Recombinant *Leishmania* Hsp90 and Hsp70 Are Recognized by Sera from Visceral Leishmaniasis Patients but Not Chagas' Disease Patients

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Approximately 70% of the cDNA clones identified by immunoscreening *Leishmania donovani* expression libraries with serum from a patient with visceral leishmaniasis (kala-azar) were found to encode the highly conserved Hsp90 and Hsp70 members of the heat shock protein family. Recombinant fusion proteins containing the C-terminal portions of *L. donovani* Hsp90 and Hsp70 were used as target antigens in enzyme-linked immunosorbent assays of various sera. Sera from four patients with visceral leishmaniasis recognized recombinant *Leishmania* Hsp90 and Hsp70, while sera from seven patients with Chagas' disease did not, despite the fact that *Trypanosoma cruzi* Hsp90 and Hsp70 share more than 80% amino acid identity with their counterparts in *Leishmania* spp. Thus, *Leishmania* Hsp90 and Hsp70 elicit strong humoral responses and are potential candidates for specific serodiagnostic assays capable of distinguishing between *L. donovani* and *T. cruzi* infections.

Visceral leishmaniasis, which is caused by several subspecies of the protozoan parasite *Leishmania donovani*, is an insect-borne disease that causes considerable morbidity and mortality in tropical and subtropical countries (32). Although signs and symptoms often suggest visceral leishmaniasis in patients infected with *L. donovani*, a definitive diagnosis depends on microscopic identification of amastigote forms of the parasite in tissue specimens, isolation of parasites in culture, or hybridization of DNA probes to tissue samples (16, 26). These parasitologic approaches are cumbersome and often lack sensitivity. Moreover, some procedures for obtaining tissue samples, such as spleen and liver biopsies, expose patients to the risk of life-threatening hemorrhage.

In efforts to simplify the diagnosis of visceral leishmaniasis, several serologic tests have been developed (1, 8, 13). Enzyme-linked immunosorbent assays (ELISAs) and indirect immunofluorescence tests are used widely, but these assays and others, all of which employ material derived from parasites grown in culture as target antigens, can lack sensitivity and specificity, are not standardized, and are not available commercially. False-positive reactions in serologic tests for *L. donovani* infection often occur with sera from patients infected with *Trypanosoma cruzi*, the cause of Chagas' disease, and the converse of this is true as well (5). This presents a practical problem in those areas of Latin America in which both infections are endemic.

The goal of the research described here was to identify *L. donovani* protein antigens that are recognized by antibodies in sera from patients with visceral leishmaniasis but not from patients with Chagas' disease. Our intent was to find antigens that, when produced by recombinant means, can func-

tion as targets in ELISAs for visceral leishmaniasis and do not cross-react with sera from Chagas' patients. For this purpose, we first immunoscreened *L. donovani* cDNA expression libraries for fusion proteins recognized by sera from a patient with visceral leishmaniasis. We found that 12 of 17 recombinant proteins identified by this approach were one of two members of the heat shock family (Hsp) of stress proteins. The ability of recombinant forms of each of these proteins to function as target antigens was tested in ELISAs, and the results suggest that these recombinant forms may be useful as target antigens in a sensitive and specific assay for the diagnosis of visceral leishmaniasis.

MATERIALS AND METHODS

Parasites. The Sudan S1 strain of *L. d. donovani* (MHOM/SD/00/IS-2D) was obtained from Dennis Dwyer at the National Institutes of Health, Bethesda, Md. Promastigotes of *L. d. donovani* were maintained at 26°C by serial passage in medium 199 supplemented with 30% fetal calf serum and 20 µg of hemin per ml as described elsewhere (23).

cDNA libraries. The construction in bacteriophage λZAPII of a cDNA library of poly(A)⁺ RNA from cultured promastigotes of *L. d. chagasi* has been described elsewhere (34). A cDNA library of total RNA from stationary-phase promastigotes of *L. d. donovani* was prepared in bacteriophage λZAP (17). cDNAs were synthesized by using oligo(dT) and Moloney murine leukemia virus reverse transcriptase in the BRL Synthesis System according to the protocol recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.) (3). After treatment with *Eco*RI methylase and attachment of *Eco*RI linkers, the cDNAs were ligated with λZAP arms (Stratagene, San Diego, Calif.) (40). The concatenated DNAs were packaged with GigaPack Gold extracts (Stratagene), and about 2 × 10⁶ phage were amplified in *Escherichia coli* Y1088 (41).

