Characterization of a *Leishmania tropica* antigen that detects immune responses in Desert Storm viscerotrophic leishmaniasis patients

(parasite/diagnosis/repetitive epitope/subclass)

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ABSTRACT A chronic debilitating parasitic infection, viscerotrophic leishmaniasis (VTL), has been described in Operation Desert Storm veterans. Diagnosis of this disease, caused by *Leishmania tropica*, has been difficult due to low or absent specific immune responses in traditional assays. We report the cloning and characterization of two genomic fragments encoding portions of a single 210-kDa *L. tropica* protein useful for the diagnosis of VTL in U.S. military personnel. The recombinant proteins encoded by these fragments, recombinant (r) Lt-1 and rLt-2, contain a 33-amino acid repeat that reacts with sera from Desert Storm VTL patients and with sera from *L. tropica*-infected patients with cutaneous leishmaniasis. Antibody reactivities to rLt-1 indicated a bias toward IgG2 in VTL patient sera. Peripheral blood mononuclear cells from VTL patients produced interferon γ, but not interleukin 4 or 10, in response to rLt-1. No cytokine production was observed in response to parasite lysate. The results indicate that specific leishmanial antigens may be used to detect immune responses in VTL patients with chronic infections.

An alternative diagnostic strategy is to identify and apply immunodominant recombinant antigens to increase assay sensitivity and specificity. We report herein the cloning, expression, and evaluation of an immunodominant *L. tropica* antigen capable of both specific antibody detection and elicitation of interferon γ (IFN-γ) production in peripheral blood mononuclear cells (PBMCs) from VTL patients. These results demonstrate the danger of relying on crude immunological assays for the diagnosis of subtle, albeit serious, VTL in Desert Storm patients.

MATERIALS AND METHODS

Parasites. *L. tropica* isolates MHOM/SA/91/WR1063SS, MCAN/SA/91/WR1091SS, MHOM/SA/91/WR1092SS, MHOM/Q/C1/91/WR1095SS, MHOM/SA/91/WR2044SS; *L. tropica* (Rupert); *L. tropica* (Azad); *Leishmania amazonensis* IFLA/BR/67/PH8; *Leishmania braziliensis* MHOM/BR/75/M2903; *Leishmania chagasi* MHOM/BR/82/BA-2, Cl; *L. donovani* MHOM/EI/67/HU3; *Leishmania guyanensis* MHOM/BR/75/M1447; *L. infantum* IPT-1; *L. major* LTM p-2; *L. major* (Friedlander); and Trypanosoma cruzi MHOM/CH/00/Tulahuen C2 were used. *Leishmania* promastigotes and *T. cruzi* epimastigotes were cultured in axenic media. *L. major* amastigotes were isolated from infected C.B-17 scid mice.

Patient Sera and PBMCs. The VTL patient group included eight culture-positive individuals, seven with confirmed *L. tropica* infection and one with insufficient parasites available for isoenzyme analysis. Four others were culture-negative but either PCR or monoclonal antibody (mAb)-smear-positive. CL patient sera were from M. Grogl (Walter Reed Army Institute of Research, Washington, DC). Normal sera were from the American Red Cross (Portland, OR).

Isolation of Lt-1 and Lt-2. *L. tropica* MHOM/SA/91/WR1063C genomic DNA was isolated and sheared by passage through a 30-gauge needle to 2–6 kb. The library was constructed in Lambda ZapII (Stratagene) by using EcoRI adapters. Expression screening was performed with a pool of preadsorbed patient sera (8).

Expression of Recombinant *L. tropica* Antigens. Induced bacterial pellets were lysed, and recombinant (r) Lt-1, rLt-1r, and rLt-2 were recovered from the inclusion bodies. rLt-1 and rLt-2 were solubilized in 8 M urea and rLt-1r in 4 M urea. The recombinant proteins were purified by ammonium sulfate precipitation and preparative gel separation by SDS/PAGE in

Abbreviations: VTL, viscerotrophic leishmaniasis; VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; PBMC, peripheral blood mononuclear cell; IL, interleukin; mAb, monoclonal antibody; TNF-α, tumor necrosis factor α; IFN, interferon; r, recombinant.

