

THE SERA FROM INDIVIDUALS SUFFERING FROM CUTANEOUS LEISHMANIASIS DUE TO *LEISHMANIA BRAZILIENSIS* PRESENT ANTIBODIES AGAINST PARASITIC CONSERVED PROTEINS, BUT NOT THEIR HUMAN COUNTERPARTS

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Summary:

Sera from individuals suffering from leishmaniasis have been shown to strongly react against conserved proteins from the parasite, such as ribosomal, histones and heat-shock proteins. Some of these proteins have also been described as immunogenic in several auto-immune syndromes, and the detection of antibodies against them is considered to be indicative of disorder of the immune system. In this paper, we investigate whether there is any relationship between the recognition of some conserved proteins from *Leishmania braziliensis* by individuals suffering from cutaneous (CL) and mucocutaneous (MCL) leishmaniasis, and the recognition of the human homologues of these antigens found in sufferers from autoimmune diseases. Our findings reveal that the immune response generated during CL and MCL is elicited specifically by the parasitic histone H1 and Hsp70, since the CL and MCL sera do not react against their human counterparts. In addition, evidence is presented showing the specific recognition of human proteins by the autoimmune sera, showing only a weak cross-reaction with the most divergent regions of the parasitic proteins.

KEY WORDS : autoimmunity, immune response, histone, chaperone, *Leishmania braziliensis*.

Résumé : LES SÉRUMS DE PERSONNES SOUFFRANT DE LEISHMANIOSE CUTANÉE PRÉSENTENT UNE FORTE RÉACTION CONTRE LES PROTÉINES CONSERVÉES DES PARASITES, MAIS NE RÉAGISSENT PAS CONTRE LEURS HOMOLOGUES HUMAINS

Les sérums de personnes souffrant de leishmaniose présentent une forte réaction contre les protéines conservées des parasites, comme par exemple les protéines ribosomales, les histones et les protéines de choc thermique (Hsp). Quelques-unes de ces protéines ont aussi été décrites comme immunogéniques dans plusieurs syndromes autoimmunitaires, et la détection d'anticorps contre elles est considérée comme un indicatif d'autoimmunité. Dans ce travail, nous avons cherché s'il existe une relation entre la reconnaissance de certaines protéines conservées de *Leishmania braziliensis* par les personnes malades de leishmaniose cutanée (CL) et mucocutanée (MCL), et la réponse générée contre les mêmes antigènes humains durant les pathologies autoimmunitaires. Nos résultats démontrent que la réponse immunitaire observée durant la CL et la MCL est générée spécifiquement par l'histone H1 et la protéine Hsp70 des parasites, mais que les sérums de CL et MCL ne réagissent pas contre leurs homologues humains. Il existe en plus une identification spécifique de protéines humaines par des sérums autoimmunitaires, et une faible réaction croisée avec les régions les plus divergentes des protéines parasitaires.

MOTS CLÉS : autoimmunité, réponse immunitaire, histone, chaperone, *Leishmania braziliensis*.

INTRODUCTION

Leishmaniasis is a disease caused by species belonging to the genus *Leishmania*, which currently threatens 350 million men, women and children in 88 countries around the world. Of the two million new cases discovered every year, about 1.5 million are cutaneous leishmaniasis (CL) or mucocutaneous leishmaniasis (MCL). *L. braziliensis* is one of the major causative agents of CL and MCL in wide areas of Central and South America.

The term 'conserved proteins' has been traditionally applied to a series of protein families that show remarkable evolutionary conservation, and are generally essential for the preservation of the cellular function.