Antibody probes and immunoscreening of cDNA libraries.

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Sera used in this study were derived from four patients with visceral leishmaniasis diagnosed by demonstration of parasites in stained smears of bone marrow aspirates (FHC2-III, Eloy 2, Pet, and FCG-1). Also included were seven serum specimens from patients with Chagas' disease (sera 1 through 7), whose infections with *T. cruzi* were diagnosed serologically by immunofluorescence assay (4) and radioimmune precipitation assay (22). Serum from one patient with serologically demonstrated infection with *Toxoplasma gondii* was also used (31). The cDNA libraries were initially immunoscreened with a 1:500 dilution of the FHC2-III serum by using a procedure described previously (17, 18). Binding of antibodies to fusion proteins in phage plaques was detected by using goat anti-human immunoglobulin G conjugated to horseradish peroxidase as the second antibody (Organon Teknika-Cappel, Durham, N.C.). Seventeen phage producing fusion proteins recognized by the FHC2-III serum and chosen from >200 positives were plaque purified in three or four additional rounds of immunoscreening and studied further.

Characterization of cDNAs. The cDNA inserts of the selected recombinant phage were recovered in pBluescript plasmid by using the protocol suggested by Stratagene (40, 41), and the recombinant plasmid DNAs were isolated for use as templates in DNA sequence determinations (15). Oligonucleotides containing sequences that flank both sides of the *Eco*RI site of pBluescript were used as primers for T7 Sequenase (U.S. Biochemicals, Cleveland, Ohio) in the dideoxynucleotide reactions (15), and sequences of 250 to 300 nucleotides were determined on both sides of the cDNA inserts. The complete sequence of one of the 90-kDa heat shock protein (Hsp90) cDNA inserts was determined by using additional oligonucleotides whose sequences were predicted from cDNA sequences previously determined. Oligonucleotides were synthesized by the University of Iowa Recombinant DNA Core Facility, which also conducted some of the DNA sequence determinations on an automated DNA sequencer (Applied Biosystems Inc., Foster City, Calif.). DNA sequences were compared with sequences in the GenBank data base by using the University of Wisconsin GCG Sequence Analysis Software Package.

Preparation of cellular extracts. Overnight cultures of *E. coli* transformed with recombinant plasmids were harvested and solubilized in sample buffer containing 0.125 M Tris HCl (pH 6.8)–4% sodium dodecyl sulfate (SDS)–20% glycerol–10% 2-mercaptoethanol. Proteins were separated on 10% SDS-polyacrylamide gels (24). Gel regions containing the fusion proteins to be used in ELISAs were excised, and the proteins were then electroeluted in an IBI UEA unidirectional electroeluter (International Biotechnologies, Inc., New Haven, Conn.) and concentrated in a Centricon-10 Microconcentrator (Amicon, Danvers, Mass.) (14). Protein concentration was determined by the Bio-Rad Protein assay (Bio-Rad Laboratories, Richmond, Calif.). In all cases, the procedures recommended by the manufacturers of the apparatuses were used.

ELISAs. The reactions were conducted in 96-well, flat-bottom Immulon I microtiter plates (Dynatech, Chantilly, Va.) coated with 0.1 ml of the partially purified antigen (125 ng per well) in phosphate-buffered saline (18.3 mM, pH 7.2) (PBS) as outlined in an earlier report (21). Nonspecific binding sites were blocked by addition to the wells of 1% bovine serum albumin in PBS with 0.05% Tween 20.

