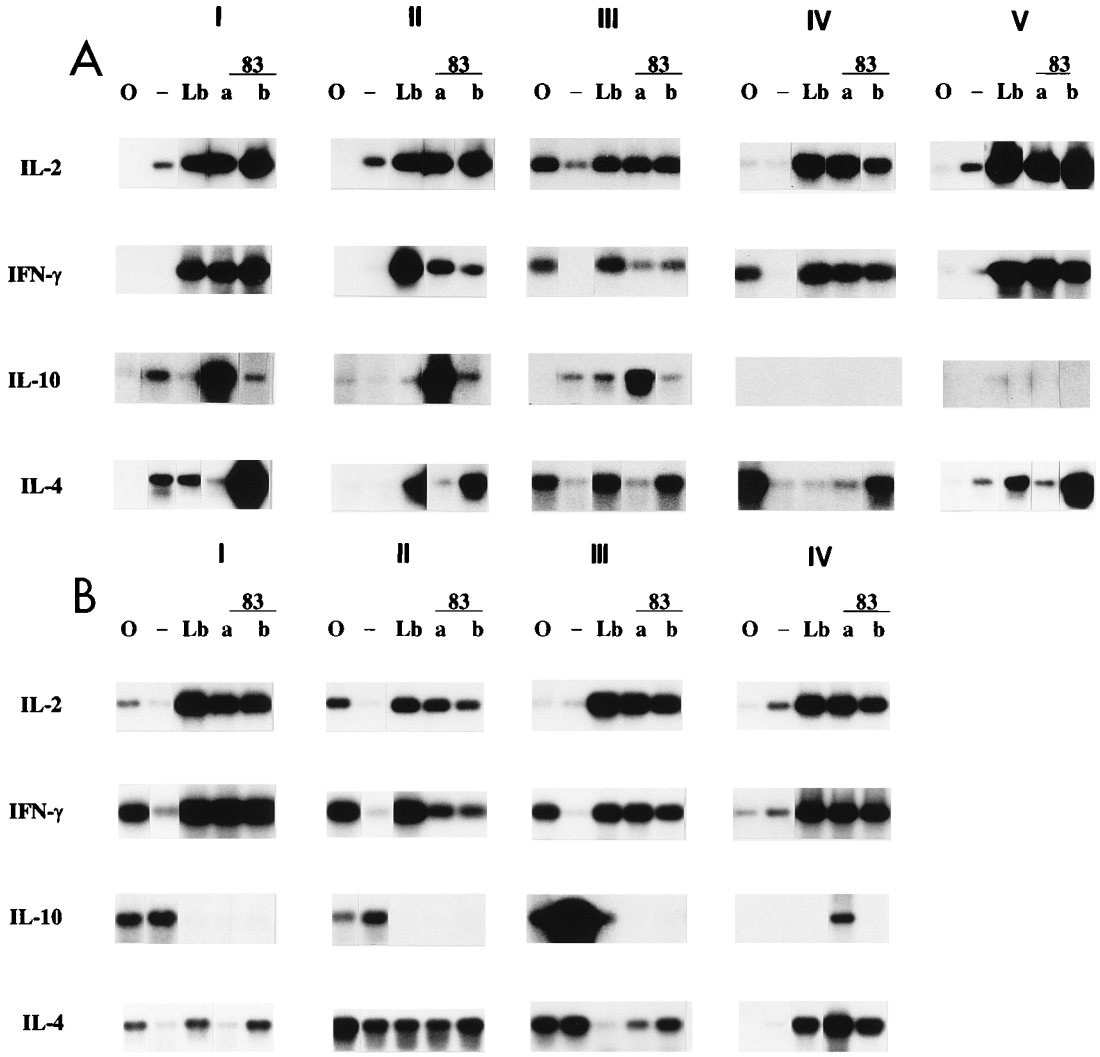

FIG. 4. PCR amplification of cytokine (IL-2, IFN-γ, IL-10, and IL-4) mRNAs isolated from ML (A) and CL (B) patient PBMC before and after stimulation with the indicated antigens. The amounts of cDNA synthesis reaction mixtures used in the cytokine reverse transcriptase PCR were normalized to the β-actin PCR product (not shown). Lanes O and −, PCR products at the initiation of culture or after 72 h of culture in the absence of Ag, respectively; lanes Lb, 83a, and 83b, PCR products following culturing of PBMC with *L. braziliensis* lysate and rLbhsp83a and rLbhsp83b Ags, respectively.

