Antibody Response against a *Leishmania donovani* Amastigote-Stage-Specific Protein in Patients with Visceral Leishmaniasis

ELODIE GHEDIN,1 WEN WEI ZHANG,1 HUGUES CHAREST,2 SHYAM SUNDAR,3 RICK T. KENNEY,4 AND GREG MATLASHEWSKI1*

Institute of Parasitology, Macdonald Campus, McGill University, Ste.-Anne de Bellevue, Quebec H9X 3V9, Canada; Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-2187; Institute of Medical Sciences, Banaras Hindu University, Nagar, Varanasi 221 005, India; and Food and Drug Administration, Rockville, Maryland 20852-1448

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*Corresponding author. Mailing address: Institute of Parasitology, McGill University, 2111 Lakeshore Rd., Ste.-Anne de Bellevue, Quebec H9X 3V9, Canada. Phone: (514) 398-7727. Fax: (514) 398-7857. E-mail: Greg_Matlashewski@Maclan.McGill.Ca.

*Leishmania* species are responsible for a wide spectrum of diseases, including self-healing skin ulcers (cutaneous leishmaniasis due to species of the *Leishmania tropica* group and *L. mexicana* complexes), mutilating lesions of the oronasal-pharyngeal mucosa (mucocutaneous leishmaniasis due to *L. braziliensis*), and fatal visceral infections (visceral leishmaniasis due to species of the *L. donovani* complex). Leishmaniasis is considered by the World Health Organization to be one of the six major tropical diseases of developing countries (22). *Leishmania* is a dimorphic protozoan which exists as a flagellated promastigote in the sandfly vector and as an intracellular amastigote in the mammalian host. The cellular transformation of the *Leishmania* parasite into an amastigote occurs within the phagolysosomal compartment of host macrophages, where it also multiplies. It is the amastigote stage which is responsible for the pathology in susceptible vertebrate hosts (for a review, see reference 14).

We have previously isolated and characterized a gene, termed A2, which is specifically expressed in the amastigote stage of *L. donovani*, the causal agent of visceral leishmaniasis (4, 5). We have also identified the A2 protein and shown that it is specifically expressed at high levels in amastigotes but not in promastigotes (23). The A2 gene is one of the few amastigote-stage-specific genes identified in *Leishmania* (4).

The A2 gene product is composed mostly of a highly conserved repetitive element which has identity with an S antigen of *Plasmodium falciparum* (4). The A2 locus is comprised of at least seven genes, which differ with respect to the lengths of the sequences encoding the repeat peptide unit (5, 23). A2 protein range in size from 45 to approximately 100 kDa (23) and were shown to be recognized by serum from a patient with visceral leishmaniasis (4). Since A2 is amastigote-stage-specific and is immunogenic (4), we investigated its potential as a diagnostic antigen.

Diagnosis of visceral leishmaniasis cannot be made solely on the basis of clinical signs and symptoms because of its resemblance to other causes of febrile splenomegalies such as malaria, typhoid fever, and tuberculosis, to name a few. Initial assessment based on symptoms is confirmed through culture of parasites from aspirates of spleen, bone marrow, or lymph node. Methods used for the diagnosis of visceral leishmaniasis may also rely on immunological techniques which detect circulating antibodies. Serological test procedures include the direct agglutination test, which involves the detection of agglutinating antibodies against *Leishmania* (7, 19); the immunofluorescent-antibody test (IFAT), with whole organisms used as antigen; the enzyme-linked immunosorbent assay (ELISA); and PCR. However, immunodiagnostic methods using whole parasites as the source of antigen are often limited by the problem of cross-reactivity between species (reviewed in reference 12). Thus, there is a need for specific antigens in diagnostic tests, particularly in the case of visceral leishmaniasis.