Nucleotide sequence accession number. Nucleotide and amino acid sequence data in this paper have been submitted

FIG. 1. SDS-polyacrylamide gel stained with Coomassie blue, showing the protein extracts from cultures of *E. coli* transformed with recombinant pBluescript plasmids derived from a λ ZAP cDNA library of promastigote mRNA from either *L. donovani* (donovani) or *L. d. chagasi* (chagasi). Each culture produced a fusion protein recognized by serum FHC2-III from a Brazilian patient with visceral leishmaniasis. Abundant fusion proteins are apparent in 12 of the 17 extracts, as exemplified by the ~30-kDa protein in the first lane and the ~40-kDa protein in the last lane. The fusion proteins in lanes marked with solid squares contain part of *Leishmania* Hsp90; in lanes marked with open circles, they have part of *Leishmania* Hsp70. Arrows indicate lanes containing fusion proteins encoded by cDNAs for which DNA sequences were determined. Numbers on the left are molecular sizes in kilodaltons.

to the GenBank data base with the accession number M73492.

RESULTS

Identification of *Leishmania* fusion proteins. Serum from a patient with visceral leishmaniasis (FHC2-III) was used to immunoscreen the two *Leishmania* cDNA expression libraries. Approximately 0.13% (1 in 800) of the recombinant phage in the two libraries encoded fusion proteins recognized by antibodies in this serum. Of the >200 phage that were positive during the first immunoscreenings, 17 that were distant from other positive phage plaques on the original plates were purified to homogeneity by additional rounds of immunoscreening. Eleven of these were from the *L. d. chagasi* library, and six were from the *L. d. donovani* library. The cDNA inserts encoding *Leishmania* proteins were recovered in pBluescript and introduced into *E. coli* DH5 α . The profiles of proteins extracted from transformed *E. coli*, separated electrophoretically, and stained with Coomassie blue are shown in Fig. 1. Abundant fusion

hsp90 cDNA clones

hsp70 cDNA clones

FIG. 2. Diagrams showing the locations of the 5' ends of three Hsp90 cDNAs and two Hsp70 cDNAs whose fusion proteins are recognized by serum FHC2-III. Open rectangles indicate the coding regions of full-length cDNAs, and horizontal lines indicate the 5' and 3' untranslated regions.

proteins, ranging in size from 18 to 90 kDa, are apparent in 12 of the 17 lysates.

With the intent of utilizing one or more of these recombinant proteins as target antigens in a diagnostic assay, we investigated 6 of the 17 recombinant plasmids further by determining the first 250 to 300 nucleotides on both ends of their inserts. Comparison of sequences obtained with those in the GenBank data base (release 26.0, September 1990) revealed that three of them encode the highly conserved Hsp90 and two code for the highly conserved Hsp70. The sequence of the sixth cDNA is not significantly similar to any sequence in the GenBank data base and was not characterized further. The locations of the 5' ends of the five Hsp cDNAs relative to those of the full-length Hsp90 and Hsp70 DNA sequences reported for other protozoan species are shown in Fig. 2. As indicated, the 5' ends of the five Hsp cDNAs lie in the coding regions for the two proteins and extend beyond the termination codons into the 3' untranslated regions. Three of the cDNAs end in runs of adenosines that are probably from the poly(A) tail of the mRNAs from which the cDNAs were derived.

To determine the frequency of the cDNAs encoding these two Hsps among the 17 purified recombinant phage, the Hsp90-3 and Hsp70-2 cDNAs were radiolabeled in vitro and hybridized to Southern blots of all 17 plasmids. Five of the cDNAs (29%) hybridized to the Hsp90 probe, and seven (41%) hybridized to the Hsp70 probe.

Sequence determination of the Hsp90-3 cDNA. At the time that this work was undertaken, the nucleotide sequence encoding a *Leishmania* Hsp90 was not available. Thus, we determined the complete sequence of the Hsp90-3 cDNA, which encodes the smallest fusion protein of the three original Hsp90 cDNAs detected (Fig. 1). While sequence analysis of the Hsp90-3 cDNA was in progress, the sequence of an Hsp90 cDNA from *Leishmania mexicana amazonensis* was reported elsewhere (39). For ease of comparison, the sequence of our *L. donovani* Hsp90-3 cDNA is shown in Fig. 3 with the numbering system of that reference. The sequence begins at nucleotide 753 and encodes the final 453 amino acids of the 703-residue Hsp90. The coding region is followed by a 3' untranslated region of 858 nucleotides which ends in a run of 9 adenosines that are likely derived from the poly(A) tail of the mRNA.