= 1.5; SEM = 0.2) than in self-healing CL patients ($\bar{x}$ = 0.35; SEM = 0.056), although their T-cell proliferative responses were similar. In fact, anti-Lbhsp83 antibody titers in serum from self-healing CL patients were comparable to those from uninfected controls ($\bar{x}$ = 0.24; SEM = 0.028). By using 2 standard deviations greater than the mean absorbance value of uninfected controls (0.484) as a criterion for positive reactivity to Lbhsp83, eight of nine of the self-healing patient serum samples tested were negative.

A similar serological comparison with rLbhsp70 revealed immunoglobulin G antibody titers in all three patient groups with absorbance values comparable to those detected for anti-rLbhsp83 antibody. All ML (n = 17) and CL (n = 13) patient serum samples tested had anti-Lbhsp70 antibody titers with absorbance values ranging from 0.21 to >3.00 ($\bar{x}$ = 1.4; SEM = 0.21) and 0.11 to 1.7 ($\bar{x}$ = 0.9; SEM = 0.21), respectively (Fig. 7). The absorbance values of sera from self-healing CL patients in response to rLbhsp70 were significantly lower than those observed with sera from ML and CL patients. In agreement with the absence of a proliferative response of ML patient PBMC following antigenic stimulation with rHuhsp70, no significant levels of anti-rHuhsp70 antibody titers were detected in sera from ML, CL, or self-healing CL patients. These results indicate that in *Leishmania*-infected individuals, patient immune responses (B and T cell) to *Leishmania* hsp70 did not result in the generation of an anti-self hsp70 autoreactive process. Studies from other laboratories have also demonstrated significant antibody titers to both *Leishmania* hsp70 and hsp83 from visceral leishmaniasis patients caused by infection with parasites of the *L. donovani* complex (9, 18).

DISCUSSION

In the current study, we have evaluated the leishmaniasis patient immune response to recombinant *Leishmania* hsps. As an effective cellular immune response would necessarily be one which would be elicited by amastigotes following infection, it would be highly desirable to induce the response by immunizing with Ags which are abundant in the amastigote stage. In this regard, Lbhsp83 and Lbhsp70 were upregulated following