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§The sequence reported in this paper has been deposited in the GenBank database (accession no. U31221).

Infection by the parasite *Leishmania* can result in a broad spectrum of pathological outcomes in the human host, ranging from simple self-healing cutaneous lesions to acute visceral leishmaniasis (VL), commonly referred to as kala-azar. The differing pathologies usually correlate with infection by differ-ent species. *Leishmania donovani* and *Leishmania infantum* usually cause VL, with symptoms including fever, emaciation, hypergammaglobulinemia, hepatosplenomegaly, and pancyto-penia. *Leishmania major* and *Leishmania tropica* generally cause cutaneous leishmaniasis (CL). Exceptional cases have been described, such as visceral outcomes in individuals infected with *L. tropica* (1, 2).

More recently, exposure of U.S. soldiers to *L. tropica* has resulted in a variant form of visceral disease in several individuals with confirmed infection with this organism (3, 4). Additional confirmed cases continue to arise at this time. Referred to as viscerotrophic leishmaniasis (VTL), these infections differ from classical VL in the variable pathology observed, with several patients lacking both fever and hepatospleno-megaly (4). In addition, serum anti-leishmanial antibody titers are much lower than those observed in patients with classical VL. Diagnosis of classical VL has utilized the elevated antibody response to parasite antigens in tests involving serological reactivity to whole or lysed promastigotes (5, 6) or to recombinant antigens (7). Confirmation is achieved by the isolation of live parasites from spleen, liver, bone marrow, or lymph nodes. Serological reactivity to promastigotes in VTL patients is usually low or absent (4).

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10% gels. Recombinant proteins were eluted from the gels and diazymed in phosphate-buffered saline (PBS), the concentration was measured by the Pierce BCA assay, and purity was assessed by SDS/PAGE followed by Coomassie blue staining.

**Molecular Analysis of Lt-1 and Lt-2.** Exonuclease III digestion was used to create overlapping deletions of the clones (9). Single-strand template was prepared and sequenced with Applied Biosystems automated sequencer model 373A or by Sanger dideoxynucleotide sequencing (10). Both strands of the coding portion of the Lt-1 clone were sequenced.

Genomic DNA was digested with a variety of enzymes, separated by agarose gel electrophoresis, and blotted on Nytran (Scheicher & Schuell). The L. tropica inserts were labeled with [32P]dCTP by random oligonucleotide primers (Boehringer Mannheim) and used as probes. Hybridizations involving the Lt-1 or the Lt-2 probes were performed at 65°C, and the hybridizations involving the rLt-1 probe were performed at 60°C in 0.2 M NaH2PO4/3.6 M NaCl/0.2 M EDTA overnight and washed to a stringency of 0.075 M NaCl/0.0075 M sodium citrate (0.15 M NaCl/0.0150 M sodium citrate was used with Lt-1 probe), pH 7.0/0.5% SDS at the temperature of hybridization.

**Serology.** ELISAs were performed as described (6). In competition experiments, sera were preincubated for 30 min with 5 μg of rLt-1r at room temperature. Plates were washed five times with PBS-T (phosphate-buffered saline/0.1% Tween 20). For IgG subclass ELISA tests, plates were coated, blocked, and incubated with sera as described (6). After plates were washed five times with PBS-T, mouse anti-human IgG1, IgG2, IgG3, and IgG4 mAb (Calbiochem) was added (50 μl of a 1:1000 dilution) and incubated 30 min. Plates were washed five times with PBS-T and bound antibodies were detected by goat anti-mouse IgG1-herosderid peroxidase (Zymed; 1:500 dilution) and ABTS substrate. The two-sample t test was used in statistical analysis.