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753  G ACG AAG AAG GTG AAG GAG GTG ACG AAG GAG TAC GAG GTC CAG AAC AAG CAC AAG CGC TTC TGG ACG CGC GAC 825
CCG AAG GAG GTG ACG AAG GAG GAG TAC GCG GGC TTC TAC AAG GGC ATC TCC AAC GAC TGG GAG GAC CGG ATG GCG 900
ACG AAG CAC TTC TCT GTG GAG GGC CAG GTG GAG TTC CGC TCG ATC ATG TTC CGG AAG CGC CCA TTC GAC 975
ATG TTC GAG CGC AAG AAG AAG CGC AAC AAC ATC AAG CAG TAC GCG CGC GTG TTC ATC AAG GAG AAC TGC GAG 1050
GAC CTG TGC CGC GAG TGG CTC GGC TTC GTG AAG GGC GTC GTG GAC AGC GAG GAC CTG CGC CTG AAC ATC TGC CGC 1125
GAG AAC CTG CAG CAG AAC AAG ATC CTG AAG GTG ATC CGC AAG AAC ATC GTG AAG TGC CTG GAA ATG TTC GAC 1200
GAG GTG GCG GAG AAC AAG GAG GAC TAC AAG CAG TTC TAC GAG CAG TTC GGC AAG AAC ATC AAG CTG GGC ATC CAC 1275
GAG GAC ACT GCG AAC CGC AAG AAG CTG ATG GAG TTC CTG GGC TTC TAC AGC ACC GAG TGC GCG CTA GAG ATC ACT 1350
ACT CTG AAG GAC TAC GTG ACC GGC ATG AAG GAG GGC CAG AAG TCG ATC TAC TAC ATC AGC GGC GAC AGC AAG AAG 1425
AAG CTG GAG TCG TCG CGC TTC ATT GAG CAG GCG AGG CGC GGC CTT GAG GTG CTG TTC ATG ACG GAA CGC ATC 1500
GAG GAG TAC GTG ATG CAG CAG GTG AAG GAC TTC GAG GAG AAG TTT GCG TGC CTG ACG AAG GGC GTG CAC 1575
TTC GAG GAG TCG GAG GAG AAG CAG CAG CGC GAG GAG AAG GCG GCG TGC GAG AAG CGC TGC AAG ACG ATC 1650
AAG GAG GTG CTG GGC GAC AAG GTG GAG AAG GTG ACC GTG TCG GAT CGC CTG TCG ACC TCG CGC TGC ATT CTG GTG 1725
ACA TCG GAG TTC GGG TGG TCG CGC CAC ATG GAG CAG CAC ATC GCG AAC CAG CGC GTG CGC GAC TCC AGC ATG TCT 1800
CGG TAC ATG ATG TCC AAG AAG ACG ATG GAG CTC AAC CGC AGA CAC CCC ATC ATC AAG GTG CTG CGC CGC CGC GAG 1875
GAA GCG GAG AAG GAC AAG GCG GTG AAG GAC CTC GTC TTC CTG CTC TTC GAG ACG TCG CTG CTC ACG TCC GGC 1950
TTC CAG CTG GAG GAC CCC ACC GGC TAC GCG AAG GCG ATC AAC CGC ATG ATG AAG CTC GGC CTG TCG CTC GAG GAG 2025
GAG GAG GAG GCA GCG GAG GCG ACG GTG GTC GAG ACG CGC CGC GCG GAG GTC ACC GTC GGC ACC TCC AGC ATG GAG 2100
CAG GTG GAC TGA GCGGATGCGAGCGGCGAGCGCGCAGCTCCGCAAGCTGCGAGCGGCGGCTCTGAGAGGCTCTTCTCCGCGGACATCTCTCCG 2175
GTTCGCGGAGCTGAGGACACATCTGCGGCGAGTCTGCGGCTCTGTTGGGATGAGTCAAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGG 2250
GAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGG 2325
CTCCGCTTCTCTCTCCGCGGCTGCGGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 2400
TTTCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGG 2475
TGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 2550
CTTTCAGAGGGGAGGCGCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 2625
CGATCGCTTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTGATTTG 2700
TTGCGCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 2775

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FIG. 3. Nucleotide sequence of *L. donovani* Hsp90-3 cDNA. The sequence begins at position 753 (in codon 251) (the nucleotide numbers of reference 39 are used) and extends to the poly(A) tail.

