Identification of the C-terminal region of 70 kDa heat shock protein from *Leishmania (Viannia) braziliensis* as a target for the humoral immune response

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Abstract A *Leishmania (Viannia) braziliensis* (Lb) promastigote cDNA library in λ gt11 was screened with patients' sera with the aim of identifying antigens specifically related to mucocutaneous leishmaniasis (MCL). One of the clones isolated, 133P, consistently reacted with MCL sera; it was sequenced and found to encode the C-terminal three-quarters of a protein belonging to the highly conserved Hsp70 family. Since Hsp70 proteins from different species tend to be less conserved through the C-terminal end, it was predicted that this region would be more antigenic and was likely to bear the discriminatory epitopes. In order to test this hypothesis, the N- and C-terminal halves of the polypeptide encoded by 133P, 133P-N and 133P-C, respectively, were expressed in *Escherichia coli*. Immunoblotting analysis demonstrated that 133P-C reacted more strongly with a pool of MCL sera than 133P-N, and both recombinant proteins reacted faintly with pools of cutaneous (CL) and visceral (VL) leishmaniasis sera. These results confirmed the predicted epitope location in the C-terminal region. The 133P-C fragment was also expressed as a fusion protein with glutathione-S-transferase (GST-133P-C), affinity-purified with glutathione-agarose and tested by ELISA with individual sera. From 46 Lb-infected patients, 41 sera (89%) were positive, no cross-reactivity was observed with healthy, *Trypanosoma cruzi*- or *L. amazonensis*-infected individuals. Despite a relatively high cross-reactivity with VL sera, the enhanced humoral response of MCL as compared with CL patients might be interesting for studies on disease aggravation.

INTRODUCTION

About 20 species in the genus *Leishmania* infect humans, causing a spectrum of clinical manifestations generically called leishmaniasis. Approximately 350 million people are at risk of infection, and 12 million is a conservative estimate of the number of known cases (WHO 1990). In South America, *Leishmania chagasi* (*L. donovani*

complex) accounts for visceral leishmaniasis (VL) and members of the *L. braziliensis* and *L. mexicana* complexes are responsible for the often self-healing cutaneous form (CL) of the disease. For as yet unknown reasons, in some cases of CL severely aggravated clinical patterns can emerge and there is currently no means to predict these aggravations (Barker 1989). For instance, *Leishmania (Leishmania) amazonensis* patients may develop diffuse cutaneous leishmaniasis (DCL) and *Leishmania (Viannia) braziliensis* CL, after an apparent cure, relapses as mucocutaneous leishmaniasis (MCL), a condition characterized by mutilating lesions with intense mononuclear cell