**Immunoblot Analysis.** *Leishmania* parasite lysate, purified rLt-1, and purified rLt-1r were subjected to SDS/PAGE in a 7.5 or 12% gel and transferred to nitrocellulose (15 min at 50V and 1 h at 100V). Filters were blocked with PBS containing 5% (w/vol) nonfat milk at 4°C overnight, washed three times in PBS-T, and incubated 1 h in human sera (diluted 1:50 in PBS-T) or rabbit sera (diluted 1:250 in PBS-T) on a rocker at room temperature. Filters were washed three times with PBS-T and bound antibody was detected with 125I-labeled protein A at 105 cpm/ml followed by autoradiography.

Rabbit antiserum to rLt-1 was raised in an adult New Zealand White rabbit (R & R Rabbity, Stanwood, WA) by an initial s.c. delivery of 200 μg of rLt-1 in 1 ml of Freund's incomplete adjuvant (Bethesda Research Laboratories) and 100 μg of muramyl dipeptide (Calbiochem), followed by two successive s.c. immunizations of 100 μg of rLt-1 in 1 ml of incomplete Freund's adjuvant at 3-week intervals. A final i.v. boost of 25 μg of rLt-1 was delivered after an additional 4 weeks and serum was collected 2 weeks later.

**Proliferative Assays and Cytokine Production.** Purified PBMCs were cultured as described (11). Both parasite lysate and rLt-1 were used at 10 μg/ml. Endotoxin levels of all antigens were <5 pg/μg. Supernatants were collected at 72 h for cytokine assays. Capture ELISAs to detect interleukin (IL)-4, IL-10, and IFN-γ were performed as described (11). Capture ELISAs to detect tumor necrosis factor α (TNF-α) used an anti-human TNF-α mAb (Immunex) and a polyclonal rabbit anti-human TNF-α sera (Immunex). Human rTNF-α was used to generate a standard curve with a sensitivity of 40 pg/ml.

Six patient and 6 normal donors were assayed for IL-4 production in response to rLt-1, and 3 patient and 3 normal donors were assayed in response to parasite lysate. Three patient and 5 normal donors were assayed for IL-10 production in response to rLt-1, and 5 patient and 8 normal donors were assayed in response to lysate. Eleven patient and 17 normal donors were assayed for IFN-γ in response to rLt-1, and 9 patient and 13 normal donors were assayed in response to lysate. Five patient and 9 normal donors were assayed for TNF-α production in response to rLt-1, and 2 patient and 4 normal donors were assayed in response to lysate.

Triplicate cultures for proliferation assays were cultured in the presence or absence of antigen for 5 days. Samples were then pulsed with 1 μCi of [3H]thymidine (ICN; 1 Ci = 37 GBq) and incubated 18 h before harvesting. Six patient and six normal donors were assayed for proliferation.

**Fig. 1.** Partial DNA sequence of the Lt-1 clone and deduced amino acid sequence of the open reading frame. The DNA sequence of the nonrepeat coding portion of the Lt-1 insert is shown, and the deduced amino acid sequence of both the repeat portion (boldface type) with degeneracies present and the nonrepeat portion. The rLt-1r recombinant protein includes one and one-third of the repeat shown and contains the upper residues shown at the degenerate positions.
RESULTS

Isolation of Recombinant *L. tropica* Clones. Approximately 43,000 recombinant phage were screened by using a pool of preadsorbed patient sera, resulting in the identification of three immunoreactive clones containing inserts of 3.3, 1.4, and 2.7 kb, encoding recombinant antigens of 75, 70, and 110 kDa. These were referred to as Lt-1, Lt-2, and Lt-3, respectively. The recombinant antigens encoded by Lt-1 and Lt-2 were expressed and purified.