Within each family, the proteins generally share domains that are responsible for their biochemical function, and present a certain structural similarity, although the sequence may present divergence. Among these proteins, the families of histones, ribosomal and chaperones (also called heat-shock proteins) have been extensively studied. Histones are evolutionarily conserved proteins which associate with DNA to form the nucleosome. The name histone H1 is applied to a family of small basic proteins which take part in the stabilization of the nucleosomes and facilitate the assembly of chromatin into higher-order structures. Histone H1 proteins have been described in different trypanosomatids like *Trypanosoma cruzi* (Aslund *et al.*, 1994), *Trypanosoma brucei* (Burri *et al.*, 1993), *Leishmania major* (Fasel *et al.*, 1994), and *Leishmania braziliensis* (Martinez *et al.*, 2002). Humoral immune response against parasite histones during infection has been described for *Leishmania infantum* H2A during canine visceral leishmaniasis (CVL) (Soto *et al.*, 1995), against histone H3, histone H2B and a fragment of histone H4 from

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L. infantum (Soto *et al.*, 1996; Soto *et al.*, 1999), and also against *L. braziliensis* histone H1 (Carmelo *et al.*, 2002). The researchers mapped the linear epitopes of histones using synthetic peptides, and their findings led to the conclusion that the humoral immune response against the *Leishmania* histones was triggered by the less conserved regions of the molecule (reviewed in Requena *et al.*, 2000; Carmelo *et al.*, 2002).

Chaperones, and particularly the members of the Hsp70 family, are among the most widely distributed proteins along the evolutionarily scale, and present a high degree of conservation regarding their primary structure, their regulation and their biochemical functions, playing a crucial role in numerous life-preserving processes in the cells (Young *et al.*, 1990). Besides this, Hsp70 has been described as a major immunogen in several parasitic infestations, such as leishmaniasis (MacFarlane *et al.*, 1990; Amorim *et al.*, 1996; Quijada, *et al.*, 1996a; Quijada *et al.*, 1996b; Zurita *et al.*, 2003), trypanosomiasis (Engman *et al.*, 1990; Krautz *et al.*, 1998), malaria (Ardeshtir *et al.*, 1987), schistosomiasis (Hedstrom *et al.*, 1987), o filariasis (Rothstein *et al.*, 1989, Selkirk *et al.*, 1989). Hsps have also been described as immunogenic in several auto-immune syndromes and pathological conditions, such as chronic inflammatory disease (Young, 1992) neurodegenerative processes and cancer (Oka *et al.*, 2001).

The aim of this paper is to determine whether individuals suffering CL and MCL present any of the classical signatures of tissue-destructive autoreactive disease, such as the presence in the sera of antibodies against human nuclear and cytoplasmic antigens. Similarly, the presence in a collection of sera from patients suffering autoimmune disorders, of antibodies that react with the chaperone Hsp70 from *L. braziliensis* (which presents a remarkable sequence identity with its human counterpart (Zurita *et al.*, 2003)) or the histone H1 from *L. braziliensis* will be investigated.

Our findings reveal that the immune response generated during CL and MCL is elicited specifically by the parasitic Histone H1 and Hsp70, since the CL and MCL sera do not react against their human counterparts. In addition, we provide evidence to the conclusion that the autoimmune sera react specifically to the human proteins, showing only a weak reaction with the least conserved regions of the parasitic proteins.

MATERIALS AND METHODS

EXPRESSION AND PURIFICATION OF RECOMBINANT *L. BRAZILIENSIS* ANTIGENS

The recombinant Histone H1 from *L. braziliensis* was purified as previously described (Carmelo *et al.*, 2002). Briefly, the coding gene was

inserted in the pQE31 expression vector (Qiagen) and the overexpression conditions optimised using the *Escherichia coli* Topp3 prokaryotic expression system (Stratagene). The purification of H1 was performed by Ni²⁺ ion-exchange chromatography in 8 M urea, 10 mM Tris-HCl, 100 mM sodium phosphate at pH 12.

The *HSP70* gene from *L. braziliensis* and five overlapping fragments covering residues 1-114, 109-245, 240-357, 352-518 and 513-663 respectively from the *L. braziliensis HSP70* gene were inserted in the pQE30/31 expression vectors (Qiagen). The recombinant protein and the fragments were purified by Ni²⁺ ion-exchange chromatography under denaturing conditions, as described in Zurita *et al.*, 2003.