In Fig. 4, the deduced C-terminal 453 amino acids of this Hsp90 are compared with the Hsp90 sequences of *L. m. amazonensis* and *T. cruzi* (10, 39). Within the region of comparison, the two *Leishmania* Hsp90s are the same at 93% of positions, while the *T. cruzi* Hsp90 has 85% identity with the *L. mexicana* Hsp90 and 82% identity with the *L. donovani* protein. The C-terminal regions are the most divergent.

The complete sequence of a cDNA encoding the *L. donovani* Hsp70 (Fig. 1 and 2) was not determined, since Hsp70 sequences of *L. donovani*, *Leishmania major*, and *T. cruzi* have already been reported elsewhere (12, 25, 27), and the partial sequences of the *L. donovani* Hsp70-1 and Hsp70-2 cDNAs are nearly identical to those reported earlier (27). A comparison of the *L. donovani* and *T. cruzi* Hsp70 protein sequences (not shown) demonstrates that most of the sequence divergence between these two Hsp70s also occurs in the C-terminal region.

ELISAs with *L. donovani* Hsp90 and Hsp70 fusion proteins. To assess the reactivities of eluted recombinant *L. donovani* Hsp90 and Hsp70 with sera from patients, we used these proteins as target antigens in ELISAs. Figure 5 shows the results obtained when the partially purified Hsp90-3 (panel A) and Hsp70-2 (panel B) fusion proteins were tested against sera from four patients with visceral leishmaniasis, seven patients with *T. cruzi* infections, and one person infected with *T. gondii*. None of the sera from patients with Chagas' disease displayed a substantive reaction with the recombinant fusion proteins, even when undiluted, and a representative tracing obtained with one of these specimens is shown in each panel. The reactivities of the sera from the patient with *T. gondii* were similar to those of sera from the Chagas' disease patients.

In contrast, three of the four sera from patients with visceral leishmaniasis reacted strongly with the Hsp90 fusion protein, while the fourth was less reactive, although still more reactive than the comparison specimens. Furthermore, all four showed a high degree of reactivity with the Hsp70 fusion protein. The most striking difference between the sera from patients with visceral leishmaniasis and the other

<i>L. donovani</i>	252	TKKVKETKEYEVQNKHKPLWTRDPKDVTKEEYAAFYKAISNDWEDPMATKHFSVEGQLEFRSIMPVFKRAPFDMFEPNK	332
<i>L. mexicana</i>		P.....A.....L.....	
<i>T. cruzi</i>		Q.FV.....E.LS.....A.L.....S.....	
<i>L. donovani</i>		KRNNIKLYVRRVFIMDNCELDLPWLFVKGVVDSDELPLNISRENQQNKILKVIKKNIVKKCLEMFDEVAENKEDYKQ	412
<i>L. mexicana</i>		E.....	
<i>T. cruzi</i>		E..A..R.....A..L..E..I.....K.....	
<i>L. donovani</i>		FYEQFGKNIKLGIHEDTANPKKIMEFLRFYSTESGLEMTTLKDYVTRMKEGQKSIYYITGDSKKKLESSPPIEQARRRGL	492
<i>L. mexicana</i>		R.....L.....EV.....AE.N.....K.....F.....	
<i>T. cruzi</i>		V.....S..R.....L..H.S...ED.....C..V.....T.....F.....	
<i>L. donovani</i>		EVLFMTEPIDEYVMQQVKDFEDKKFACLTKEGVHFESEEEKQREEEKAACEKCKTMKEVLGDKVEKVTVSDRLSTSP	572
<i>L. mexicana</i>		Y.....R.....T...L.....E.....	
<i>T. cruzi</i>		T...K.....TAY.RL..A..D.....V..E..A...	
<i>L. donovani</i>		CILVTSEFGWSAHMEQIMRQAVRDSSMSAYMSSKKTMLNPRHPIIKVLRVRVEADENDKAVKDLVFLFDTSLLTSGF	652
<i>L. mexicana</i>		M.....L.....AQ.....K.....E.....	
<i>T. cruzi</i>		L.....A.....I..A...V.E.K.....Y.....A.....	
<i>L. donovani</i>		QLEDPTGYAKRINRMIKLGLSLDEEEE--AAEATVVTETAPAEVTVGTSSMEQVD	706
<i>L. mexicana</i>		E.....EE.V...A.A.....A.....L...	
<i>T. cruzi</i>		T.D...S..E..H.....D.DNGNE...EPAAV...PVA.....N...	