We report here an evaluation of patient antibody responses against the A2 protein by screening sera with a recombinant A2 protein fused to glutathione S-transferase (GST) in Western blot assays and ELISAs. We have also combined immunoprecipitation and Western blot analyses to screen sera. We present results which show that A2 may be a valuable diagnostic antigen for serodiagnosis of visceral leishmaniasis.
Materials and methods

Western blot analyses. An A2-GST fusion protein that lacks the putative N-terminal signal sequence was produced from the pGEX-2T/A2R vector as previously described by Zhang et al. (23). Recombinant protein expression was induced from the lac promoter by addition of IPTG (isopropyl-d-thiogalactopyranoside) (Promega, Montreal, Canada). Total proteins from lysates of Scherichia coli cells were loaded on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. Western blotting was performed with an anti-A2 monoclonal antibody (C9) (25) on nitrocellulose membranes in a 1:2,500 dilution of antibody, a biotinylated goat anti-mouse immunoglobulin G (IgG) Fe (for the monoclonal antibody) or an anti-human IgG Fe ( Gibco BRL, Burlington, Canada) as used in the second antibody. Bound antibodies were detected with streptavidin–conjugated horseradish peroxidase (Amersham, Oakville, Canada) and 3,3-diaminobenzidine (Sigma, Montreal, Canada) in a 0.03% H2O2 solution.

ELISA. The A2-GST fusion protein produced in E. coli cells was affinity purified according to the method described by Smith and Johnson (20). An overnight culture of E. coli cells transformed with pGEX-2T/A2R was diluted 1:100 in 800 ml of fresh medium and grown at 37°C for 3 h, at which point protein expression was induced by addition of IPTG to a 1 mM concentration. Incubation was prolonged for 2 h. The cells were pelleted and resuspended in a 1:100 culture volume of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4). The cells were lysed on ice by mild sonication (four times for 20 s each) and centrifuged at 13,000 × g for 20 min at 4°C. The supernatants were kept and 1 volume of 50% glutathione-Sepharose beads (Pharmacia Biotech, Montreal, Canada) was added; adsorption was complete after 1 h of mild agitation at room temperature. After adsorption, the beads were collected by a 500 × g spin for 30 s and washed 5 or 6 times with 10 volumes of PBS. Elution of the protein was performed by incubating a 10 mM solution of free reduced glutathione (50 mM Tris–HCl [pH 8.0]) for 30 s at room temperature.

Flat-bottom microtiter immunooassay plates (Immulon4; Dynatech Laboratories) were coated overnight at 4°C with 0.5 μg of A2-GST fusion protein or the same amount of control GST protein in 100 μl of PBS. The coating solution was flicked out of the plates (no washing), and free binding sites were blocked with 200 μl of 5% milk in PBS containing 0.05% Tween 20 (T-PBS) for 1 h at 37°C. The plates were then washed three times in T-PBS. Sera diluted 1:250 in 1% bovine serum albumin-T-PBS were added in 100-μl aliquots with duplicates for each sample. The plates were incubated for 1 h at 37°C. After three washes, horseradish peroxidase-conjugated goat anti-human IgG (Bio-Rad, Mississauga, Canada) diluted 1:2,000 in 1% bovine serum albumin-T-PBS was added in 100-μl aliquots. The plates were washed three times. Finally, ABTS [2,2′-azino-bis(3-ethylbenzthiazolin-sulfonic acid)] substrate diluted in citrate buffer and activated with 0.01% (vol/vol) H2O2 was added. The reaction was left to proceed for 30 min and then stopped by addition of 10 μl of a 10% SDS solution per well. The optical density of the reaction mixture was measured at 405 nm in an ELISA reader (Microplate autoreader; Bio-Tek Instruments). A reference positive serum was used in all plates. The lower limit of positivity (cutoff) was determined by the mean of the negative controls plus three standard deviations.

