

Immunoblot Analysis of the Humoral Immune Response to *Leishmania donovani* Polypeptides in Cases of Human Visceral Leishmaniasis: Its Usefulness in Prognosis

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Sera from Indian patients with parasitologically confirmed visceral leishmaniasis were studied by immunoblot analysis in order to identify a specific pattern for *Leishmania* infection. A soluble extract of *Leishmania donovani* was used as antigen. At diagnosis the sera from patients with visceral leishmaniasis specifically recognized fractions represented by bands of 201 kDa (50% of serum samples), 193 kDa (60%), 147 kDa (50%), 120 kDa (60%), 100 kDa (50%), 80 kDa (80%), 70 kDa (70%), 65 kDa (100%), 50 kDa (50%), 36 kDa (50%), 20 kDa (70%), and 18 kDa (50%). The 65-kDa band, common to all patients infected with *Leishmania* parasites, was found at the time of diagnosis. However, the immunoblot pattern changed after patients were treated and cured with sodium antimony gluconate (SAG; $n = 10$) or miltefosine ($n = 10$), as was evident from blots of sera obtained pretreatment and at 1, 3, and 6 months posttreatment. At 6 months posttreatment, immunoblots of sera from patients on the SAG regimen showed the disappearance of all bands except the 70-kDa band. Similarly, sera from those on the miltefosine regimen showed the disappearance of all bands except the 65- and 70-kDa bands. This study shows that Western blot analysis is a sensitive test for detection of anti-*Leishmania* antibodies. Moreover, the persistence of reactivity with the 65- and 70-kDa bands in the sera of all groups shows its promise as a diagnostic and prognostic tool.

Human visceral leishmaniasis is caused by a protozoan parasite of the *Leishmania donovani* complex, namely, *Leishmania donovani*. This infection is characterized clinically by fever, hepatosplenomegaly, anemia, and weight loss. A marked hypergammaglobulinemia and absence of detectable cell-mediated immunity are the principal immunological features of the disease. Although patients generate high levels of nonspecific immunoglobulin as well as specific antileishmanial antibodies, they fail to respond to parasite antigen in the delayed-type hypersensitivity skin test (33).

A kinesin-related protein-encoding gene has been discovered in *Leishmania chagasi* and contains a repetitive 117-bp sequence encoding 39 amino acid residues (K39) conserved at the C-terminal end in all of the visceral leishmaniasis-causing isolates examined so far (5). The recombinant product of K39 (rK39) has proved to be a very sensitive and specific antigen in an enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of visceral leishmaniasis from the foci of endemicity in Brazil, China, Pakistan, and Sudan (5, 26). Kumar et al. (19) reported extremely high levels of anti-rK39 antibodies in patients with visceral leishmaniasis, suggesting the application of rK39 for a sensitive and specific means of serodiagnosis and the potential of rK39 ELISA in the monitoring of drug therapy and the detection of disease relapses.

To date, several *L. donovani*-specific proteins, including spe-

cies-specific (2, 9, 11, 14, 25, 33) and subspecies-specific (21) molecules, have been identified with monoclonal antibodies or polyvalent antisera. Several of these proteins elicit a strong humoral immune response during visceral leishmaniasis, allowing the specific diagnosis of visceral leishmaniasis by a competitive ELISA (15) or a direct dot blot assay (16). Immunoblots have been useful in studies of host serological responses during infection, and a few antigens with potential diagnostic value have been selected (7, 24, 27).

The humoral immune response occurs during the active phase of infection, with the appearance of low titers of antibodies that disappear some months after the end of treatment representing a temporary response (4). This fact has made the assessment of antibody titers a useful tool for patient follow-up after therapy and for defining the period during which clinicians should observe patients in order to make a prognosis.

In the present study, we have examined patients' antibody responses at the time of diagnosis and various stages posttreatment using the immunoblot technique with soluble *L. donovani* antigen. We had the double objective of identifying a particular band pattern present in the patients affected by visceral leishmaniasis (confirmed by the presence of parasites in a sample taken from the spleen) and describing any possible variation in this band pattern following antileishmanial therapy with sodium antimony gluconate (SAG) or miltefosine.