**Molecular Analysis of *L. tropica* Genes.** Sequence analysis of the two *L. tropica* clones was performed. The DNA sequence of the coding portion of the Lt-1 clone includes a repeated sequence at the 5′ portion of the clone containing eight copies of a 99-bp repeat and three copies of a 60-bp repeat unit that is part of the larger 99-bp repeat (data not shown), followed by 800 bp of nonrepeat sequence (Fig. 1). The deduced amino acid sequence of the 99-bp repeat contains limited degeneracies (Fig. 1). The molecular mass of the predicted recombinant protein is 67,060 Da. A database search with the predicted amino acid sequence of the open reading frame yielded no significant homology to previously submitted sequences. Predicted secondary structure of the repeat portion of the clone is entirely α-helical. Sequence analysis of Lt-2 revealed that the 3′ portion of the clone consisted of a mixture of 60- and 99-bp repeats that were identical, excepting occasional degeneracies, to the 60- and 99-bp repeats observed in Lt-1 (data not shown). Collectively, the sequencing data suggest that Lt-1 and Lt-2 are different portions of the same gene, Lt-2 being upstream of Lt-1, with possibly a small overlap. The nested deletion set of Lt-1 formed for sequencing included a deletion clone containing the 5′ portion of the repeat sequence. This clone, referred to as Lt-1r, consists of one and one-third repeats (Fig. 1) and was also expressed as a fusion protein and purified.

Genomic DNA from a number of *Leishmania* species including *L. tropica* were analyzed on Southern blots by using the Lt-1 insert as a probe. Collectively, various digests of *L. tropica* DNA indicated that this gene had a low copy number (Fig. 2). The comparison of hybridization intensities to *Pst I* digests of numerous species yielded a surprising result: the greatest hybridization was observed with members of the *L. donovani* complex including *L. donovani, L. chagasi,* and *L. infantum* and lower hybridization was observed with *L. major,* a species that has been considered to be closely related to *L. tropica* (Fig. 2). In addition, weak hybridization was observed with *L. amazonensis,* and none was observed with *L. braziliensis, L. guyanensis,* or *T. cruzi.* An overlapping pattern was observed by using the Lt-2 insert as a probe (data not shown), consistent with the premise that these two clones contain sequences near or overlapping one another.

Southern blot analyses of digested genomic DNA from four *L. tropica* parasite strains isolated from VTL patients and three *L. tropica* parasite strains isolated from CL patients (two human and one canine) were performed by probing with the Lt-1r insert. The seven *L. tropica* isolates yielded similar intensities and restriction patterns, with only a single restriction fragment length polymorphism among the isolates (data not shown). These data indicate strong similarity in this region among the *L. tropica* isolates.

To characterize the native *Leishmania* protein that represents the Lt-1 clone, a rabbit antiserum was raised to rLt-1 and used in an immunoblot of *Leishmania* lysates (Fig. 3). Reactivity was observed with a 210-kDa protein in *L. tropica* promastigote lysate (lane 3) and the gene was designated *Lt-210.* Due to the great difficulty of obtaining *L. tropica* amastigotes, we examined the reactivity of rLt-1 antisera with *L. major* promastigote and amastigote lysates, reasoning that the hybridization in Southern blot analysis, albeit weak, indicated the presence of an *Lt-210* homolog in this species. These results (lanes 1 and 2) demonstrated the presence of a cross-

![Fig. 2. Southern blot analysis of Lt-1 sequence. Genomic DNA (2.5 μg per lane) of *L. tropica* was digested with EcoRI (lane 1), Xba I (lane 2), Xho I (lane 3), BamHI (lane 4), HindIII (lane 5), and Pst I (lane 6). *L. major* (lane 7), *L. donovani* (lane 8), *L. infantum* (lane 9), *L. chagasi* (lane 10), *L. amazonensis* (lane 11), *L. braziliensis* (lane 12), *L. guyanensis* (lane 13), and *T. cruzi* (lane 14) were digested with *Pst I.* Blots were probed with the Lt-1 sequence. Molecular sizes in kilobases are shown.](image)