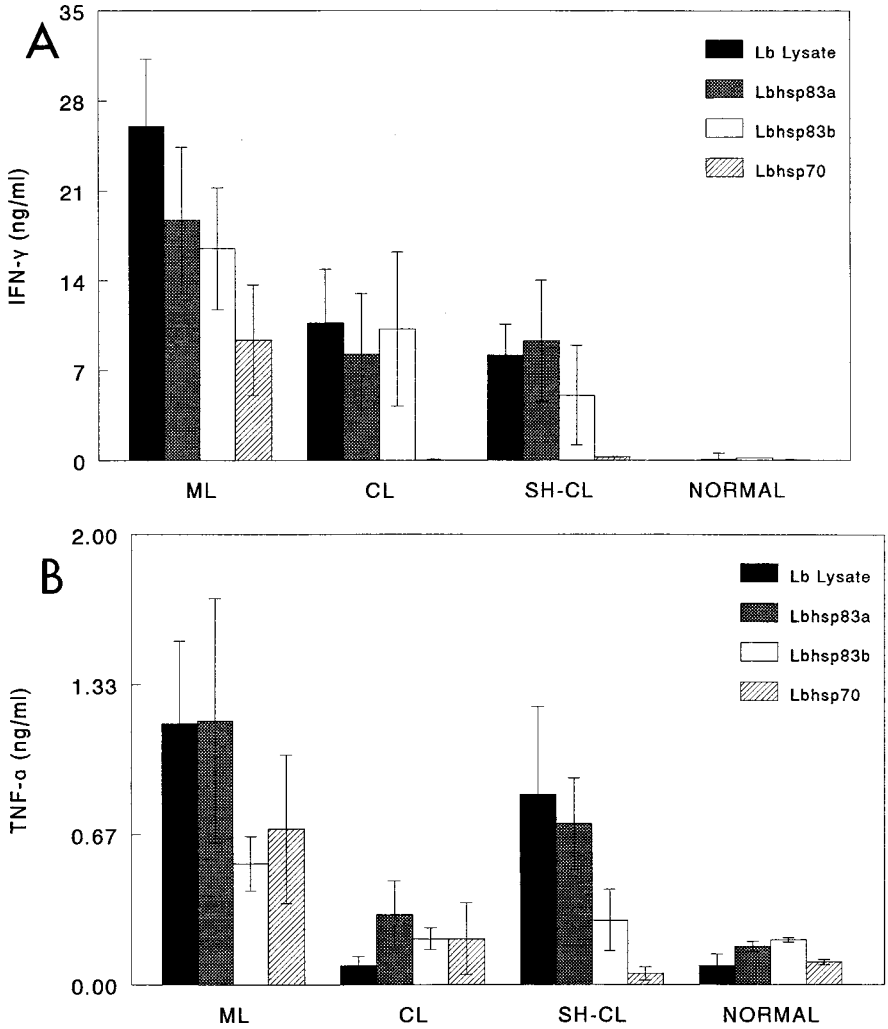

FIG. 5. Comparison of levels of IFN-γ (A) and TNF-α (B) in the supernatant as determined by ELISA from 72-h cultures of PBMC from *Leishmania*-infected and control individuals in response to stimulation with parasite lysate or recombinant *L. braziliensis* hsp83 and hsp70 Ags. The mean value ± SEM is indicated for each patient group. SH-CL, self-healing CL.

a temperature shift to mimic the parasite life cycle stages from the insect promastigote form to the intracellular amastigote stage of the infected mammalian macrophage (not shown). rLbhsp83 represents the most potent *Leishmania* human T-cell Ag reported thus far, as demonstrated by the degree of both proliferation and cytokine production. rLbhsp83 stimulated PBMC from all three patient groups (ML, CL, and self-healing CL) to proliferate with SIs that paralleled those obtained in response to whole-parasite lysate. Our in vitro data therefore suggested that major histocompatibility complex restrictions are not likely to limit the utility of Lbhsp83 Ag to induce proliferative and cytokine responses.

The proliferative responses of PBMC from self-healing CL patients following stimulation with rLbhsp83 were comparable to those of ML patients. Although the responses were significantly lower in PBMC from most CL patients compared with those from patients with ML, rLbhsp83 elicited levels of IFN-γ and TNF-α comparable to those elicited by parasite lysate. In contrast, while rLbhsp70 stimulated PBMC from most ML patients to proliferate and secrete cytokines, often with a magnitude comparable to that stimulated by rLbhsp83, it did not stimulate PBMC from the self-healing CL patients examined. We also have found that Lbhsp83 stimulated PBMC from patients infected with *L. chagasi*, *Leishmania guyanensis*, or *L. amazonensis* to proliferate and/or secrete IFN-γ (unpublished results). In addition, rLchsp70 was as effective as rLbhsp70 in stimulating PBMC from *L. braziliensis*-infected individuals to proliferate. Therefore, immunodominant T-cell epitopes appear to be conserved within the hsp70 and hsp83 Ags among different *Leishmania* species. Thus, the present study has revealed differences in patient immune responses to two different members of the *Leishmania* hsp family. In addition, the hyperproliferative responses observed in ML patients do not appear to be the result of an autoreactive response to self hsp70, since rHuhsp70 did not stimulate PBMC from ML patients to proliferate or secrete cytokines. This is despite the fact that *Leishmania* and human hsp70s share ~72% homology.