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		K	K	A	V	V	T	V	P	A	Y	F	N	D	S	Q	R	Q	A	T	K
1	G	AAG	AAG	GCT	GTG	GTG	ACG	GTG	CCG	GCC	TAC	TTC	AAC	GAC	TCG	CAG	CGC	CAG	GCG	ACG	AAG
		D	A	G	T	I	A	G	L	E	V	L	R	I	I	N	E	P	T	A	A
62		GAC	GCC	GGC	ACG	ATT	GCT	GGC	CTG	GAG	GTG	CTG	CGC	ATC	ATC	AAC	GAG	CCA	ACA	GCG	GCG
		A	I	A	Y	G	L	D	K	G	D	D	G	K	E	R	N	V	L	I	F
122		GCC	ATC	GCG	TAC	GGC	CTG	GAC	AAG	GGC	GAC	GAC	GGC	AAG	GAG	CGC	AAC	GTG	CTC	ATC	TTC
		D	L	G	G	G	T	F	D	V	T	L	L	T	I	D	G	G	I	F	E
182		GAC	CTT	GGC	GGC	GGC	ACG	TTC	GAT	GTG	ACG	CTG	CTG	ACG	ATC	GAC	GGC	GGC	ATC	TTC	GAC
		V	K	A	T	N	G	D	T	H	L	G	G	E	D	F	D	N	R	L	V
242		GTG	AAG	GCG	ACG	AAC	GGT	GAC	ACG	CAC	CTT	GGC	GGC	GAG	GAC	TTT	GAC	AAC	CGC	CTC	GTG
		T	F	F	S	E	E	F	K	R	K	N	K	G	K	D	L	S	S	S	H
302		ACG	TTC	TTC	AGC	GAG	GAG	TTC	AAG	CGC	AAG	AAC	AAG	GGT	AAG	GAC	CTT	TCG	TCG	AGC	CAC
		R	A	L	R	R	L	R	T	A	C	E	R	A	K	R	T	L	S	S	A
362		CGC	GCG	CTG	CGC	CGC	CTG	CGC	ACG	CGC	GAG	CGC	CGC	AAG	CGC	ACG	CTG	TCG	TCG	SCC	GCG
		T	Q	A	T	I	E	I	D	A	L	F	D	N	V	D	F	Q	A	N	I
422		ACG	CAG	GCG	ACG	ATC	GAG	ATC	GAC	GCG	CTG	TTC	GAC	AAC	GTC	GAC	TTC	CAG	GCC	AAC	ATC
		T	R	A	R	F	E	E	L	C	G	D	L	F	R	S	T	M	Q	P	V
482		ACG	CGC	GCG	CGC	TTC	GAG	GAG	CTG	TGC	GGC	GAC	CTG	TTC	CGC	AGC	ACA	ATG	CAG	CGC	GTG
		E	R	V	L	Q	D	A	K	M	D	K	R	S	V	H	D	V	V	L	V
542		GAG	CGC	GTG	CTG	CAG	GAC	GCG	AAG	ATG	GAC	AAG	CGC	TCC	GTG	CAC	GAC	GTG	GTG	CTG	GTG
		G	G	S	T	R	I	P	K	V	Q	S	L	V	S	D	F	F	G	G	K
602		GGC	GGG	TCG	ACG	CGC	ATC	CCG	AAG	GTG	CAG	TCC	CTC	GTG	TCG	GAC	TTC	TTC	GGC	GCG	AAG
		E	L	N	K	S	I	N	P	D	E	A	V	A	Y	G	A	A	V	Q	A
662		GAG	CTG	AAC	AAG	AGC	ATC	AAC	CCC	GAC	GAG	GCT	GTC	GCG	TAC	GGC	GCT	GCG	GTG	CAG	GCG
		F	I	L	T	G	G	K	S	K	Q	T	E	G	L	L	L	L	D	V	T
722		TTC	ATC	CTG	ACG	GGC	GGC	AAG	AGC	AAG	CAG	ACG	GAG	GGC	CTG	CTG	CTG	GAT	GTG	ACG	
		P	L	T	L	G	I	E	T	A	G	G	V	M	T	A	L	I	K	R	N
782		CCG	CTG	ACC	CTG	GGC	ATT	GAG	ACG	GCC	GGC	GGC	GTG	ATG	ACG	GCG	CTG	ATC	AAG	CGC	AAC
		T	T	I	P	T	K	K	S	Q	I	F	S	T	Y	A	D	N	Q	P	G
842		ACG	ACG	ATC	CCG	ACC	AAG	AAG	AGC	<u>CAG</u>	<u>ATC</u>	<u>TTC</u>	TCG	ACG	TAC	GCG	GAC	AAC	CAG	CCC	GGC
		<i>Bgl</i> III																			
		V	H	I	Q	V	F	E	G	E	R	A	M	T	K	D	C	H	L	L	G
902		GTG	CAC	ATC	CAG	GTC	TTC	GAG	GGC	GAG	CGC	GCG	ATG	ACG	AAG	GAC	TGC	CAC	CTG	CTG	GGC
		T	F	D	L	S	G	I	P	P	A	P	R	G	V	P	Q	I	E	V	T
962		ACG	TTC	GAC	TTG	TCC	GGC	ATC	CCG	CCA	GCG	CCG	CGC	GGC	GTG	CCG	CAG	ATC	GAG	GTG	ACG
		F	D	L	D	A	N	G	I	L	N	V	S	A	E	E	K	G	T	G	K
1022		TTC	GAC	CTG	GAC	GCG	AAC	GGC	ATC	CTG	AAC	GTG	TCC	GCG	GAG	GAG	AAG	GGC	ACC	GGC	AAG
		R	N	H	I	T	I	T	N	D	K	G	R	L	S	K	D	E	I	E	R
1082		CGC	AAC	CAT	ATC	ACC	ATC	ACC	AAC	GAC	AAG	GGC	CGA	CTG	AGC	AAG	GAC	GAG	ATC	GAG	CGC
		M	V	N	D	A	S	K	Y	E	Q	A	D	K	M	Q	R	E	R	V	E
1142		ATG	GTG	AAC	GAT	GCG	TAC	AAG	TAC	GAG	CAG	GCC	GAC	AAG	ATG	CAG	CGC	GAG	CGC	GTG	GAG
		A	K	N	G	L	E	N	Y	A	Y	S	M	K	N	T	V	S	D	T	N
1202		GCG	AAG	AAC	GCG	CTG	GAG	AAC	TAC	GCG	TAC	TCG	ATG	AAG	AAC	ACG	GTC	TCC	GAC	ACG	AAC
		V	S	G	K	L	E	E	S	D	R	S	A	L	N	S	A	I	D	T	A
1262		GTG	TCC	GGC	AAG	CTG	GAG	GAG	AGC	TCC	GCG	CTG	AAC	TCG	GCG	ATC	GAC	ATC	ACG	GCG	
		L	E	W	L	N	S	N	Q	E	A	S	K	E	E	Y	E	H	R	Q	K
1322		CTG	GAG	TGG	CTG	AAC	AGC	AAC	CAG	GAG	GCG	TCG	AAG	GAA	GAG	TAC	GAG	CAC	CGC	CAG	AAG
		E	L	E	S	T	C	N	P	I	M	T	K	M	Y	Q	S	M	G	G	G
1382		GAG	CTG	GAG	AGC	ACA	TGC	AAC	CCG	ATC	ATG	ACC	AAG	ATG	TAC	CAG	AGC	ATG	GGC	GGC	GGC
		A	G	G	M	P	G	G	M	P	D	M	S	G	M	G	G	G	A	G	P
1442		GCG	GGC	GGC	ATG	CCC	GGC	GGT	ATG	CCG	GAC	ATG	AGC	GGC	ATG	GGC	GGT	GGT	GCG	GGT	CCG
		A	A	G	A	S	S	G	P	K	V	E	E	V	D	*					
1502		GCC	GCC	GGA	GCC	TCC	TCC	GGC	CCC	AAG	GTC	GAG	GAG	GTC	GAC	TAGGCTCTG	TGTGCAACAG				
1563		CGGTGCTGTA	GTGCTCGCCG	GTGAATGCCA	TATATCCTGC	TCACGCCGCT	GATGTATGTC	TGGGTGTTGG													
1633		CGGATGCGGG	TTCGCTGATG	CCGTGGAGAT	ACTCCTCGAA	CTGATGGATA	TGGTGTCTACT	ATGTCTCTGTT													
1703		CAGGTGTTTT	TCTGCTTTCG	GTTGACGCGC	GGGTGTCTTA	CAGTGGCGCT	GCGACTCTCT	CGTCGCATAT													
1773		GCGGCGTGTC	TCTGCGTGTG	TGTATGTCTT	TTATTTTTTT	TGTGTGTTTT	ATATTTTTCT	CCTTTCGTAC													
1843		TAAGAGCGTT	TGCTCACTCC	TACCCACCCC	ACATTAAGCC	CCTCCTCCCC	TCTCCCTCC	CCGCAATTTG													
1913		TTTTGTCTTC	AAAAATTAGT	TCTCTCTTTG	TTTCCGCCCC	TCTCTCGGTG	TCTTGCATAT	GAACCTGCAG													
1983		AGAGCCATCG	CACACTCTCC	TCTTCTTAG	TCTCTCCCTC	TCTCTTTAAC	GCCCCAACCT	GTTGTGCTC													
2053		TGCTTTCAC	ACCCCACTAC	TGCGGTACAG	CTGCATGTGT	GTGTGTGTGG	GCGCAGTGCC	TTCTTGTGTT													
2123		CTCTCTGTTT	TCGCTTTTCC	CTTCCACCCG	TGGGCASTAA	AGGCGCATAA	GCCTGTGCAC	TGGTGCAGCA													
2193		GGGAGGAGAG	GACAACGAGC	CCGCTGTCT	GCTATAATCG	CCGCTGAAGT	TGTTCCGACAC	CTTCGACGA													
2263		GAGGAAGTGG	ATTGATCGCG	GCGGTGCGGG	CGGAGAGAGG	GAGCTGGTGA	GAAGGAAAG	AGAGAAAGCA													
2333		TGCAGAGTGA	GTGAGCACTC	GTTAGGCGGG	CTTTGGCAGC	GGGAATGCTC	ACCTGCATGT	CTGCAGTGA													
2403		GGCTGGCGTT	GATTGAGACA	AGCGGCCACC	GCCATCTGTG	CGTTTGTGGA	CGCGGAGAA	TCAAAAAA													