![Fig. 3. Reactivity of rabbit anti-*rLt-1* sera on *Leishmania* lysates. Bands from SDS/PAGE gels containing 15 μg of *L. major* promastigote lysate (lane 1), 15 μg of *L. major* amastigote lysate (lane 2), and 15 μg of *L. tropica* promastigote lysate (lane 3) were transferred to nitrocellulose and reacted with a 1:250 dilution of rabbit anti-*rLt-1* sera. No reactivity was observed by using a similar dilution of the preimmunization sera. Molecular masses in kDa are indicated.](image)

![Fig. 4. ELISA evaluation of *L. tropica* infection by using VTL sera or CL patient sera, *L. tropica* lysate (1 μg), and the antigens rLt-1 (50 ng) and rLt-1r (25 ng). *A*50 values (mean + SEM) are shown for VTL patient sera (*n* = 12), CL patient sera (*n* = 10), and normal sera (*n* = 18). Sera were used at a 1:50 dilution.](image)
reactive protein of similar size in *L. major* that was most abundant in amastigotes, the form occurring during human infection. An additional amastigote protein of lesser size is also recognized by the rLt-1 antisera and may represent either a related protein or a degradation product of Lt-210.

**Patient Sera Reactivity with *L. tropica* Recombinant Antigens.** To determine their diagnostic potential, rLt-1 and rLt-1r were evaluated by ELISA with sera from VTL and CL patients and normal sera (Fig. 4). Mean reactivity to rLt-1 was significantly higher in both the CL group (*P* = 0.002) and the VTL group (*P* < 0.001) compared to normal sera. A significant increase in reactivity to rLt-1r was also observed in both the CL group (*P* = 0.001) and the VTL group (*P* < 0.001) relative to normal sera. In addition, a significant increase in reactivity to rLt-2 was observed in the VTL group (*P* = 0.036) but not in the CL group (*P* = 0.279) compared to normal sera (data not shown). Reactivity to *L. tropica* promastigote lysate was significantly increased in the CL group (*P* < 0.001) but not in the VTL group (*P* = 0.594), compared to the normal group.

To determine the complexity of epitopes being recognized by patient sera, two VTL sera and one CL serum were preincubated with 5 µg of rLt-1r and tested for reactivity with rLt-1 in an ELISA. In all three cases, the reactivity to rLt-1 was eliminated by preincubation with rLt-1r (data not shown), indicating those individuals were reacting exclusively to epitopes contained within the repeated sequence.

Immunoblot analysis of parasite lysate, rLt-1, and rLt-1r was performed with sera from two VTL patients and a pool of three normal sera (Fig. 5). Increased reactivity to both recombinant antigens was observed in VTL patient sera compared to normal sera. Collectively, the ELISA and immunoblot data indicate that both rLt-1 and rLt-1r have increased specificity compared to promastigote lysate and suggest their utility in a diagnostic assay.

The reactivity of patient sera was further examined by ELISA with IgG subclass-specific mAbs. Analysis of anti-rLt-1 IgG responses (Fig. 6) demonstrated different subclass distributions in VTL and CL patient sera. A bias toward IgG2 subclass was observed in VTL patient sera, whereas a mixed profile was observed with CL patient sera. In addition, CL IgG responses to *L. tropica* lysate were biased toward IgG1 and IgG3. Normal mean IgG reactivities to rLt-1, when measurable, were biased toward IgG1 and IgG2 (data not shown). Similar results were obtained by substituting rLt-1r for the rLt-1 antigen (data not shown).