Unlike the case with ML and CL patients, individuals with self-healing CL may represent those who have developed immune responses capable of resolving their disease. Perhaps the tissue destruction observed in ML patients is in part due to a hypersensitive response to leishmanial T-cell Ags that specifically stimulate PBMC from ML but not self-healing CL pa-

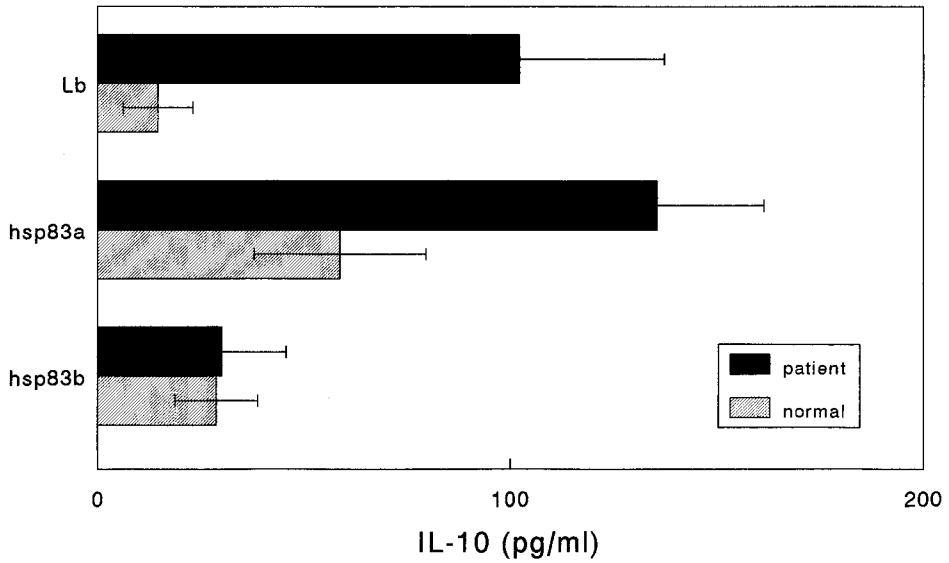

FIG. 6. Levels of IL-10 in the supernatant as determined by ELISA from cultures incubated for 72 h following stimulation of PBMC from *L. braziliensis*-infected individuals and uninfected controls with parasite promastigote lysate (Lb) or with rLbhsp83a or rLbhsp83b.

tients to proliferate, such as hsp70. Therefore, our demonstration that Lbhsp83 stimulated T cells from individuals with self-healing CL to proliferate and produce IFN-γ suggests that PBMC responses by this group of patients to Lbhsp83 are associated with protective immunity. Of interest, γδ T cells from PBMC of a patient with ML responded to stimulation with rLbhsp70 (25). Although the role of γδ T cells in leishmaniasis is not fully understood, γδ T cells isolated from patients with acute disease secrete high levels of IL-4 and IL-6 (23). Another study linked γδ T-cell expansion to parasite burden in a murine model of leishmaniasis (24). In contrast, Russo et al. proposed a possible role for γδ T cells in protective immunity (25). Comparative analysis of PBMC responses of ML and self-healing CL patients to rLbhsp83 revealed that while the proliferative and cytokine responses of ML patients to rLbhsp83a were similar to those elicited by rLbhsp83b, PBMC from all six self-healing CL patients showed a higher degree of stimulation in response to rLbhsp83a. This suggests that multiple T-cell epitopes are present within hsp83 and a bias in epitope selection is associated with the outcome of infection. Hence, in the case of self-healing CL, the production of IFN-γ and TNF-α following stimulation with Lbhsp83 may result in the induction of leishmanicidal activity in macrophages (1, 16, 32), while the hyperresponsiveness of ML may result in pathological consequences associated with mucosal lesions. This may suggest that a particular antigen plays a dual role in the induction of protective immune responses and/or in the development of tissue damage.