Fig. 1 Nucleotide and corresponding amino acid sequences of 133P. The derived amino acid sequence is shown above the nucleotide sequence. The position of the *Bgl*III site used to produce the recombinant N- and C-terminal fragments is indicated. The asterisk represents the stop codon.

infiltrate in the oro- and nasopharyngeal regions. Since the severe tissue destruction mimics tuberculoid leprosy, the same social stigma is attached to MCL sufferers. The relapse can occur from months to many years after the initial infection and, therefore, in clinical practice, a diagnostic or prognostic test for a mucocutaneous outcome would be extremely useful to assess the necessity and timing of chemotherapy, which is expensive, has undesirable side-effects and requires medical supervision (Chang et al 1985; Wilson and Pearson 1990).

Our approach to this pursuit was to screen a *L. (V.) braziliensis* cDNA expression library with sera from patients presenting either MCL or CL. One λ gt11 clone was isolated that appeared to be recognized preferentially by MCL sera. Nucleotide sequencing revealed that it encodes an antigen belonging to the 70 kDa heat shock protein family, which has long been recognized as a common immunogen in many bacterial and parasitic diseases (reviewed by Young et al 1990 and Polla 1991), including *L. donovani* and *L. major* infections. As expected, its level of identity to Hsp70-like proteins of other *Leishmania*, and even to the human sequence is high, but since sequence identity decreases towards the C-terminus, the epitopes recognized by the patients' sera were rationally predicted to be located in the variable C-terminal region. Aiming to confirm the predicted epitope location, a large amount of recombinant antigen was produced for serological studies. Here we report that MCL, CL and VL serum-reactivity is indeed directed towards the C-terminal region of Hsp70, which contains the immunodominant epitopes.

MATERIALS AND METHODS

Patients' sera

All the sera used in this study were from patients with well-characterized clinical conditions and bearing active lesions at the time of collection, irrespective of whether they had received prior chemotherapy or not. The sera from *L. (V.) braziliensis*-infected patients came either from Rio de Janeiro State, Brazil (provided by Drs S. Coutinho, Alda Cruz and F. Conceição-Silva, Fiocruz) or from Bahia State (provided by Dr C. Cuba-Cuba, University of Brasília). The sera from *L. (L.) amazonensis*-infected patients were provided by Dr Jeffrey Shaw, Evandro Chagas Institute, and the sera from visceral leishmaniasis patients by Dr José Franco da Silveira, Escola Paulista de Medicina.

Library immunoscreening

A *L. (V.) braziliensis* (MHOM/BR/M2903) promastigote cDNA library constructed in λ gt11 as described by

Gubler and Hoffman (1983) was provided by John Kelly (London School of Hygiene and Tropical Medicine). Approximately 40 000 recombinant phage clones were screened using the procedure described by Ozaki et al (1986). Prior to use, sera were diluted 1:50, preabsorbed by incubating overnight at 4°C with wild-type λ gt11 plaque lifts on nitrocellulose membranes and affinity chromatography on a total *Escherichia coli* Y1090 cell lysate immobilized on sepharose. Sera were used in a 1:100 working dilution.