SERA

The sera used in this study include seventy-two serum samples from individuals with different pathologies, as follows: 30 serum samples from patients with CL and 20 with MCL diagnosed by culture and microscopic visualization of parasites (the samples were collected by the Laboratorio de Microbiología, Facultad de Biología, San Antonio Abad University of Cuzco, Peru); 22 serum samples from Spanish patients with different autoimmune syndromes were collected by staff from the Immunology Section from the Hospital Universitario de Canarias, Tenerife, Spain, and confirmed by immunofluorescence (IF).

DETECTION OF ANTIBODIES RELATED TO AUTO-IMMUNE DISORDERS

The presence in sera of antibodies that are considered as classical signatures of autoimmune syndromes was assed using INNO-LIA ANA kit (Innogenetics) according to the manufacturer's instructions. This kit assesses the humoral response against the following antigens: nDNA: native DNA; SmB and SmD: non-histone nuclear proteins; RNP-70-K, RNP-A, RNP-C: nuclear ribonucleoproteins; Ro52 (SS-A) y Ro60(SS-A): 52 and 60 kDa proteins, RNA-associated; La(SS-B): 48 kDa phosphoprotein, associated to RNA-polymerase III transcripts; Cenp-B: kinetochore proteins, related to centromeric DNA; Scl-70 (Topo-I): 70 kDa basic non-histone protein, degradation product of native DNA topoisomerase I; Ribosomal: P0, P1 and P2 phosphoprotein; Histones: mix of human H1, H2A, H3 and H4 histones; p-ANCA (Mpo): neutrophilic myeloperoxidase; Glomerular Basement Membrane: α 3-chain of the glomerular basement membrane collagen IV; Antimitochondrial: mitochondrial antigens.

ELISA MEASUREMENTS

The recombinant *L. braziliensis* proteins were diluted in carbonate-bicarbonate buffer (pH 9.6) to 4 μ g/ml. ELISA plates (Immulon 4HBX; Dynex) were sensitised

with 100 µl of antigen per well, incubated overnight at 4° C, and washed three times with phosphate-buffered saline (PBS) 0.05 %-Tween 20 (PBS-T) buffer. All the sera were diluted 1:100 in PBS-T with 5 % nonfat dried milk, 100 µl of the mixture was added to each well, and the plates were incubated at 37° C for 1 h, after which the plates were washed again. A mixture of anti-human immunoglobulin A (IgA), IgG, and IgM (heavy and light chains) conjugated with peroxidase (Jackson ImmunoResearch) was used as the second antibody; the mixture was diluted 1:2000 in PBS-T, and 100 µl was added to each well. After incubation at 37° C for 1 h and the corresponding washings, the mixture was developed with *ortho*-phenylenediamine (Sigma) as the substrate. The absorbance was read at 450 nm. These ELISA trials were performed in triplicate, and the mean values for the three assays are presented. Only the results of the ELISAs for which there was a difference of 5 % or less among the different assays were considered.

RESULTS

The INNO-LIA ANA is a qualitative test detecting antibodies to several different nuclear and cytoplasmic antigens, most of which are recombinantly made (SmB, RNP-70k, RNP-A, RNP-C, SSA/Ro52, SSB/La, Cenp-B, Topo-I, Jo-1), with the exception of SSA/Ro60 and histones (natural), and SmD and ribosomal P (synthetic). This kit has shown a remarkable specificity combined with sensitivity in the detection of several autoimmune diseases, very similar to that of the single reference assays, but in a single test format (Pottel *et al.*, 2004)

In order to detect the presence of autoimmune disorders in the patients, whether innate or induced by the parasitic infestation, the sera were analyzed using the INNO-LIA ANA kit (Innogenetics), following the manufacturer's instructions. Table I shows the reactivity found for each of the 22 serum samples from autoimmune disorders sufferers. All of them presented reactivity against one or more of the antigens assessed by the kit, particularly RNPs, native DNA and RNA-associated proteins, all of them clinical parameters considered as classical signatures of autoimmune disorders. On the contrary, none of the CL and MCL sera assayed reacted against any of the different nuclear and cytoplasmic antigens assessed in the kit (data not shown).