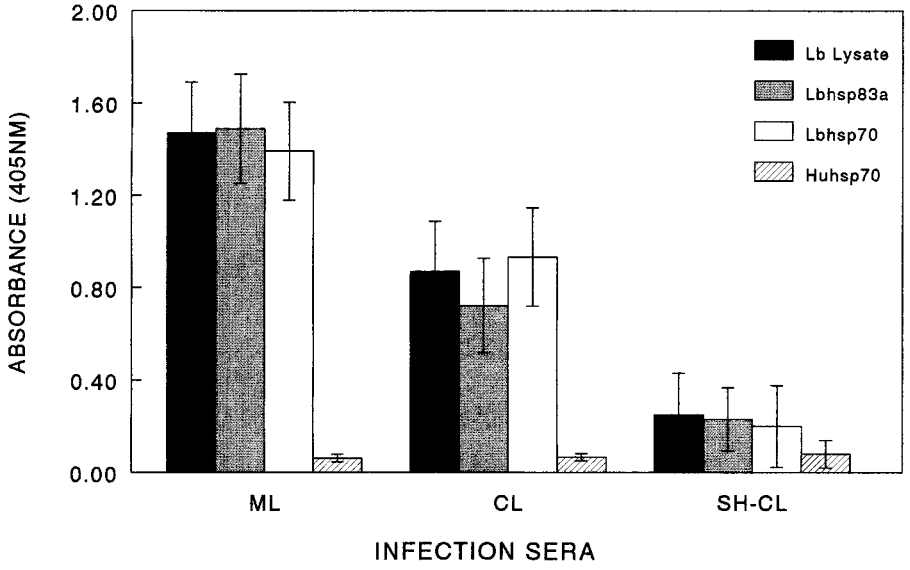

FIG. 7. ELISA evaluation showing the reactivities (mean ± SEM) of *L. braziliensis* infection sera from patients with ML, CL, and self-healing CL (SH-CL) with lysate, rLbhsp83, rLbhsp70, and rHuhsp70.

Stimulation of ML patient PBMC with rLbhsp83 elicited the expression of mRNA for IFN-γ and IL-2 as well as the secretion of IFN-γ and TNF-α. In contrast, mRNA of IL-10 and IL-4 differed depending on the antigenic stimulus. Lbhsp83a stimulated the expression of IL-10 but not IL-4 mRNA, while the C-terminal portion, Lbhsp83b, stimulated the expression of IL-4 but not IL-10 mRNA. The results suggest that (i) the ways in which individual Ags are processed and presented determine the outcome of the cytokine profile, (ii) an IL-10-promoting linear or conformational epitope is contained within the amino portion of Lbhsp83a, and/or (iii) the higher levels of TNF-α produced by patient PBMC in response to rLbhsp83a upregulate the expression of IL-10 mRNA. Studies using human monocytes have demonstrated that the addition of TNF-α can also induce IL-10 production (33). In humans, IL-10 is produced by a variety of cell types, including $CD4^+$ T cells, $CD8^+$ T cells, activated B cells, and monocyte/macrophages (20). Although we do not know the cell types that produce IL-10 in patient PBMC following stimulation with Lbhsp83a, our preliminary results indicate that macrophages and not T cells are the major source of IL-10. In contrast, it is likely that T cells are the dominant source of IL-4, on the basis of our observation on the production of IL-4 by Ag-specific T-cell lines (29a).

Serological evaluation of patient sera with parasite lysate, rLbhsp70, and rLbhsp83 revealed significant immunoglobulin G antibody titers in patients with ML and CL but not individuals with self-healing CL. This is despite the fact that the degrees of proliferative response by ML and self-healing CL patient PBMC to rLbhsp83 were comparable. We found that >95% of sera from ML and CL patients had antibody titers to both rLbhsp70 and rLbhsp83. In agreement with the absence of proliferative response by patient PBMC to rHuhsp70, no significant anti-Huhsp70 antibody titers were detected in sera of patients with ML, CL, or self-healing CL. Our serological data have demonstrated that *L. braziliensis* hsps represent potentially important diagnostic antigens in *Leishmania* infection and may be used to distinguish patients with active or cured disease. It therefore appears that a major distinction in patient immune responses to *Leishmania* hsps in the context of active disease or in the resolution of disease may be associated with antibody titers to *Leishmania* Ags.

In conclusion, we have analyzed patient immune responses to two potent leishmanial Ags. The differential cytokine elicitation patterns of Lbhsp83a and Lbhsp83b suggest the potential for designing Ags with improved immunotherapeutic or immunoprophylactic potential. In addition, the powerful patient T-cell responses to Lbhsp70, but not to the human homolog, suggest methods for selecting antigenic determinants from proteins that are highly conserved among species. Future studies will define epitopes of leishmanial hsps and evaluate them in animal models of leishmaniasis.

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