Immunoprecipitation analysis. L. donovani Sudanese strain 1SD2 cells engineered to overexpress the A2 protein were grown at 37°C, pH 5.5, in RPMI medium containing 20% fetal calf serum and G418 (100 μg/ml). A2 proteins overexpressed in L. donovani amastigotes were immunoprecipitated with human immune serum by a method previously described by Brandau et al. (2). Briefly, 107 cells were harvested by centrifugation and lysed in 50 μl of solubilizing buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, and 2.5 μg of aprotinin, leupeptin, and pepstatin per ml). Lysates were centrifuged at 13,000 × g for 15 min at 4°C, and soluble proteins were incubated with 1 μl of human immune serum for 1 h at 4°C. A 35-μl portion of protein A-Sepharose (Pharmacia Biotech) was added, and the total mix was further incubated for 2 h at 4°C. The immunosorbent was centrifuged and washed three times in Net Gel (150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 0.02% NaN3, 0.25% gelatin, 50 mM Tris [pH 7.5]). Twenty-five microliters of 2× SDS sample buffer (125 mM Tris, 4% SDS, 20% [vol/vol] glycerol, 100 mM dithiothreitol, 0.005% bromophenol blue [pH 6.8]) was added to the slurry, heated at 95°C for 5 min, and loaded on an SDS–10% polyacrylamide gel. Immunoprecipitated A2 proteins were detected by Western blot analyses with anti-A2 monoclonal antibody C9 and by the ECL Western blotting detection system (Amersham). The secondary antibody, a goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad), was used at a 1:3,500 dilution.

Karyotype analyses. Leishmania chromosomes were separated in a 1% agarose pulsed-field grade gel (Bio-Rad) with a Bio-Rad DRII pulsed-field gel electrophoresis (PFGE) apparatus. Leishmania species used in the karyotype analyses are listed in Table 1. Separating conditions were set at 150 V with 120 s of switching time for 48 h at 14°C. Genomic DNA was obtained from vitricultured promastigotes of the different strains listed in Table 1. Southern blot membranes of chromosomes separated by transversal alternating-electrophone (APE) were kindly provided by Danielle Legare and Marie-Ouelleau from Laval University. Chromosomes were separated according to procedures described by Grondin et al. (6). All Southern blot membranes were hybridized with a probe representing the A2 protein coding region obtained from a 1.4-kb XhoI–XhoI fragment of genomic clone GeC90 (4). Hybridization was performed on high stringency: 1 M NaCl, 1% SDS, and 10% dextran sulfate for 18 h at 65°C.

Table 1. Species of Leishmania used in karyotype analyses

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference stock</th>
<th>Location or ATCC* no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. donovani complex</td>
<td>MHOM/SD/00/1S-CL2D</td>
<td>Sudan</td>
</tr>
<tr>
<td>L. donovani WR657</td>
<td>MHOM/IN/80/DD8</td>
<td>India</td>
</tr>
<tr>
<td>L. donovani WR684</td>
<td>MHOM/ET/67/82</td>
<td>Ethiopia</td>
</tr>
<tr>
<td>L. infantum</td>
<td>MCA/SP/00/XXX</td>
<td>Spain</td>
</tr>
<tr>
<td>L. mexicana complex</td>
<td>MNYC/BZ/62/M379</td>
<td>Brazil</td>
</tr>
<tr>
<td>L. tropica complex</td>
<td>MHOM/SU/58/OE</td>
<td>Sudan</td>
</tr>
<tr>
<td>L. tropica WR664</td>
<td>MHOM/SU/74/K27</td>
<td>Sudan</td>
</tr>
<tr>
<td>L. major WR662</td>
<td>MHOM/IL/67/Jericho11</td>
<td>Israel</td>
</tr>
<tr>
<td>L. major</td>
<td>MRHO/SU/59/P/LV39</td>
<td>Sudan</td>
</tr>
</tbody>
</table>

a ATCC, American Type Culture Collection.

Human sera. Three different groups of immune sera were screened in this study. The first group was used to determine the specificity of anti-A2 antibodies, while the two other groups were used to determine the prevalence of anti-A2 antibodies in individuals with visceral leishmaniasis.

(i) Group A. Group A included sera from the Centers for Disease Control and Prevention (CDC) (Atlanta, Ga.) from patients with different types of Leishmania infections and Trypanosoma cruzi infections.