DNA sequencing

The 2.5-kbp insert of clone 133P was subcloned into the *EcoRI* site of the pT7T3 19U phagemid (Pharmacia) and 300–500 bp long fragments obtained by sonication of the plasmid (Baer et al 1984) were subcloned in M13mp18 and sequenced by the dideoxy-chain termination method (Sanger et al 1977) using T7 DNA polymerase. Each single base pair was sequenced six times on average and at least once in each DNA strand.

Expression in *E. coli*

Taking advantage of a suitable *Bgl*II site in position 869 of the cDNA (see Fig. 1) and the *Bam*HI site in the phagemid polylinker, *Bam*HI-*Bgl*II fragments were isolated from two clones with the 133P insert in opposite orientations and subcloned into the *Bam*HI cloning site of pET8c vector (Rosenberg et al 1987). The two resulting constructs, 133PpET-N and 133PpET-C, code for, respectively, the 288 N-terminal (133P-N) and 225 C-terminal (133P-C) amino acids of the total protein encoded by the cDNA (see diagram in Fig. 2A). Recombinant plasmids were used to transform *E. coli* BL21(DE3) (Rosenberg et al 1987) and transformants were grown at 37°C in LB medium supplemented with ampicillin until the culture reached an A_{600} of 0.3–0.5. Isopropyl- β -D-thiogalactopyranoside (IPTG) was then added to 0.02% (w/v) final and incubation carried on for a further 3 h before harvesting the bacteria. Cells from large-scale preparations (500 ml of bacterial culture) were lysed in 25 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM dithiothreitol, 2 mg/ml lysozyme), incubated on ice for 15 min and the lysate sonicated with 3 pulses of 30 s at maximum power prior to centrifugation for 5 min at 17 000 g. In the case of 133P-C, the recombinant protein remained soluble in the supernatant while 133P-N was found in the precipitate which was further washed 4 times with 20 ml of washing buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40) and resuspended in 2 ml of water. The recombinant proteins were subsequently purified by preparative SDS-PAGE and electroelution (Carroll and Laughon 1987).

Fig. 2 Design and expression of 133P-derived recombinant proteins in *E. coli* and immunoblot reactivity of pools of patients' sera. Panel A shows a diagram with the structure of 133P cDNA and constructs made in the pET expression vector. The slashed areas indicate coding regions. In panels B–F a series of identical samples containing bacterial lysates of non-recombinant *E. coli* BL 21 (track E), 133P-C (track C), 133P-N (track N) and a mixture of the two electroeluted fragments (tracks C + N) and *L. (V.) braziliensis* lysate (track L) were analyzed in replica SDS-PAGE gels. One of the gels was Coomassie blue stained (panel B) and the remaining were transferred to nitrocellulose filters and probed with a pool of 2 MCL sera from Rio de Janeiro (panel C); a pool of 11 CL sera from Rio de Janeiro (panel D); a pool of 4 VL sera (panel E) or with normal human serum (panel F). Bound antibody was detected with 125 I-protein A and autoradiography. The relative positions of molecular weight markers appear on the left-hand side of each panel.

The same *Bam*HI-*Bgl*II fragment mentioned above, coding for the C-terminus of 133P, was also subcloned in the *Bam*HI site of pGEX-3X plasmid (Pharmacia) in order to express 133P-C in fusion with glutathione-S-transferase (GST-133P-C). Induction of expression and recombinant protein purification by affinity chromatography with glutathione-agarose were performed essentially as described by Smith and Johnson (1988). GST alone was prepared in the same way from bacteria transformed with non-recombinant pGEX plasmid.

SDS-PAGE and immunoblotting

All protein samples were reduced with 5% 2-mercaptoethanol and analyzed by SDS-PAGE in 14% gels as described by Laemmli (1970). Western blots of these gels were obtained and processed by standard methods (Harlow and Lane 1988). For preabsorption of sera, a non-recombinant *E. coli* BL21 extract was obtained by resuspending the bacterial pellet of an overnight culture in 1/100 of the original volume of TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 2 mg/ml of lysozyme and sonicating until the viscosity disappeared. Undiluted sera were then rotated for 3–5 h with 10 volumes of extract and cell debris removed by centrifugation. After blocking with TBS containing 5% defatted milk (TBS/MT) for 1 h, filters were incubated overnight with preabsorbed human sera diluted 1:100 in the same buffer. Subsequent washes were 3 times 20-min in TBS/MT. Filters were then incubated for 2 h with 0.25 μ Ci of 125 I-labelled protein A (Amersham, specific activity 30 mCi/mg) in 10 ml of TBS/MT, washed as before and then autoradiographed at -70°C .