MATERIALS AND METHODS

Antigen. *L. donovani* (MHOM/IN/96/B.H.U.70) promastigotes were cultivated in tissue culture flasks with RPMI 1640 medium (Hi-Media, Mumbai, India)

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supplemented with 10% fetal calf serum (Gibco, Grand Island, N.Y.) and antibiotics (gentamicin) (14).

Parasites were taken at the late-logarithmic phase of growth, washed five times at 4°C with sterile phosphate-buffered saline (PBS), and centrifuged at 1,400 × g for 15 min. The parasite pellet was resuspended in 1 ml of PBS, and the mixture was immediately frozen at -70°C. In order to make up only one batch of antigen for Western blot analysis, all parasites were kept frozen at this temperature until there were sufficient parasites from which soluble antigen could be obtained.

To prepare the soluble antigen, a method described by Isaza et al. (13) was used, with slight modifications. Briefly, the parasites were defrosted and resuspended in 2 ml of lysis buffer (20 mM Tris HCl [pH 7.4] containing 40 mM NaCl, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride [BDH, Mumbai, India], and 0.4% sodium dodecyl sulfate [BDH]) (12, 17). The mixture was left on ice for 30 min, with vortex agitation every 10 min. It was then centrifuged at 6,000 × g for 20 min at 4°C. The supernatant was removed and kept at -70°C until use. A small sample was used for protein determination by a method modified from that of Lowry et al. (23). By this method the final antigen protein concentration was found to be 9.4 mg/ml.

Human sera. Serum samples were collected from patients with parasitologically confirmed visceral leishmaniasis (kala azar) (*L. donovani*) body score in splenic aspirate of 2⁺ to 4⁺, i.e., between >1 to 10 parasites/100 field and >1 to 10 parasites/field (6) at the time of diagnosis. Sera were obtained by venipuncture from patients and controls registered at Kala-azar Medical Research Centre, Muzaffarpur, India, and Sir Sundar Lal Hospital, Banaras Hindu University, Varanasi, India. Blood was allowed to coagulate at room temperature and was then centrifuged at 1,400 × g for 5 min. All sera were stored at -70°C until required.

Western blot analysis. SDS-polyacrylamide gel electrophoresis was done with a vertical (Bangalore Genei, Peenya Bangalore, India) gel apparatus. The antigen was boiled for 5 min in sample buffer (two times) and was immediately subjected to electrophoresis in an SDS-10% polyacrylamide gel containing 0.1% SDS as described by Laemmli (20). The slab gel was run with two lanes per comb: a 100-mm lane for the parasite antigen sample and a 7-mm lane for a wide-range molecular mass marker (kind gift of David Sacks, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.). Three hundred micrograms of protein was used for gel electrophoresis. The gels were run at 15 mA in the stacking gel and 30 mA in the resolving gel until the bromophenol blue dye migrated 1 cm from the bottom of the gel in Tris-glycine-SDS buffer (pH 8.3) (13).

Immunoblotting. Transfer of polypeptides from SDS-polyacrylamide gels onto nitrocellulose membrane (pore size, 0.45 μm; kind gift of R. T. Kenney, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, Md.) was done in a transblotting chamber at 4 V/cm and 4°C for overnight in 25 mM Tris-192 mM glycine-20% (vol/vol) methanol (30). Following the blotting, the membranes were stained with 0.5% ponceau red for 5 min and were destained with distilled water. The blotted gels were stained with Coomassie brilliant blue in order to make sure that all polypeptides had been completely transferred. Detection of antibodies from patients' sera bound to antigens was done with a 5-bromo-4-chloro-3-indolylphosphate-Nitro Blue Tetrazolium system (BCIP-NBT; Kirkegaard & Perry Laboratories, Gaithersburg, Md.). Four-millimeter-wide strips were cut from the previously blotted membrane and blocked in 5% bovine serum albumin-0.3% Tween 20 in 100 mM Tris-buffered saline (TBS; pH 7.4) for an hour at room temperature. After six washes of 15 min each in TBS-0.1% Tween 20, the strips were incubated with serum (diluted 1:100 in 1% bovine serum albumin-TBS) for 2 h at 37°C with constant stirring. After incubation with the primary antibody, the strips were washed four times for 15 min each time with Tween 20-TBS. After the last wash, immune complexes were detected with a 1:500 dilution of alkaline phosphatase (Bangalore Genei) conjugated with goat anti-human immunoglobulin G (IgG), and the mixture was incubated with constant shaking for 2 h at room temperature. After incubation the membrane was washed with Tween 20-TBS three or four times and then washed with TBS only to remove the Tween 20. The membranes were developed with the BCIP-NBT system (Kirkegaard & Perry Laboratories) for 5 min. The reaction was stopped with PBS-EDTA (20 mM EDTA).

