

Association of *Leishmania* Heat Shock Protein 83 Antigen and Immunoglobulin G4 Antibody Titers in Brazilian Patients with Diffuse Cutaneous Leishmaniasis

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Received 29 April 1997/Returned for modification 1 July 1997/Accepted 9 September 1997

Diffuse cutaneous leishmaniasis (DCL) is characterized by the presence of numerous nonulcerated nodules and plaques containing large numbers of *Leishmania amazonensis* parasites and few lymphoid elements. The immune responses of DCL patients reflect severe antigen-specific T-cell deficiencies, while the antibody response to *Leishmania* antigens is often accentuated. We report herein on the *Leishmania* antigen-specific antibody subclass distribution in DCL patients and demonstrate that a dominant antigen contributing to the biased immunoglobulin G4 antibody subclass in sera of DCL patients is *Leishmania* heat shock protein 83.

Leishmania spp. are obligate intracellular protozoan parasites of macrophages that cause a spectrum of clinical and immunological manifestations. The major clinical forms are visceral (VL), cutaneous (CL), mucosal (ML), and diffuse cutaneous (DCL) leishmaniasis (7, 8, 13, 16, 18). Human VL and DCL are associated with a strong Th2 cytokine pattern, with immunosuppression being the hallmark of these clinical forms of leishmaniasis (6, 12, 14).

DCL is a rare disease, having been reported to occur in only 300 to 500 people worldwide. It is caused predominantly by *Leishmania amazonensis* infection and is characterized by the presence of multiple chronic nodular skin lesions containing many parasites (5, 9, 16). The lesions sometimes resemble those of lepromatous leprosy and may persist for a lifetime. DCL is also characterized by an absence of T-cell proliferation or delayed-type hypersensitivity response to parasite lysate and by a predominant Th2 cytokine profile. These patients are generally resistant to chemotherapy and usually relapse after treatment, in spite of transient improvement and apparent healing in some cases. Anti-*Leishmania* antibody levels are high in these patients, with titers of 1:4,800 or higher (9). The present study was undertaken to determine the antibody subclass distribution in sera of DCL patients and to define antigens that may be involved in the pathological consequences associated with DCL.

Peripheral blood and serum samples were obtained from individuals living in areas in which epidemiological, clinical, and immunological studies of leishmaniasis have been performed (13). DCL patients were characterized by the presence of disseminated plaques or nodules (mainly on the face and extremities) which were filled with *Leishmania* organisms (1). One patient (No. 8) had had prior treatment with pentavalent antimony and gamma interferon (IFN- γ).

For serological studies, microtiter plates (Probind; Falcon) were coated overnight with 250 ng of recombinant antigen or *Leishmania* lysate (1 μ g) per well. The recombinant antigens used in this study were from genomic clones of *Leishmania braziliensis* heat shock protein 70 (hsp70), hsp83, and *Leishma-*

nia eukaryotic initiation factor 4A (LeIF) (25). For immunoglobulin G (IgG) subclass determination, the plates were coated with the antigen(s), incubated with serum, and then reacted with mouse anti-human IgG1, IgG2, IgG3, and IgG4 monoclonal antibodies (50- μ l aliquots of 1:1,000 dilutions; Calbiochem) as described previously (10). Bound antibodies were detected with goat anti-mouse IgG-horse radish peroxidase (Zymed; 1:500 dilution), using 2,2'-azinobis(3-ethylbenzothiazolinesulfonic acid) (ABTS) as the substrate.

Serological responses of patients with DCL are dominated by the IgG4 subclass. Antibody reactivities of serum specimens from 10 DCL patients to a parasite lysate were analyzed and compared to those of samples obtained from 22 ML and 23 CL patients. The IgG absorbance values of DCL patient sera were similar to those of ML patient sera and significantly higher than those observed for serum samples from CL patients (Fig. 1). Sera from all 10 DCL patients analyzed were positive with the lysate, and seven specimens had absorbance values of >2 . The lowest titer was for a sample from a donor who had undergone cytokine therapy (1, 2). The sera were further examined to determine their IgG subclass (IgG1, IgG2, IgG3, and IgG4) specificities. In general, and in agreement with a recent report (21), our results (Fig. 1) indicate that the serological responses of patients with DCL to parasite lysate were dominated by the IgG1 and IgG4 subclasses (with IgG4 being significantly dominant), while those of ML and CL patients were biased toward IgG1 and IgG3. Eight of 10 DCL serum samples tested had antibodies of the IgG4 subclass, and 5 had titers with absorbance values of >2.0 to >3 . The remaining two samples (patients 1 and 10) had unusually high IgG1 (absorbance values of 1.8 and 2.1) and IgG3 levels but were negative for the IgG4 subclass. This is reminiscent of the pattern seen with ML patients and may suggest that the latter two "DCL" patients are progressing toward or have underlying ML.