ELISA with patients' sera

Recombinant proteins (GST-133P-C or GST) were initially diluted to 1 μ g/ml in 0.01 M Na_2CO_3 , 0.03 M NaHCO_3 , pH 9.6, and 50 μ l per well were applied to 96-well plates (Coastar 3590). The plates were then incubated at 37°C for 2 h followed by overnight incubation at 4°C and 3 washes with PBS-T (10 mM NaH_2PO_4 , 1.5 mM KH_2PO_4 , 150 mM NaCl, 0.1% Tween-20, pH 7.5). Each well was blocked with 100 μ l per well of PBS-T containing 5% defatted milk for 2 h at 37°C . Plates were washed as above and then incubated with 50 μ l per well of non-preabsorbed patient sera diluted in PBS-T containing 1% milk, for 1 h at 37°C . After the washes, 50 μ l per well of peroxidase-coupled anti-human immunoglobulin (Sigma A-8400), diluted 1:500 in PBS-T/1% milk, were added and plates incubated for 1 h at 37°C . After the final washes, 100 μ l per well of freshly prepared developing solution (25 ml of 0.1 M citric acid, 0.1 M sodium phosphate, pH 4.5 containing 0.4 mg/ml o-phenylene diamine and

0.4 μ l/ml 30 vol hydrogen peroxide) were added, reaction carried out for 10 min and then stopped by the addition of 50 μ l per well of 2 M sulfuric acid. The absorbance at 492 nm was measured with an ELISA reader (Labsystems). In each ELISA, sera were tested in triplicate and a negative control, normal human serum, was included as well as a known positive human serum.

Nucleotide sequence accession number

Nucleotide sequence data reported in this paper have been submitted to GenBank with the accession number M87878.

RESULTS

Isolation of recombinant λ gt11 clones

Two immunoscreenings of the *L. (V.) braziliensis* λ gt11 library were performed. In the first, clone 133P was selected with serum from one MCL patient from Três Braços, Bahia State, Brazil. In the second screening, an additional 16 clones were isolated using a pool of two MCL and one CL sera from Rio de Janeiro. The reactivity of all these clones, including two controls, clone 24a1 isolated from a *L. (V.) braziliensis* library with MCL sera, and clone Ld30a1 from a *L. donovani* library with VL sera, were tested against several sera, as shown in Figure 3. With the exception of clones 13–16 and 24a1, all clones, including 133P, reacted with the CL+MCL pool used in the second library screening (Fig. 3B), but only clone 133P was positive for another pool of 7 MCL sera from Rio de Janeiro (Fig. 3C). Two sera, which were part of the mixed pool mentioned in Figure 3B, were tested individually and it was found that clones 11, 12 and 133P reacted only with the MCL serum (Fig. 3D) and clones 1–10 only with the CL serum (Fig. 3E). With the exception of the positive control (clone Ld30a1), no reaction with any of the 18 clones was observed either with a pool of 4 VL sera (Fig. 3F), or with one chagasic serum (Fig. 3G). Considering these results, it was decided to further characterize clone 133P, which appeared to be promising as a marker for MCL.

133P cDNA sequence

The nucleotide sequence of 133P revealed that the 2472 bp cDNA is not full length and has an ORF of 1546 bp followed by a 926 bp-long 3' untranslated region (Fig. 1). The derived amino acid sequence indicated that the polypeptide encoded belongs to the 70 kDa heat-shock protein family (Hsp70) and corresponds approximately to the C-terminal three-quarters of the whole native protein.

Fig. 3 Serum-reactivity of the isolated recombinant clones. Panel A shows a key to the plaque dot-blot positions of the clones on the nitrocellulose filters tested against a pool of 2 MCL and 1 CL sera from Rio de Janeiro (panel B); a pool of 7 MCL sera from Rio de Janeiro (panel C); an individual MCL serum (panel D); an individual CL serum (panel E); a pool of 4 VL sera (panel F) and one chagasic serum (panel G). All sera were preabsorbed and filters were processed exactly as described in the text for library screening. Bound antibodies were detected with goat anti-human Ig-alkaline phosphatase conjugate.

A measurement of amino acid sequence identity using the DNASTAR computer program showed that 133P is highly conserved with Hsp70s from *L. amazonensis* (91%) (Bock and Langer 1993), *L. donovani* (93%) (MacFarlane et al 1990) and *L. major* (97%) (Lee et al 1988); *Trypanosoma cruzi* (84%) (Requena et al 1988); *Trypanosoma brucei* (85%) (Glass et al 1986) and even with the human 70 kDa cognate protein (72%) (Dworniczak and Mirault 1987). Since these proteins are less conserved in the C-terminal region, there is a high probability that any immunodominant, and possibly species-specific, epitopes are

located towards the C-terminus as there is less sequence conservation in this region.

Expression in *E. coli* and analysis of recombinant Hsp70

In order to confirm the prediction that the C-terminal region bears the main epitopes recognized by the patients, two constructs were made to express separately the N- and C-terminal halves of the protein encoded by clone 133P, as shown in Figure 2A.

Fig. 4 Expression of GST-133P-C using vector pGEX and affinity-purification of the recombinant fusion protein. Coomassie blue-stained 10% SDS-PAGE gel showing total recombinant bacterial lysates before (track 1) and after (track 2) IPTG induction and the 51 kDa GST-133P-C affinity-purified with glutathione-agarose (track 3).

Upon induction by IPTG, *E. coli* produced both recombinant polypeptides at high levels, as shown in the Coomassie blue-stained SDS-PAGE in Figure 2B. The migration patterns of both polypeptides is consistent with the predicted molecular weights of 36 kDa for the

N-terminal (133P-N, track N) and 25.5 kDa for the C-terminal (133P-C, track C) constructs.