Statistical analysis. Analysis of the corresponding polypeptides in the blotted membranes was done by using an exponential regression curve with the molecular mass markers of each membrane, calculating the relative mobility (R_f) of each protein (10), and looking for its corresponding molecular mass.

RESULTS

Study population. The clinical and laboratory features of the visceral leishmaniasis patients on admission are summarized in

TABLE 1. Clinical and laboratory features of patients with visceral leishmaniasis

| Characteristic | Study entry | |
|---|--------------|---------------|
| | Pretreatment | Posttreatment |
| No. of patients | 20 | 20 |
| Age (yr) | 21.10 ± 3.80 | |
| Duration of illness (mo) | 4.30 ± 0.30 | |
| Wt (kg) | 43.50 ± 5.70 | 44.30 ± 5.60 |
| Karofsky score ^a | 74.00 ± 1.60 | 84.00 ± 1.60 |
| Spleen size (cm) | 8.30 ± 1.30 | 4.20 ± 0.60 |
| Splenic aspirate score | 2.25 ± 0.30 | |
| Leukocyte count (10 ³ /mm ³) | 3.63 ± 0.88 | 4.52 ± 1.5 |
| Hemoglobin concn (g/dl) | 8.90 ± 0.50 | 10.40 ± 0.50 |
| Platelet count (10 ³ /mm ³) | 0.70 ± 0.10 | 1.30 ± 0.10 |

^a The Karnofsky performance scale is as follows: 100, able to carry on normal activity and no special care is needed, with no complaints and no evidence of disease; 90, able to carry on normal activity, with minor signs or symptoms of disease; 80, normal activity with effort, with some signs or symptoms of disease; 70, unable to work but able to live at home and care for most personal needs, with various amounts of assistance needed.

Table 1. The response to SAG or miltefosine therapy was prompt, and the splenic aspirate smears of all patients showed no parasites (*L. donovani* bodies) at the end of treatment (the patients were parasitologically cured). These patients were subsequently monitored.

Pretreatment sera. In general, the sera from patients ($n = 10$) with visceral leishmaniasis specifically recognized polypeptides ranging from 18 to more than 201 kDa, with the frequently occurring bands being of 201 kDa (50% of serum samples), 193 kDa (60%), 147 kDa (50%), 120 kDa (60%), 100 kDa (50%), 80 kDa (80%), 70 kDa (70%), 65 kDa (100%), 50 kDa (50%), 36 kDa (50%), 20 kDa (70%), and 18 kDa (50%). We found one band of 65 kDa in sera from 100% of patients with active disease (Fig. 1).

Immunoblot evaluation. (i) SAG treatment group. We studied the sera of patients ($n = 10$) who were being treated with SAG. We did antibody tests at regular intervals for up to 6 months posttreatment. All patients were cured after the end of treatment, as confirmed by laboratory and parasitological tests (Table 1). At 1 month after treatment, the bands of 201, 193, 100, and 36 kDa disappeared. At 3 and 6 months posttreatment all bands except the 70-kDa band disappeared (Fig. 2).

(ii) Miltefosine treatment group. We also studied patients ($n = 10$) who were being treated with the oral drug miltefosine. We compared pretreatment sera with sera obtained at 1, 3, and 6 months posttreatment. At 1 month posttreatment only the 20- and 80-kDa bands had disappeared. At 3 months posttreatment all bands except the bands at 36, 65, and 70 kDa had disappeared; and at 6 months posttreatment all bands except the 65- and 70-kDa bands had disappeared. The 65- and 70-kDa bands remained at all time points. Sera from all patients had antibodies to the 65- and 70-kDa bands in common at pretreatment and 1, 3, and 6 months (follow up) posttreatment (Fig. 3).