FIG. 4. Comparison of the C-terminal amino acid sequences beginning at position 252 of Hsp90s from *L. donovani* (derived from nucleotide sequence in Fig. 3), *L. mexicana* (39), and *T. cruzi* (10). Dots indicate identity with the *L. donovani* sequence.

specimens occurs at a dilution of 1:32 (2^5) for Hsp90-3 protein and a dilution of 1:128 (2^7) for Hsp70-2. At the lower dilution of 1:16 (2^4), the responses of the sera from patients with visceral leishmaniasis to both target proteins are less than they are at higher dilutions. This dilution effect is likely

to be due to interference with the specificity of the ELISA by an excess of nonspecific proteins in the sera.

A final experiment was performed to provide insight into the apparent conflict between the markedly different reactivities of the *Leishmania* and *T. cruzi* sera with the recombinant *Leishmania* Hsps we observed and earlier reports of cross-reactivities (1, 5, 13, 36). The seven sera from patients with Chagas' disease were reacted with *T. cruzi* and *L. d. chagasi* antigens in Western blots (immunoblots). The results of this experiment are shown in Fig. 6, where the strong reactivities of the sera from patients with Chagas' disease confirm the presence of specific antibodies directed against many *T. cruzi* antigens (lanes T in panels 1 through 7). The cross-reactivities of these sera with *Leishmania* antigens are

FIG. 5. Plots of A_{492} versus serum dilution in exponents of 2 (i.e., $2^6 = 1/64$ dilution, etc.) in ELISAs using the Hsp90-3 fusion protein (A) and the Hsp70-2 fusion protein (B) shown in Fig. 1. The sera examined include sera from four Brazilian patients with visceral leishmaniasis (FHC2-III, Eloy 2, Pet, and FCG-1), sera from seven patients with Chagas' disease (only serum 2 is shown; see Fig. 6), and serum from a patient infected with *T. gondii* (Toxo).

FIG. 6. Western blots of protein extracts from *T. cruzi* (T) and *L. d. chagasi* (L) probed with sera from seven Chagas' disease patients (sera 1 through 7), showing those proteins in both organisms that are recognized by the sera. Parallel Western blots were also probed with serum from an uninfected individual (negative serum) and with serum FHC2-III from a patient with visceral leishmaniasis. Numbers on the left are molecular sizes in kilodaltons.

shown in the second lane of each pair (lanes L), and it can be seen clearly that three sera (no. 2, 3, and 7) have antibodies whose reactions with antigens in the range of 30 to 60 kDa are comparable to their reactions with *T. cruzi* antigens. The other four anti-*T. cruzi* sera react to a lesser extent with a few *Leishmania* antigens. None of the seven sera, however, show substantive reactivity with antigens above the level of ~60 kDa, which is where Hsp70 and Hsp90 are located.

DISCUSSION

Our results indicate that patients with visceral leishmaniasis generally have strong antibody responses against *Leishmania* Hsp90 and Hsp70, a finding consistent with earlier reports that parasite Hsps are prominent targets of host immune responses. Antibodies against the parasite Hsp90 have been found in sera of patients with malaria and trypanosomiasis, and anti-Hsp70 antibodies occur in patients with malaria, trypanosomiasis, schistosomiasis, filariasis, and leishmaniasis (reviewed in references 20, 37, and 42).