The recombinant proteins were purified by electroelution from preparative SDS-PAGE gels and mixed, as shown in Figure 2B (track C + N). Replicas of the gel shown in this Figure were transferred to nitrocellulose membranes and probed with pools of mucocutaneous (Fig. 2C), cutaneous (Fig. 2D) and visceral (Fig. 2E) leishmaniasis sera, as well as with a normal human serum from a non-endemic country (Fig. 2F).

The pattern of proteins recognized in the native *L. (V.) braziliensis* lysate by the patients with the different clinical conditions is clearly distinct regarding both intensity and specificity (Fig. 2C, D and E, L tracks). With exception of a set of bands in the region of 10–20 kDa, in all cases the overall reactivity predominated towards the bands with molecular weights higher than 50 kDa. None of the tested sera recognized proteins in the non-recombinant *E. coli*, an observation that, when compared to similar immunoblot patterns of non-preabsorbed sera (data not shown), confirmed the necessity of the pre-absorption step.

Fig. 5 Quantitative analysis of patient serum-reactivity against GST-133P-C. ELISA using GST-133P-C or GST on solid phase was performed as described and the resulting ODs at 492 nm for each *L. braziliensis* patient with MCL (▲), MCL by contiguity (△), CL (○) and disseminated CL (■) are shown on the left side, as indicated. Patients bearing VL by *L. chagasi* (▶), CL by *L. amazonensis* (□), Chagas' disease (▼) as well as healthy individuals (◇) are shown on the right. Each patient serum has also been assayed against GST (♦) and the result is shown under each GST-133P-C record. All sera were tested in triplicate and in a 1:160 dilution.

Table Summary of patient serum-reactivity against GST-133P-C in ELISA *

Clinical form	A ₄₉₂ > 0.2	A ₄₉₂ > 0.5
MCL	18/20 (90%)	11/20 (55%)
CL	15/18 (83%)	3/18 (17%)
CL (disseminated)	4/4 (100%)	3/4 (75%)
MCL (contiguity)	4/4 (100%)	0/4 (0%)
VL (<i>L. chagasi</i>)	4/6 (67%)	4/6 (67%)
CL (<i>L. amazonensis</i>)	1/7 (14%)	0/7 (0%)
Chagas' disease	0/3 (0%)	0/3 (0%)
Normal sera	2/12 (17%)	0/12 (0%)

* The values express the number of positive sera, for each established A₄₉₂ cut-off, over the total number of sera tested for each clinical condition. Percentage of positive sera is indicated beside each value.

As expected, the MCL sera reacted strongly to the C-terminal polypeptide but a weak binding to the N-terminal fragment is also seen in Figure 2C. The same faint level of reaction was found for CL sera towards both N- and C-terminal electroeluted polypeptides though the binding is still stronger in the latter (Fig. 2D, track C + N). Interestingly, when the C-terminal fragment was present in a total bacterial lysate (Fig. 2D, track C), no reaction with CL sera was observed, an indication that the electroelution procedure may disclose non-exposed epitopes. On the other hand, the VL pool of sera (Fig. 2E) reacted only faintly to the N-terminal polypeptide and not at all to the C-terminal. The negative control showed no reaction of the recombinant antigens with a normal human sera (Fig. 2F).

Due to the need of using purified antigen for analysis of a large number of non-preabsorbed patient sera and to the difficulty previously experienced in purifying large amounts of proteins expressed using vector pET, the same 133P-C fragment was expressed in fusion with GST, using a pGEX vector. As shown in Figure 4, the expected fusion protein (GST-133P-C) of 51 kDa was soluble and could be subsequently affinity-purified from cell lysates.

Serology

The purified GST-133P-C was tested against patients' sera by ELISA, as shown in Figure 5 and Table. To rule out the possibility of serum reaction against the GST fragment present on the fusion protein, each individual serum was tested with isolated GST, obtained from expression of non-recombinant pGEX plasmid. The level of reaction of patients' sera against isolated GST in ELISA was less than 0.2 of absorbance, approximately the same number obtained by testing normal human sera against GST-133P-C, so establishing the value of 0.2 as the assay's background (Fig. 5).

Thus, considering 0.2 as the assay cutoff, 41 out of 46 *L. (V.) braziliensis*-infected patients (89%) were positive for GST-133P-C (Fig. 5 and Table). On the other hand, if we consider an assay cutoff of 0.5, it was observed that approximately half of the MCL sera tested (11/20 or 55%) was positive, whereas only 3 out of 18 CL sera (17%) reacted with GST-133P-C. A Fisher's exact test indicated that the difference in GST-133P-C reactivity between CL and MCL sera was statistically significant, with a *P* value of 0.02. Sera from *L. (V.) braziliensis*-infected patients with disseminated cutaneous lesions and patients with mucous lesions due to contiguity with primary cutaneous lesions were also tested. In the first case, 3 out of 4 (75%) displayed values over 0.5 and, in the second group, all 4 sera (100%) reacted over 0.2 but under 0.5 of absorbance (Fig. 5 and Table).

Lastly, chagasic and CL sera from *L. (L.) amazonensis*-infected patients were negative, but among 6 visceral leishmaniasis patients (*L. chagasi*), 4 were positive (67%) with values higher than 0.5 (Fig. 5 and Table).