(ii) Group B. Group B included sera from the CDC collected from patients admitted to a Sudanese hospital. These were patients with kala-azar—acute visceral leishmaniasis—admitted for treatment (see Table 2). Sera were screened at the CDC by IFAT with whole parasites as antigen. Some of the sera were also subjected to a direct agglutination test. Three samples from hospital patients admitted for reasons other than leishmaniasis that tested negative on the IFAT represent the control group.

(iii) Group C. Group C included sera collected in India from patients in various stages of treatment or recovery from kala-azar. They had been diagnosed with visceral leishmaniasis based on clinical signs of the disease (hepato-splenomegaly, fever, weight loss, hair loss, and hypergammaglobulinemia) and on parasite culture of splenic and/or bone marrow aspirates. Samples were divided into two categories: (i) patients with active disease and (ii) patients in healing and remission from whom blood was drawn at different times after treatment. For most of the patients, a standard intravenous antimycosis treatment of 20 mg/kg of amphotericin B per day for 30 days was used. Five samples from a control group representing unexposed hospital workers with no history of visceral leishmaniasis were used as negative controls.

We deemed important the use of endemic control sera for this study. Clearly we did not expect to observe anti-A2 antibodies in individuals not infected with L. donovani, since the A2 genes are present only in L. donovani and L. mexicana strains, as demonstrated in Fig. 1.

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RESULTS

Karyotype analyses of A2 genes. The A2 genes were originally isolated from *L. donovani donovani* (4). To determine if the A2 gene is conserved in other *L. donovani* subspecies and *Leishmania* species, karyotype analysis of *Leishmania* chromosomes was performed. Analyses revealed that the A2 genes were conserved only in species from the *L. donovani* complex (New World and Old World visceral agents) and *L. mexicana* (New World cutaneous agent) (Fig. 1). The A2 gene was not detected in the *L. tropica* complex (*L. major*, *L. tropica*, and *L. aethiopica*) or the *L. braziliensis* complex. These data show that the A2 genes are specific to *L. donovani* and *L. mexicana*. It is possible that the A2 genes have diverged in the other species to such an extent that they do not hybridize with the A2 probe at the high-stringency conditions used.

Specificity of the anti-A2 antibody response. Because of the specificity of the A2 gene to visceral agents and its absence in most cutaneous species, we determined whether there was a similar specificity in the anti-A2 antibody response. Western blot analyses of immune sera against a recombinant A2-GST fusion protein was performed. Figure 2 shows an example of the Western blot procedure used to screen the serum samples. Total proteins from *E. coli* cells expressing the recombinant A2-GST fusion protein (Fig. 2A, lane a) and control recombinant GST alone (lane b) were subjected in parallel to SDS-polyacrylamide gel electrophoresis. Purified versions of the recombinant proteins were also run in parallel (Fig. 2A, lanes c and d). The GST protein alone ran at 22 kDa, while the GST-A2 fusion protein ran at 44 kDa. As positive controls for Western blotting (Fig. 2B), A2 was detected with the anti-A2 monoclonal antibody C9 (23) or immune serum which was previously shown to react strongly against *L. donovani* antigens from a young Iranian patient suffering from visceral leishmaniasis (4). Figure 2B demonstrates that this Western blot analysis could be used to identify sera containing anti-A2 antibodies.

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**FIG. 1.** Karyotype analyses and detection of the A2 gene in *Leishmania* species associated with pathology in humans. Chromosomes were separated by PFGE (A) or TAFE (B). Equal loading was verified by agarose gel staining. Southern blot membranes were hybridized at high stringency with a probe representing the A2 protein coding region (a 1.1-kb *Xba* I-*Xho* I fragment purified from a genomic clone). Lanes: 1, *L. donovani*; 2, *L. donovani* WR657; 3, *L. donovani* WR684; 4, *L. infantum*; 5, *L. mexicana*; 6, *L. tropica* WR683; 7, *L. tropica* WR664; 8, *L. major* WR662; 9, *L. major*; 10, *L. donovani*; 11, *L. chagasi*; 12, *L. infantum*; 13, *L. amazonensis*; 14, *L. mexicana*; 15, *L. braziliensis*; 16, *L. guyanensis*; 17, *L. panamensis*; 18, *L. major*; 19, *L. tropica*; 20, *L. aethiopica* (see Table 1 for details of *Leishmania* spp. used). Molecular weights were determined with yeast chromosomes. ( Autoradiographs were scanned by using Adobe Photoshop 3.0.)