The striking, and somewhat unexpected, observation is that sera from patients with Chagas' disease do not react with recombinant *Leishmania* Hsp90 and Hsp70, despite more than 80% identity over the full *Leishmania* and *T. cruzi* Hsp sequences. This contrasts with earlier reports of cross-reactivity in serodiagnostic assays for antibodies to *Leishmania* spp. and *T. cruzi* based on native parasite antigens (1, 5, 13, 36). Our results are supported by the findings of MacFarlane et al. (27), who identified an Hsp70 cDNA in a cDNA library made from an African strain of *L. donovani* by screening with a patient serum and then examined the reactivity of the resultant Hsp70 fusion protein with a variety of other serum specimens. In Western blots, their fusion protein did not react with 1:100 dilutions of sera from 20 patients with Chagas' disease or with the same dilutions of sera from 27 patients with cutaneous or mucocutaneous leishmaniasis. They also found that only 9 of 17 sera from patients with visceral leishmaniasis recognized their Hsp70 fusion protein, whereas all four of the specimens we tested were reactive. More sera from patients with visceral leishmaniasis will need to be tested in our ELISAs to determine their sensitivities.

In contrast to our results and those of MacFarlane et al. (27), previously published Western blots from several laboratories have suggested that Hsp70s from *Leishmania* spp. and *T. cruzi* might be cross-reactive. Extracts of *Leishmania* spp. were shown to contain an antigen(s) of about 70 kDa that reacted with sera from patients infected with *T. cruzi* (7, 9, 35), and conversely, *T. cruzi* extracts possessed a 70-kDa antigen(s) that was recognized by sera from leishmaniasis patients (2, 7, 28). Cross-reactivities among a number of different *Leishmania* and *T. cruzi* antigens have also been shown by using immunofluorescence assays (6). Furthermore, when we recently immunoscreened a *T. cruzi* cDNA library with serum from a patient with visceral leishmaniasis, the most frequently detected fusion protein contained the *T. cruzi* Hsp70 sequence (29).

Several factors may have contributed to the specificity of the reactions we observed in our Hsp90 and Hsp70 ELISAs. First, the Hsp fusion proteins that we used as target antigens contain the C-terminal sequences, i.e., the regions where the most sequence diversity among Hsps of *Leishmania* and *T. cruzi* occurs (30). Thus, the more highly conserved N-terminal portions of the Hsps, with which cross-reacting antibodies would be expected to react, are not present in the fusion proteins we used. Second, in those *Leishmania* species and

T. cruzi isolates for which sequence information is available, the C termini of the *T. cruzi* Hsp70 molecules have more tandem copies of the tetrapeptide GGMP than do the *Leishmania* Hsp70 molecules (12, 27, 38). Since repetitive epitopes of *T. cruzi* are known to be particularly antigenic (17, 19), the anti-Hsp70 response in *T. cruzi* infections may be directed more toward these GGMP repeats than they are in *Leishmania* infections. This observation suggests that anti-*Leishmania* sera are more likely to recognize *T. cruzi* Hsp70 than the converse, although we have not tested this possibility. A third consideration is that within the fusion proteins themselves, the highly conserved Hsp linear and/or conformational determinants may not be exposed as prominently as they are in the native antigens. A final technical factor that may contribute to the specificity of our ELISA results is that native Hsps associate with a number of other proteins, an observation that led to a proposal that they function as molecular chaperones (11, 33). Thus, positive signals on some of the early Western blots might have been due to nonspecific binding of native Hsp to antibody. Fusion proteins containing only a portion of the Hsp70 sequence would be expected to participate relatively less in these nonspecific protein-protein interactions.

In conclusion, more work is required to determine the molecular basis for the immunospecificity of recombinant leishmania Hsp70 and Hsp90 fusion proteins reported here and by MacFarlane et al. (27). Nevertheless, our results support the growing literature suggesting that recombinant versions of Hsps react with antibodies from patient sera in a species-specific manner and deserve consideration as potential target antigens in assays for parasitic infections.

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