DISCUSSION

In this study the isolation of 17 clones from a *L. (V.) braziliensis* cDNA library in λ gt11 using sera from patients with cutaneous (CL) and mucocutaneous (MCL) leishmaniasis is reported. In a preliminary evaluation, these clones were divided in three groups, according to their reactivity on plaque dot-blot (Fig. 3): non-reactive (clones 13–16), CL-reactive (clones 1–10) and MCL-reactive (clones 133P, 11 and 12). Since in these dot-blot assays clone 133P reacted specifically with MCL sera, it was further characterized. Its sequence corresponded to the C-terminal three-quarters of an Hsp70-related antigen, with high identity at the amino acid level with both parasite and human Hsp70-related proteins.

The *L. braziliensis* Hsp70 contains two repeats of the tetrapeptide GGMP (Fig. 1), characteristic of both human


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1 ----- Lb
1 MTFDGAIGIDLGTTSYSCVGVWQNERVDIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVAMNPHNTVFDA Ld
1 MTFDGAIGIDLGTTSYSCVGVWQNDRVEIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVAMNPHNTVFDA La
1 MTFDGAIGIDLGTTSYSCVGVWQNERLDIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVAMNPHNTVFDA Lm
1 MTEGAIGIDLGTTSYSCVGVWQNERVEIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVAMNPHNTVFDA Tc
1 MSKGAIGIDLGTTSYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVAMNPHNTVFDA Hu

1 ----- Lb
71 KRLIGRKFNDSVVSQDMKHWPFFKVTTKGDDKPMIAVQYRGEKFTTPEEISSMVLLKMKETAAYLGKQV Ld
71 KRLIGRKFNDSVVSQDMKHWPFFKVTTKGDDKPVISVQYRGEKFTTPEEISSMVLLKMKETAAYLGKQV La
71 KRLIGRKFNDSVVSQDMKHWPFFKVTTKGDDKPVISVQYRGEKFTTPEEISSMVLLKMKETAAYLGKQV Lm
71 KRLIGRKFNDSVVSQDMKHWPFFKVITKGDDKPVIVQYRGETKTFNPEEVSSMVLSKMKETAEAYLGKQV Tc
71 KRLIGRRFDDAVVSQDMKHWPFFMVNDAGR-PKVQVEYKGETKSFYPEEVSSMVLTSMKETAAYLGKTV Hu

1 KKAUVTVPAYFNDSQRQATKDAGTIAGLEVLRINEPTAAAIAYGLDKGDDGKERNVLIFFDLGGGTDFVT Lb
141 .....R-.....S Ld
141 .....S.....La
141 .....V.....Lm
141 .....VE.....Tc
140 TN.....N.....KV-A.....S Hu

71 LLTIDGGIFEVKATNGDTHLGGEDFDNRLVTFSEEFKRKNKGKDLSSSHRALRRLTACERAKRTLSSA Lb
210 .....T.....N-A.....G.....Ld
211 .....T.....N-A.....S.....La
211 .....R.....T.....N-A.....Lm
211 .....AH-TD.....TNL.....Tc
209 I-ED.....S-A.....M-NH-IA.....H-...I-ENK-V.....S Hu

141 TQATIEIDALFDNVDFQANITRARFEELCGDLFRSTMQPVERVLQDAKMDKRSVHDVVLVGGSTRIPKVQ Lb
280 .....E.....T.....I.....Ld
281 .....T-N.....I.....La
281 .....E-I.....T.....I.....Lm
281 A.....T.....E-G-L.....A.....M Tc
278 ..S...S-YEGI-YTS.....NA...G-LD...KA-R...L-SQI-I.....I Hu

211 SLVSDFFGGKELNKSINPDEAVAYGAAVQAFILTTGGKSKQTEGLLLLDVTPLTTLGIETAGGVMTALIKRN Lb
350 .....Ld
351 .....La
351 .....Lm
351 Q.....R.....K...Q.....S.....Tc
348 K-LQ...N.....A-S-D-ENVQD.....S.....V.....Hu

281 TTIPTKKSQIFSTYADNQPGVHIQVFEGERAMTKDCHLLGTFDLGIPAPRGVPQIEVTFDLANGILN Lb
420 .....Ld
421 .....La
421 .....Lm
421 .....E.....P.....Tc
418 .....QT-T-T-S.....L-Y.....NN...K-E-T.....I.....Hu

351 VSAEEKGTGKRNHITITNDKGRLSKDEIERMVNDASKYEQADKMQRERVEAKNGLENYAYSMMKNTVSDTN Lb
490 .....Q.....M...AD-RA-D.....LG-S Ld
491 .....Q.....M...AD-A-AD.....LG-S La
491 .....Q.....Lm
491 .....Q-VL.....RA.....RE-A-AE-D-VRQID.....F...A-N-P Tc
488 ..VD-S...E-K.....ED...QE-E-KAE-EK-DK-SS-S-S-FN-A-E-EK Hu

421 VSGKLEESDRSALNSAIDTALEWLNNSQEAKEEYERQKELESTCNPIMTKMYQSMGGGAGGMPGMPD Lb
560 .....DD-KAT-KE-VT...S...T...K...V.....AG.....Ld
561 .....DDT-K-T-KE-EA...S...T...K...NV.....A-...La
517 ----- Lm
561 -A-I-A-KKTIT-VEE.....N.....NL-T...N...G-A-A-...[GGMP]10Tc
558 LQ-INDE-KQKILDKCNEIIN-DK-T-E-F-Q...KV...I-L-A-MP.....F-Hu

491 MSGMGGGAGPAAGASSGPKVEEVD* Lb
630 ...S.....G.....* Ld
630 ...S-S--R-G.....* La
517 ----- Lm
664 -----N-SS-----E.....* Tc
627 -----P-SG.....TI.....* Hu

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Fig. 6 Sequence comparison between parasite and human Hsp70s. Alignment of amino acid sequences of: *L. (V.) braziliensis* (Lb), *L. donovani* (Ld), *L. amazonensis* (La), *L. major* (Lm), *T. cruzi* (Tc), *T. brucei* (Tb) Hsp70s and human Hsc70, obtained by Clustal method. Gaps (-) were introduced to optimize alignment and dots represent residues identical to the corresponding Lb (133P) sequence. Lb and Lm sequences are incomplete and the asterisks indicate the C-terminal end of sequences. References to the sequences analyzed are mentioned in the text.