**FIG. 2.** (A) Coomassie blue staining of total proteins from *E. coli* transformed with the GST-expressing pGEX-2T plasmid alone (lane a) and the pGEX-2T plasmid containing A2 insert DNA (lane b). Lanes c and d contained 2 μg each of purified GST and A2-GST fusion protein, respectively. (B) Western blot analyses of total proteins present in lanes a and b were performed with a monoclonal antibody against A2 and positive immune serum from a visceral leishmaniasis patient, as indicated. (Gels and membranes were scanned with Adobe Photoshop 3.0.)
Sera from patients with *T. cruzi*, *L. tropica*, *L. braziliensis*, *L. mexicana*, or *L. donovani* infections were next screened for reactivity against the recombinant A2 protein. Positive samples displayed a pattern similar to that for the positive control shown in Fig. 2B (positive serum). Anti-A2 antibodies were detected only in individuals infected with *L. donovani* or *L. mexicana*. Five of seven *L. donovani*-infected individuals and 3 of 5 *L. mexicana* cases were positive for anti-A2 antibodies. Individuals infected with *T. cruzi* (four samples), *L. tropica* (four samples), and *L. braziliensis* (two samples) were all negative for anti-A2 antibodies. Therefore, there was no cross-reactivity to trypanosome or other *Leishmania* infections. These results are consistent with the karyotype analyses, which revealed that A2 genes were conserved only in *L. donovani* and *L. mexicana* strains.

**Prevalence of anti-A2 antibodies in *L. donovani*-infected individuals.** Based on the results of Western blot screening for anti-A2 antibody response (above), we examined the prevalence of anti-A2 antibodies in sera from a larger population of *L. donovani*-infected patients. In Western blot analyses, a positive response against the A2 recombinant protein was detected in 41% of *L. donovani*-infected individuals in the Sudan and 48% of those in India, as well as in 33% of individuals in a posttreatment stage (Table 2). In ELISAs using the purified recombinant A2-GST and GST alone as a negative control (as shown in Fig. 2A), 18 of the 22 Sudanese sera tested and 15 of the 25 Indian sera tested from infected individuals showed IgG reactivity with the recombinant A2 above the cutoff level (Table 2 and Fig. 3), while there was no reactivity with GST alone (data not shown). This corresponds to, respectively, 60 and 82% of positive cases detected. As shown in Fig. 3B, patients in posttreatment were separated according to the time at which serum samples were collected after treatment. Group III represents patients up to 12 months after treatment, and group IV represents patients from 24 to 106 months after treatment.

Surprisingly, one patient who had been treated for visceral leishmaniasis more than 8 years before (106 months) tested positive in the ELISA (Fig. 3B).

Immunoprecipitation analysis was also performed to examine reactivity against conformational epitopes on A2. It was thought that this assay might provide better sensitivity for detecting anti-A2 antibodies. Total solubilized proteins derived from cultured *L. donovani* were immunoprecipitated with immune human sera, and the immunoprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis. A2 proteins in the immunoprecipitates were then identified with the anti-A2 monoclonal antibody by Western blot analysis. Figure 4 shows representative data for several samples. Figure 4, lane A, shows the pattern of the various A2 proteins expressed in lysates of *L. donovani* cells which overexpress two A2 protein species at 53 and 33 kDa. When both the 53- and 33-kDa bands were observed, a sample was considered positive for anti-A2 antibodies. Identification of the 33-kDa band was particularly helpful since, in some samples, the antibody heavy chain tended to comigrate with the 53-kDa band, overwhelming its effect. Figure 4, lane b, shows a pool of five negative-control serum samples. Lanes c and d represent samples from two individuals in posttreatment in which sample c was negative and sample d was positive for anti-A2 antibodies. Lanes e through h are from four individuals with kala-azar and range from weakly positive (lane h) to strongly positive (lane f). Immunoprecipitations were performed only with the sera collected in India (group C). Ninety-two percent of active disease cases tested positive for anti-A2 antibodies by this analysis (Table 2), as opposed to 48% positive with Western blotting only and 60% with ELISA only. These data demonstrate that the immunoprecipitation analysis was the most sensitive approach for identifying anti-A2 antibodies. This also indicates