Interestingly, all patients in both the SAG and miltefosine treatment groups showed the exact same response pattern after treatment and follow-up.

DISCUSSION

Parasitological methods have always been considered first-choice procedures for the diagnosis and prognosis of leishman-

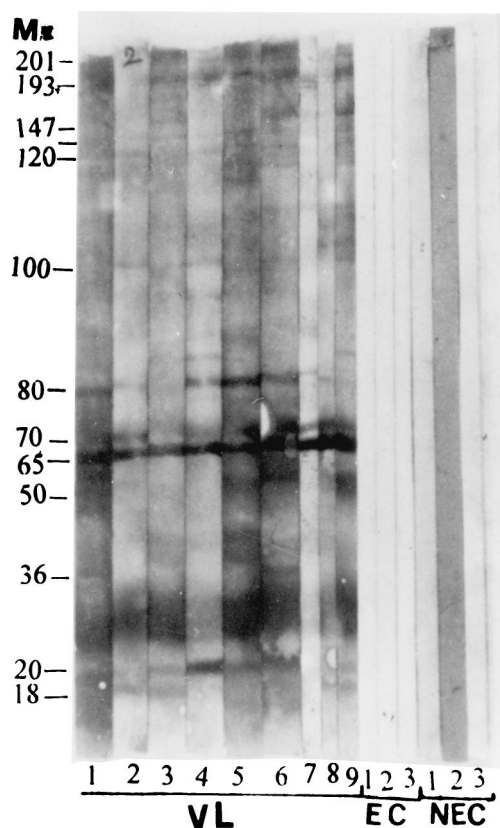


FIG. 1. Immunoblotting with *L. donovani* soluble antigen with the alkaline phosphatase system. VL lanes 1 to 10, sera from patients with visceral leishmaniasis at the time of diagnosis; EC lanes 1 to 3, sera from controls from an area of endemicity; and NEC lanes 1 to 3, sera from controls from an area of nonendemicity.

iasis due to their 100% specificities, although their sensitivities may vary depending upon the experience of the diagnostic team. However, in some instances it is very difficult to demonstrate the presence of the parasite, such as in patients with mucocutaneous leishmaniasis and visceral leishmaniasis. In these patients, serodiagnosis becomes an important alternative for demonstrating the presence of the parasite (18). Specific serological techniques are based on the determination of antibodies produced against the circulating *Leishmania*-specific antigens.

Both clinical and epidemiological data provide presumptive evidence in the diagnosis of visceral leishmaniasis. However, on the basis of these same criteria, other diseases must be considered in the differential diagnosis; among these are malaria, typhoid fever, tuberculosis, and leukemias. Definitive diagnosis is based on demonstration of the parasites, which may be found in aspirates of the spleen (98% of the aspirates are positive), (sternal) bone marrow (85% of the aspirates are positive), and liver (60% of the aspirates are positive). Splenic aspiration is the most reliable procedure, but it is a high-risk procedure. Because in many cases demonstration of parasites is difficult, serological tests have provided a useful alternative.

Among the most recently described methods, the Western

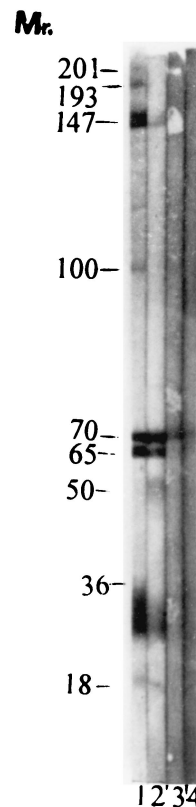


FIG. 2. Immunoblotting with soluble *L. donovani* antigen with the alkaline phosphatase system. Lane 1, serum from patients at the time of diagnosis; lanes 2, 3, and 4, patterns obtained for all patients ($n = 10$) with serum taken on days 30, 90, and 180, respectively, after treatment with SAG.

blot technique is highly sensitive and specific and provides broader information about the parasite's antigenic profile (18).

In the present study, a 65-kDa antigenic component was recognized by 100% of serum specimens from patients with clinically and parasitologically confirmed visceral leishmaniasis. It was never identified in the control sera tested (100% specificity). There was little variation in the patterns of the bands recognized by each of the serum specimens (Fig. 1).