hsp83 is an immunodominant antigen recognized by sera from DCL patients. The specificity of the antibody response of DCL patients to defined antigens was further evaluated by using three immunodominant leishmanial antigens (hsp70, hsp83, and LeIF [24]). The reactivities of all 10 DCL patient sera with the three recombinant antigens revealed an antibody specificity pattern that was predominantly restricted to hsp83 (Fig. 2). Two DCL patient sera also had antibody titers to

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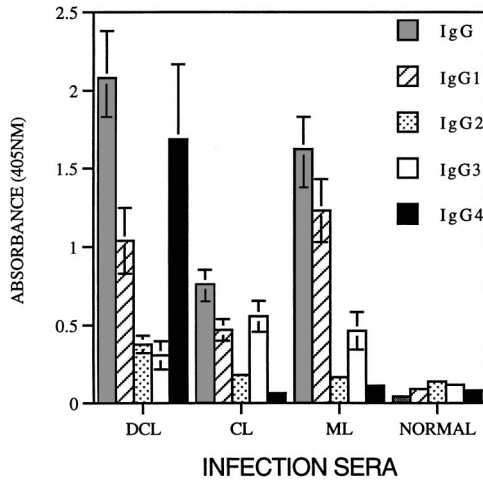


FIG. 1. Enzyme-linked immunosorbent assay results showing total IgG and IgG subclass reactivities (absorbance values [means ± standard errors of the means]) of sera from patients with DCL, CL, and ML to leishmanial lysate.

hsp70 and LeIF. Interestingly, these were the same individuals (patients 1 and 10) with high IgG1 and IgG3 reactivities but no IgG4 subclass reactivity to the lysate. Unlike those of DCL patients, sera from CL and ML patients had significant levels of antibody to hsp70 as well. However, sera from all three patient groups were only weakly reactive with LeIF.

Serological responses of DCL patients to hsp83 are dominated by the IgG4 subclass. Having demonstrated that DCL patients elicit a high-level antibody response to hsp83, the IgG subclass specificity was further examined. The results (Fig. 3) demonstrate that the major anti-hsp83 IgG antibody subclass in sera of DCL patients is IgG4, with absorbance values comparable to those for parasite lysate. Eight of the 10 DCL patient sera tested had IgG4 subclass antibodies to hsp83, with absorbance values ranging from 0.5 to >3.0. Five of these had anti-hsp83 IgG4 antibody subclass absorbance values of >2.0 to >3.0. Similar to the results observed for parasite lysate, sera from patients 1 and 10 were also negative for IgG4 subclass antibodies to hsp83. In contrast, however, patients 1 and 10

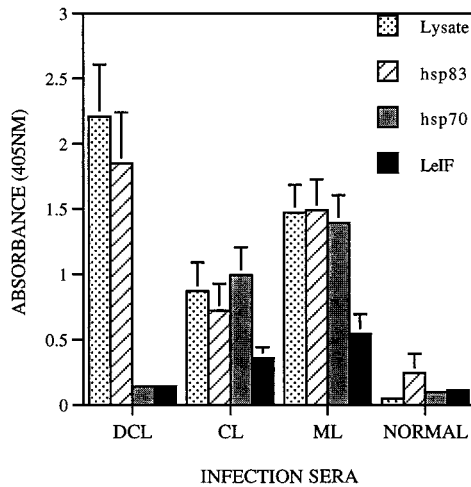


FIG. 2. Antibody reactivities (absorbance values [means ± standard errors of the means]) of sera from patients with *Leishmania* infections to lysate, rhsp83, rhsp70, and rLeIF.