<table>
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<th>Serum group</th>
<th>Clinical condition</th>
<th>No. of positive samples/no. tested (%) by:</th>
<th>Western blotting</th>
<th>ELISA</th>
<th>Immunoprecipitation</th>
</tr>
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<tbody>
<tr>
<td>B</td>
<td>Active disease</td>
<td>13/32 (41)</td>
<td>18/22 (82)</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
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<td>15/25 (60)</td>
<td>25/27 (92)</td>
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<tr>
<td></td>
<td>Posttreatment</td>
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<td>10/27 (37)</td>
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<td></td>
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<td>0/5 (0)</td>
<td>ND</td>
<td>0.5 (0)</td>
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</tbody>
</table>

*ND, not done.*

![FIG. 3. ELISA of the IgG antibody response of visceral leishmaniasis patients and other groups against the *L. donovani* A2 protein. A short horizontal line marks the arithmetic mean of each group. The cutoff values were obtained by calculating the means of the negative control serum samples plus 3 standard deviations. (A) Sudanese samples (group B): I, control sera that tested negative on IFAT for leishmaniasis from patients in a Sudanese hospital; II, sera from patients with active disease. (B) Indian samples (group C): I, control sera from unexposed hospital workers; II, sera from patients with active disease; III, sera collected from treated patients up to 12 months posttreatment; IV, sera collected from treated patients more than 12 months posttreatment. p36 and p106 correspond to serum samples collected from patients 36 and 106 months, respectively, after treatment.](http://cvi.asm.org/)
that many of the antibodies produced against A2 recognize conformational epitopes.

**DISCUSSION**

Karyotype analyses of species from the four *Leishmania* complexes (*L. donovani*, *L. mexicana*, *L. tropica*, and *L. braziliensis*) revealed that A2 genes are conserved in *Leishmania* strains of the *L. donovani* and *L. mexicana* complexes but not in strains of the *L. tropica* or *L. braziliensis* complexes. From a phylogenetic point of view, the conservation of A2 genes in both *L. donovani* and *L. mexicana* establishes an interesting link between these two groups, etiological agents of different types of leishmaniasis. *L. donovani* and its subspecies are responsible for visceral leishmaniasis in the Old and New World, while *L. mexicana* is an exclusively New World species that causes cutaneous lesions.

Since the expression of A2 is amastigote specific and this stage is associated with pathology in humans, we were interested in examining antibody response against the A2 protein. We demonstrated by immunoblotting that the A2 antibody response was specific for patients with visceral leishmaniasis and *L. mexicana* infections and that there was no cross-reactivity with sera from individuals with other parasitic diseases. The anti-A2 antibodies were not detected in patients with cutaneous leishmaniasis caused by *L. major*, *L. tropica*, or *L. braziliensis*, nor in individuals with nonleishmanial diseases. This was consistent with the karyotype data showing that the A2 genes were conserved in *L. donovani* and *L. mexicana*.

Patients infected with *L. donovani* were screened for anti-A2 antibodies by using a recombinant A2 as well as native A2 proteins. Immunoprecipitation of A2 protein and then detection of immunoprecipitated A2 with anti-A2 monoclonal antibodies in 92% of patients with active visceral leishmaniasis. Western blotting alone and ELISA using the recombinant A2 were less sensitive (48 and 60%, respectively). This result shows that A2 holds considerable potential as an antigen in serodiagnosis and that further studies should focus on the development of A2 as a serodiagnostic antigen for visceral leishmaniasis.