Comparing our results with those of other groups, we found differences in the band patterns recognized by sera from patients with visceral leishmaniasis and cutaneous leishmaniasis using several *Leishmania* isolates as antigen. The studies of Dos Santos et al. (7) showed reactivities with the 119- and 123-kDa bands when an extract of *L. infantum* was used as the antigen with sera from patients with visceral leishmaniasis. In addition, Rolland-Burger et al. (28) found that the sera of patients with visceral leishmaniasis reacted to a 119-kDa band when *L. infantum* was used as the antigen. If antibodies remain in the patient for years and the patient's serum has a Western blot pattern similar to the one obtained at the time of diagnosis, then serious doubts arise as to whether a patient is ever completely cured (i.e., the patient has no remaining parasites) or whether treatment has induced a chronic subpatent infection, leaving the patient susceptible to relapse later on; for example, immunosuppression can lead to the reemergence of

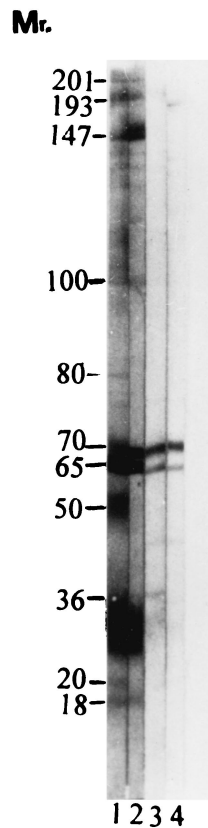


FIG. 3. Immunoblotting with soluble *L. donovani* antigen with the alkaline phosphatase system from a patient with visceral leishmaniasis. Lane 1, serum from patients at the time of diagnosis; lanes 2, 3, and 4, the patterns obtained for all patients ($n = 10$) with serum taken on days 30, 90, and 180, respectively, after treatment with miltefosine.

active infection, as in the case of visceral leishmaniasis in patients with AIDS (1, 3, 8). A test as sensitive as Western blotting may be able to identify immunosuppressed patients who could be at risk of a relapse of leishmaniasis.

The only difference between the pre- and posttreatment sera of the groups treated with SAG and miltefosine was the disappearance of all protein bands except those of 65 and 70 kDa, respectively. We could detect a 65-kDa polypeptide in 100% of the pretreatment sera from kala azar patients (Fig. 1). Vinayak et al. (31) reported on a 63- to 68-kDa complex which is an immunodominant surface molecule of *L. donovani* that can be used as an immunoprophylactic agent. Investigations have indicated that major surface glycoproteins with molecular masses ranging from 63 to 68 kDa (known as the glycopeptide 63 [gp63] complex) are important ligands in cell-to-cell interactions and cell infectivity (29). Vinayak et al. (32) have shown that promastigotes of *L. donovani* use the gp66 molecule not only for identification but also for attachment to macrophages. Sera from patients with confirmed leishmaniasis have been shown to identify the gp63 complex molecule in Western immunoblots of *Leishmania* crude antigen (22). Further work is needed to define the possible value of the 65-kDa band as a prognostic indicator. We suggest that the band of 65 kDa could be useful for diagnosis of Indian visceral leishmaniasis.

Furthermore, the results of our studies suggest that Western

blotting could be a sensitive technique for differentiation between the acute and chronic stages of disease. The presence of bands of 201, 193, 147, 100, 80, 70, 65, 50, 36, 20, and 18 kDa (Fig. 2 and 3) and the subsequent disappearance of all bands except those of 65 and 70 kDa during the course of treatment suggest the usefulness of Western blotting in making a clear-cut prognosis. The most interesting observation was the disappearance of protein bands unique to both the SAG and the miltefosine treatment groups, as the sera of all patients in the trial groups showed the same band patterns at the respective time points. This suggests that the type of drug used for treatment probably has a bearing on the immune response. Our data also suggest that Western blotting could successfully be used when there is no splenomegaly. Western blotting could also be a prognostic tool, as the disappearance or reappearance of specific bands could predict cure or relapse. Moreover, Western blotting analysis showed a large diversity in the antibody response to visceral leishmaniasis and permitted detection of antibodies to *Leishmania* in serum.

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