Hsc70 and other parasite Hsp70-related proteins (Young et al 1990), including *T. cruzi*, where 10 repeats are present in a tandem array (Fig. 6 and Requena et al 1988). It is not known at present if the *L. (V.) braziliensis* protein encoded by the 133P cDNA is a heat-inducible or a constitutively expressed member of the Hsp70 family.

The observation that Hsp70s are less conserved towards the C-terminus (Fig. 6) as well as data from other groups (reviewed by Young et al 1990) led to the prediction that the immunogenic epitope(s) should be located on the C-terminus. Indeed, such an epitope was identified in the C-terminus of *L. donovani* Hsc70 (Wallace et al 1992). In order to test this prediction, the N- and C-terminal halves of 133P were expressed in *E. coli* and the reactivity of patient sera with the recombinant proteins was tested by immunoblots (Fig. 2). MCL sera reacted more strongly with the C-terminal fragment (133P-C), whereas CL and VL sera weakly recognized both fragments. Given the high level of identity between *L. (V.) braziliensis* and *L. donovani* Hsp70s, the observed lack of cross-reactivity in plaque dot-blots between MCL and VL sera comes as a surprise, but could be an operational consequence of the stringent preabsorption step in which cross-reacting antibodies could have bound to DnaK protein, the *E. coli* Hsp70 counterpart. On the other hand, an apparently contradictory finding was observed in ELISA, in which 4 out of 6 VL sera reacted with GST-133P-C (Fig. 5 and Table). As the same results were observed at least in three separate experiments, this contradiction could be partially attributed to the fact that the VL sera tested by ELISA were not the same ones tested by immunoblot, and also because the recombinant antigens were obtained and processed by quite different procedures.

Cross-reactivity between highly homologous parasitic Hsp70s has been previously documented. For instance, *L. donovani* recombinant Hsp70 reacted specifically with VL but not with MCL and CL sera (MacFarlane et al 1990; Wallace et al 1992), but cross-reactivity with sera from chagasic individuals was observed (Andrade et al 1992). Conversely, recombinant Hsp70 from *T. cruzi* was shown to react with both VL (Nafziger et al 1991) and MCL (Yeyati et al 1991) sera. It must be taken into account that these observations were made in different laboratories, with sera from various sources and recombinant antigens obtained and analyzed by multiple systems. It is therefore conceivable that such discrepancies might not be confirmed if immunoassays were conducted in strictly comparative protocols or using synthetic peptides instead of recombinant proteins.

The majority of the sera from *L. (V.) braziliensis*-infected patients (89%) reacted with GST-133P-C above the established ELISA cutoff ($A_{492} > 0.2$), and little reactivity was observed with sera from either *L. amazonensis*-infected,

chagasic or healthy individuals (Fig. 5 and Table). In addition, as it is shown in the Table, if an A_{492} of 0.5 is considered as the cutoff point, 55% of the MCL patients are positive, against only 17% of CL, an observation which may reflect the possibility that Hsp70 recognition could correlate with either the stage of infection or the evolution of a more severe clinical condition. In this direction, the reactivity was greater with sera from patients with disseminated CL, a condition which is considered an aggravated form of CL. Moreover, in patients in which primary cutaneous lesions on the face spread to neighboring mucous tissues (MCL by contiguity), the reactivity was at the same level as with CL patients, with A_{492} between 0.2 and 0.5. It is, therefore, conceivable that the increase in Hsp70 serum reactivity in MCL is just a consequence of the overall increase in the humoral response previously reported (Mendonça et al 1988).

Because Hsp70s are immunodominant antigens and display amino acid sequences that are remarkably conserved, the possibility of molecular mimicry between host and parasite Hsps leads to speculation on autoimmunity, particularly in diseases in which only a few parasites are found in the lesions (Petry and Eisen 1989; Polla 1991). However, this kind of argument does not seem to hold true in the case of Chagas' disease, where anti-*T. cruzi* Hsp70 antibodies do not seem to recognize the human counterpart on HeLa cells (Engman et al 1990).

Another interesting question to be explored is the cellular response against Hsp70 in MCL and CL. Indeed, recombinant *L. (V.) braziliensis* Hsps, including Hsp70, were shown to stimulate proliferation of MCL patients' T-lymphocytes (Carvalho et al 1993).

Despite the fact that no diagnostic claims can be drawn at this point, these data may represent a first step in developing a diagnostic or prognostic test for a MCL outcome. It also indicates the relevance of a more detailed evaluation of *L. (V.) braziliensis* Hsp70 humoral and cellular responses, including detailed deletion mapping of the C-terminal region, in order to search for discriminatory epitopes and to better understand the pathogenesis of mucocutaneous leishmaniasis.

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