A large number of patients potentially infected with *L. donovani* can be tested by serodiagnosis, replacing techniques that rely mainly on the identification of parasites from tissue biopsy. The A2 antigen can be added to the list of parasite antigens potentially useful for specific immunodiagnosis of visceral leishmaniasis. This list includes native proteins such as gp63, which can permit a distinction between ongoing and previous infections to be made (15), a p32 membrane protein of *L. donovani infantum* promastigotes which is suitable for the specific diagnosis of Mediterranean visceral leishmaniasis (21), and the 70- and 72-kDa proteins purified from *L. donovani* promastigotes (9, 10), among others. Recombinant proteins have also proven useful, among them rK39, a recombinant protein that contains a 39-amino-acid repeat part of a 230-kDa protein predominant in *L. chagasi* tissue amastigotes (3), and recombinant gp63 antigens from *L. chagasi* and *L. donovani* (18).

Contrary to proteins such as gp63, A2 is specific to *L. donovani* and *L. mexicana*, which eliminates misdiagnosis due to cross-reactivity. A2 also has two characteristics that resemble the *L. chagasi* 230-kDa protein whose 39-amino-acid repeat was shown to be a useful antigen for serodiagnosis of visceral leishmaniasis (3). A2 is amastigote stage specific, and it contains a repeat sequence. rK39 was shown to be an early surrogate marker for disease progression in visceral leishmaniasis, and rK39 seroreactivity correlates with active disease. Ninety-eight percent of active disease cases were detected with this marker (1). As shown in the present study, high levels of anti-A2 antibodies also occur in cases of acute visceral leishmaniasis, demonstrated by the detection of 92% of individuals with active disease by using the A2 antigen.

A2 is a protein that is developmentally expressed in the amastigote stage of the parasite, the stage at which the *Leishmania* organism is in the phagolysosomal compartment of host macrophages. As previously suggested (1), during the acute phase of the disease the host may produce specific antibodies, including the A2 and K39 antigens, against replicating *Leishmania* organisms. We suggest that A2 could be employed in conjunction with the recently developed rK39 antigen in the serodiagnosis of visceral leishmaniasis. It could, for example, be used as a second antigen when positive results are equivocal with the rK39 antigen.

The anti-A2 monoclonal antibody which was raised against a recombinant A2 protein (23) was also shown in this study to be capable of reacting strongly on Western blots with the A2 protein. The A2 monoclonal antibody may be useful for distinguishing *L. donovani* and *L. mexicana* from other *Leishmania* strains. Monoclonal antibodies have previously been used in serodiagnostic assays. For example, Jaffe and McMahon-Pratt (8) have developed a competitive serodiagnostic assay for visceral leishmaniasis using species-specific *L. donovani* monoclonal antibodies. The assay is based on the specific inhibition of monoclonal antibody binding to a crude parasite homogenate by serum from patients with visceral leishmaniasis. Monospecific antibodies are also suited for the taxonomic identification of *Leishmania* species (11, 16), and several species-specific leishmanial proteins have been identified with monoclonal antibodies (9, 11). The anti-A2 monoclonal antibody could, for example, be used to differentiate between visceral leishmaniasis due to *L. donovani* or *L. tropica* infections. In a recent study (17), *L. tropica* was found to visceralize in some individuals, confirming that *L. tropica* is a coendemic agent of visceral leishmaniasis in India. The same visceralizing effect of *L. tropica* was observed in soldiers returning from Operation Desert Storm (13).

In summary, we have examined the antibody response against the amastigote-specific antigen A2, which is present in members of the *L. donovani* and *L. mexicana* complexes. The antibody response to the *L. donovani* and *L. mexicana* complexes was specific enough to hold potential in serodiagnostic assays for visceral leishmaniasis. A2 is one of the only *L. donovani* amastigote-specific markers identified to date, and we suggest that it may prove valuable in a serodiagnostic test that uses a spectrum of antigens specific to different stages of the *Leishmania* parasite and/or to different species.
ACKNOWLEDGMENTS

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