**Patient PBMC Response to Lt-1.** Patient and normal PBMCs were analyzed for proliferation and production of IFN-γ, TNF-α, IL-4, and IL-10, in response to *L. tropica* lysate or purified rLt-1. No significant proliferative responses to either lysate or rLt-1 were observed in patient compared to normal PBMCs. However, rLt-1, but not promastigote lysate, elicited the production of IFN-γ from patient, but not normal, PBMCs (Fig. 7). Collectively, 11 VTL patient and 17 normal PBMC preparations cultured with rLt-1 were assayed, and a significant increase in IFN-γ production was observed with patient PBMCs (*P* = 0.008). Neither *L. tropica* lysate nor rLt-1 elicited production of detectable levels of IL-4 in patients or normal PBMCs. Similarly, no increase in IL-10 or TNF-α production was observed in patient PBMCs compared to normal PBMCs cultured with lysate or rLt-1.
DISCUSSION

We have characterized immune responses of VTL patients to recombinant *L. tropica* antigens, rLt-1 and rLt-1r. These antigens appear to be serologically immunodominant, detecting antibodies in both *L. tropica*-infected VTL and CL patient sera. The molecular characterization of the two *L. tropica* genomic clones analyzed in this study indicated that they shared a domain of repeated sequence. Previous work has demonstrated the presence of serological repeat epitopes within other antigens of *Leishmania* (7) and a related parasite, *T. cruzi* (12, 13). Competition experiments with several patient sera using the Lt-1r gene product demonstrated that the serological epitope of rLt-1 was confined to the repeat portion of the clone.

The data presented herein indicate that the VTL patients are not unresponsive to leishmanial antigens but that the crude lysate most often used in diagnostic assays was unable to detect such responses. In fact, specific serological and cellular responses to rLt-1 indicate that these individuals are responding to at least one immunodominant antigen. To further characterize the serological response to rLt-1 and rLt-1r, the relative contributions of individual IgG subclasses were assessed. It was found the antibody responses to rLt-1 and rLt-1r in VTL patient sera were biased toward IgG2, while CL patient sera showed a mixed IgG profile to the recombinant antigens. CL patient reactivity to *L. tropica* lysate was predominantly of the IgG1 and IgG3 subclasses. These results argue that the contributing factors controlling antibody response in these patients may include properties inherent to the antigen, as demonstrated by the difference between the CL reactivity to rLt-1 and the reactivity to the heterogenous mixture of antigens present in the promastigote lysate. However, the different responses to rLt-1 by VTL and CL sera also suggest that the overall pathology contributes to the serological response to individual antigens.

The observed bias toward IgG2 reactivity observed is unusual since this subclass has been associated with nonprotein T-cell-independent antigens such as bacterial polysaccharides (14, 15). Whether or not the rLt-1 repeat is mimicking a carbohydrate antigen or is operating through a T-cell-dependent mechanism is unclear. The significance of antigen-specific IgG subclass expression in humans is not known, but some associations have been made between cytokine and isotype profiles. In a T-cell-dependent assay, the addition of IL-4 to human PBMCs induced production of IgE (16, 17). In addition, IL-4 has been implicated in the switching of sIgM+ B cells to IgG4 (18). More recent findings have demonstrated that the addition of IL-10 to sIgD+ sIgM+ B cells activated through CD40 results in secretion of IgG1 and IgG3 (19), while addition of IFN-γ to unstimulated PBMCs results in an increased production of IgG2 (20).

In addition to serological responses, we examined cytokine profiles in PBMCs stimulated with *L. tropica* antigens. Analyses of patient cytokine profiles have been useful for immunological characterization of different clinical forms of leishmaniasis (21, 22). The examination of the cytokine profile from VTL patient and normal PBMCs revealed that rLt-1 elicited significantly higher levels of IFN-γ from VTL patient PBMCs compared to normal PBMCs. These results indicated that rLt-1 was able to elicit the production of a predominantly T helper Th1-associated cytokine, IFN-γ, consistent with a rLt-1-specific IgG2 bias in these individuals, rather than the production of subclasses more closely associated with T helper Th2 cytokines. Finally, it is interesting to speculate that antigen-specific IFN-γ production may be contributing to the symptoms reported by veterans with unexplained Gulf War-related illnesses, sometimes referred to as Gulf War Syndrome (23).

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