Given the conservation of histones and Hsp70s along the evolutionarily scale, we were interested to determine whether the sera from individuals with autoimmune disorders recognise our antigens, both the proteins and the recombinant fragments. For this purpose, the sera from individuals suffering autoimmune disorders were tested for their reactivity against recombi-

Code	ANA (IF)	Autoantigens
AI 1	+	RNP-70, RNP-A, RNP-C, nDNA
AI 2	+	Ro52, Ro60, La, nDNA.
AI 3	+	nDNA
AI 4	+	La
AI 5	+	Cenp-B
AI 6	+	RNP-70, RNP-A, RNP-C, Rheumatoid factor
AI 7	+	Ro52
AI 8	+	Scl-70
AI 9	+	Ro52, nDNA, Rheumatoid factor
AI 10	+	RNP-A, RNP-C, Ro52, Ro60, La, nDNA.
AI 11	+	Glomerular basement membrane, p-ANCA (Mpo)
AI 12	+	Rheumatoid factor
AI 13	+	SmB, RNP-C, Histones, nDNA
AI 14	+	nDNA
AI 15	+	Rheumatoid factor
AI 16	+	Scl-70
AI 17	+	Ro52, Ro60, La, Rheumatoid factor
AI 18	+	RNP-A, Ro52, La, nDNA
AI 19	+	Ro52, Ro60, nDNA
AI 20	+	SmB, SmD, RNP-70, RNP-A, RNP-C, Ro60, Ribosomal, Rheumatoid factor
AI 21	+	Cenp-B, Antimitochondrial
AI 22	+	SmB, SmD, RNP-C, nDNA

Table I. – Reactivity of the autoimmune sera against the nuclear and cytoplasmic antigens assessed in the INNO-LIA ANA kit (Innogenetics). These parameters are the classical signatures of a number of autoimmune disorders.

nant *L. braziliensis* histone H1, Hsp70 and the fragments from the Hsp70 by ELISA, as described above. None of these sera reacted against the H1 from *L. braziliensis* (data not shown). A similar result was found for the Hsp70 (Fig. 1). Out of the 22 sera assayed, only three of them showed a weak reaction to some of the recombinant fragments used as antigens. Serum number 11 reacted weakly against the full-length rLbHSP70 and sera numbered 1 and 10, both of them belonging to SLE sufferers, reacted against rLb70 (109-245), rLb70 (352-518) and rLb70 (513-663).

DISCUSSION

The recognition of conserved parasitic proteins by sera from individuals suffering from leishmaniasis is a well established fact (reviewed in Requena *et al.*, 2000). Examples of this phenomenon are the high reactivity (66.6 %) observed in CL and MCL sera against the recombinant H1 from *L. braziliensis*, particularly localized in a 54-residue region located between amino acids 36 and 90 (Carmelo *et al.*, 2002). Similarly, the recombinant Hsp70 from *L. braziliensis* was recognized by 84 % of the CL and MCL sera assayed, with particular incidence in the C-terminal fragment of the protein, rLB70 (513-663) (Zurita *et al.*, 2003). The antigenic reaction displayed by the CL and MCL sera against the parasitic H1 and Hsp70 prompted us to