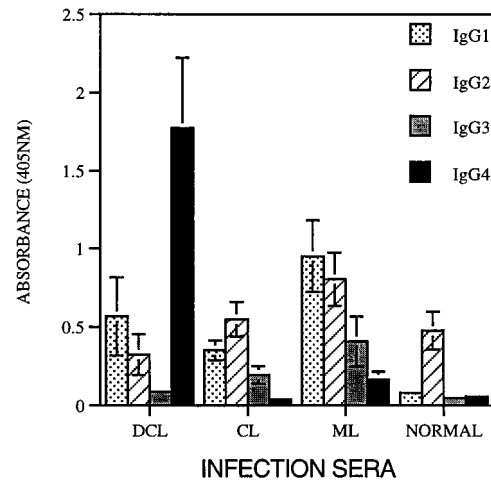


FIG. 3. Serological response of DCL patients to rhsp83 is dominated by the IgG4 subclass. Shown are IgG subclass reactivities (absorbance values [means ± standard errors of the means]) of sera from patients with DCL, CL, and ML to rhsp83.

had restricted IgG1 reactivities to hsp83 but were positive for both the IgG1 and IgG3 subclass with lysate. The background reactivities of the 19 normal sera were predominantly of the IgG2 type.

We have identified *Leishmania* hsp83 as a dominant antigen contributing to the associated IgG4 subclass antibody response in sera of DCL patients from Brazil. Although the significance of antigen-specific IgG subclasses in humans is not fully understood, some associations between antibody isotype and cytokine profile have been made. For example, the addition of interleukin-4 (IL-4) to human peripheral blood mononuclear cells (PBMC) induced the production of IgE (19, 22, 23, 27), and IL-4 has been implicated in the switching of surface-IgM-positive B cells to the production of IgG4 (11). IL-10 appears to represent a switch factor for IgG1 and IgG3 (4), while the addition of IFN-γ (a Th1-associated cytokine) to pokeweed mitogen-stimulated PBMC resulted in an increased production of IgG2 while dramatically suppressing the production of IgG1 (15, 26). IL-4 also induces the production of IgE by peripheral blood lymphocytes, and IL-4-induced IgE production is blocked by IFN-γ and IFN-α (17, 19). Examination of the cytokine profiles from both skin lesions and resting peripheral blood lymphocytes of DCL patients revealed that the levels of IL-4 and IL-10 mRNAs are significantly elevated (6, 20, 24, 25). It is generally accepted that an antibody subclass bias toward IgG4 and IgE reflects a Th2-associated profile and a pathological outcome in human leishmaniasis.

In sera from DCL patients, antibody specificities toward defined leishmanial antigens that were evaluated included those for the 70- and 80-kDa heat shock proteins and a constitutively expressed ribosomal protein, LeIF (24, 25). These antigens were previously demonstrated to stimulate human PBMC from three different groups of leishmaniasis patients (ML, CL, and self-healing CL) to proliferate and produce cytokines. For example, recombinant hsp-83 (rhsp83) stimulated T cells from individuals with self-healing CL to proliferate and produce IFN-γ, suggesting that PBMC responses to *L. braziliensis* hsp83 in this group of patients may be associated with protective immunity (25). Hence, in the case of self-healing CL, the production of IFN-γ and tumor necrosis factor alpha following stimulation with *L. braziliensis* hsp83 may re-

sult in the induction of leishmanicidal activity in macrophages (3, 28), while the production of IgG4 of DCL may result in pathological consequences. This suggests that a particular antigen may play a dual role in the induction of protective immune responses and the development of tissue damage.

Of particular interest is the apparent isotype switching seen in sera from patient no. 8, who was previously treated for DCL (1, 2). This was the only serum specimen with a predominant IgG2 subclass specificity (absorbance, 1.44) toward hsp83. Therefore, the IgG subclass specificity toward hsp83 in patients with DCL reflects the severity of the disease and may be a reliable marker for predicting the outcome of therapy.

Serological evaluation of patient sera with parasite lysate, recombinant *L. braziliensis* hsp70 and hsp83 and rLeIF revealed the presence of significant IgG antibody titers in patients with ML and CL but not in individuals with self-healing CL (25). While >95% of serum samples from ML and CL patients had antibody titers to both recombinant *L. braziliensis* hsp70 and hsp83, sera from DCL patients showed an antibody response bias toward rhsp83. Our serological data have demonstrated that *Leishmania* hsp83 is potentially an important antigen in the diagnosis of the outcome of infection with *L. amazonensis* and that hsp83 is comparable or superior to lysate for use as a diagnostic marker in leishmaniasis infection and, in particular, in the identification of individuals with DCL.

We thank Antonio Campos-Neto, Heather Secrist, Peter Probst, and John Webb for critically reviewing the manuscript and Jeff Guderian for excellent technical assistance.

This work was supported by grant AI25038 from the National Institutes of Health. Yasir A. W. Skeiky was a Centennial Fellow of the Medical Research Council of Canada.

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