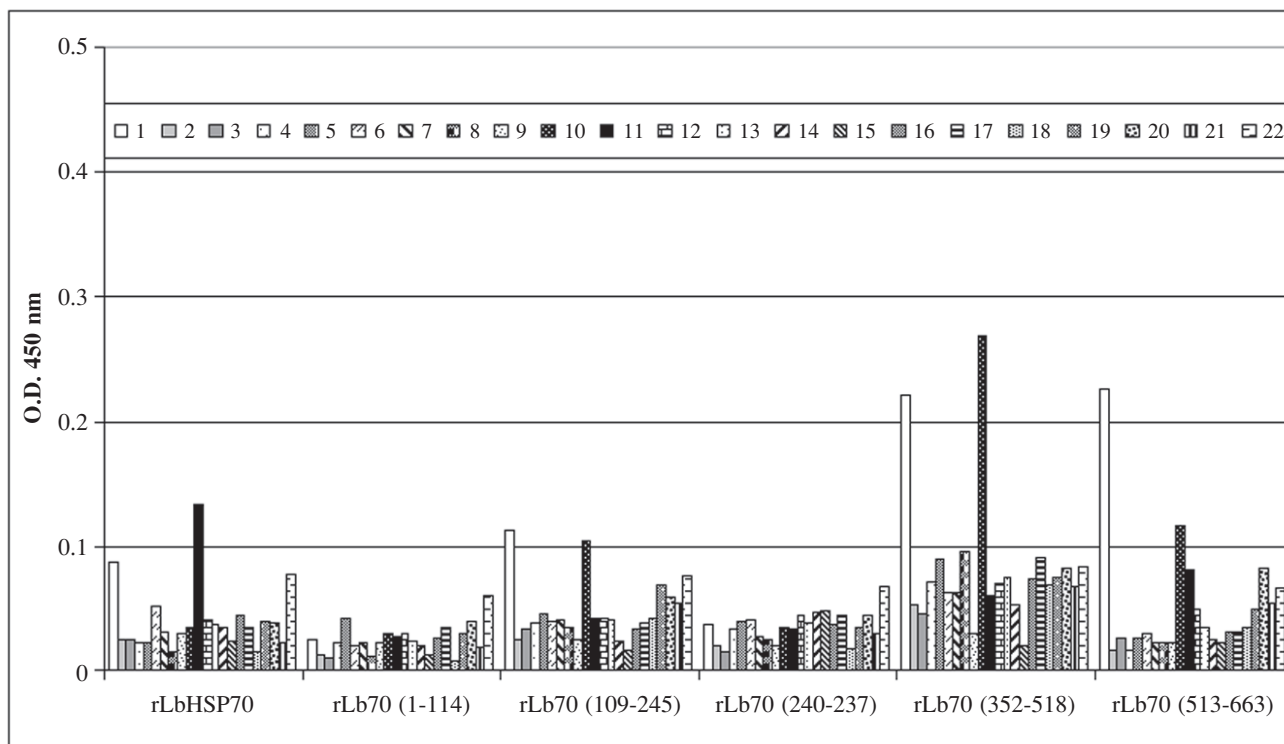


Fig. 1. – The graph shows the reactivity, assayed by ELISA, of the 22 sera from autoimmune-disease sufferers against the full-length Hsp70 and its fragments. The cut-off value was defined as the mean absorbance value of the control sera + 3 standard deviations (SD). Since this value was always below 0.1, it was established at 0.1. The legend in the graph shows the pattern assigned to each sera, numbered 1 to 22 for simplicity.

consider if those antibodies were specifically directed against the parasitic proteins or just the consequence of cross-recognition with their human counterparts, in a process analogous to what happens during Chronic Chagas' Disease. One of the most characteristic phenomena induced by *T. cruzi* infection in humans and experimental animals is the development of humoral and cellular autoimmunity against a diverse group of autoantigens, including cardiac myosin (Leon *et al.*, 2004). Moreover, there is a long-standing debate among scientists considering whether this autoimmunity is pathogenic, and the mechanisms by which this immunity is induced (Kierszenbaum, 1999; Girones & Fresno, 2003; Leon & Engman, 2003; Tarleton, 2003). These autoimmune processes have not been found during the infection with *Leishmania* (Leon *et al.*, 2004), apparently ruling out the intervention of autoimmunity in the pathology of the leishmaniasis. Our results are in good agreement with this aspect, since none of the CL and MCL sera assayed contained antibodies that bound any of the antigens in the INNO-LIA ANA kit, indicating that the antigenic response against the *L. braziliensis* H1 and Hsp70 detected in the sera is not a consequence of autoimmune disorders in the patients, and suggesting that the parasitism by *L. braziliensis* does not induce autoimmune processes in the host. Likewise, our findings establish that the immune res-

ponse against these two antigens induced in the individuals is specific to the parasitic H1 and Hsp70 proteins.

There are several reports in the literature of similar findings. Skeiky *et al.* (1995) observed that a *L. braziliensis* HSP70 recombinant fragment lacking the 131 N-terminal end, stimulated proliferation of Peripheral Blood Mononuclear Cells (PBMC) from MCL patients, but not from individuals who had recovered from the disease. Similarly, PBMC from MCL patients weren't stimulated by recombinant human HSP70. These results suggest that the PBMC response in LMC patients is unrelated to any autoimmune disorder, since the response against human HSP70 is null.

The presence in the sera of antibodies against endogenous human proteins, such as chaperones and histones, is an attribute of disruption of the immune system structure and functions. Anti-Hsp70 antibodies have been detected in individuals suffering from multiple sclerosis (Birnbaum & Kotilinek, 1993), ulcerative colitis and Lyme Disease (Jarjour *et al.*, 1991). Similarly, anti-H1 antibodies have been shown to be present in several autoimmune disorders like Systemic Lupus Erythematosus (SLE) (Gabler *et al.*, 2003), ulcerative colitis (Eggena *et al.*, 2000) and localized scleroderma (Sato *et al.*, 2004). In our experiments, none of the sera from individuals suffering autoimmune disorders presented

antibodies against *L. braziliensis* histone H1, suggesting that the immune response elicited during these autoimmune processes is specific to the human histones, among other autoantigens, excluding cross-reactivity with the parasitic H1.

A similar result was found for the Hsp70, since only three of the 22 sera assayed recognised some of the protein's fragments. Serum number 11 showed some reaction against the full-length rLbHSP70; this sera belongs to an individual suffering from Goodpasture Syndrome, characterized by glomerulonephritis, pulmonary hemorrhage, and autoantibodies to the glomerular and alveolar basement membranes (Fox & Swann, 2001), and is the only serum with this syndrome. Sera numbered 1 and 10 reacted against rLb70 (109-245), rLb70 (352-518) and rLb70 (513-663), and both of them belong to SLE sufferers, in which Hsp90 and inducible-Hsp70 (but not the constitutive counterpart) are overexpressed from PBMC (Twomey *et al.*, 1992, Dhillon *et al.*, 1993), a feature that may be related to the production of autoantibodies. Several studies have described the presence of autoantibodies against Hsp70 during SLE (Minota *et al.*, 1988; Ghoreishi *et al.*, 1993; Conroy *et al.*, 1996). Therefore, it is likely that the reaction of those two sera against fragments of *L. braziliensis* Hsp70 is a consequence of cross-reaction with the human counterpart, due to the high sequence identity with the human cognate Hsp70 (71.5 %) (Zurita *et al.*, 2003), and the presence in those regions of two repetitions of the tetrapeptide GGMP, that has been found in most HSP70 from parasites, human and rat (Young *et al.*, 1990).

The antigens we have used in our study are among the most evolutionarily conserved proteins. Besides this, some of these proteins have been described as antigens during a number of infectious and autoimmune diseases, and are commonly found in the cells as part of multiprotein complexes. This picture catalogues these proteins in a group that has been called panantigens by Requena and co-workers in 2000, and that includes some ribosomal proteins, beside histones and heat-shock proteins. These authors propose that the strong recognition of these antigens in humans and animals with leishmaniasis could be the result of three main factors: their abundance as circulating complexes during *Leishmania* infection, their stability as nucleoprotein particles and their capacity to be processed by antigen-presenting cells. As shown in this paper, in spite of the high sequence conservation observed for some of these proteins, as shown by the high sequence identity among Hsp70s, the immunological response found in infected individuals is generally specific against the parasitic proteins and not their human homologues, since it is normally directed against the most divergent regions of the proteins.

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