Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis

(repetitive epitope/motor protein/parasite/diagnosis)

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**ABSTRACT** We report the cloning of a *Leishmania chagasi* antigen gene and an evaluation of leishmaniasis patient antibody responses to the recombinant protein, rK39. rK39 contains a 39-amino acid repeat that is part of a 230-kDa protein predominant in *L. chagasi* tissue amastigotes. Sequence analyses showed this protein, LeKin, to be related to the kinesin superfAMILY of motor proteins. Southern blot analyses demonstrated LeKin-related sequences in seven species of *Leishmania*, with conservation of the repeat between *L. chagasi* and *Leishmania donovani*. Serological evaluation revealed that 98% (56 of 57) of Brazilian and 100% (52 of 52) of Sudanese visceral leishmaniasis patients have high antibody levels to the rK39 repeat. Detectable anti-K39 antibody was virtually absent in cutaneous and mucosal leishmaniasis patients and in individuals infected with *Trypanosoma cruzi*. The data show that rK39 may replace crude parasite antigens as a basis for serological diagnosis of visceral leishmaniasis.

Protozoan parasites of the genus *Leishmania* are widely distributed and transmitted by the bite of sandflies. In the vertebrate host, the infecting promastigotes differentiate into and replicate as amastigotes within macrophages. Symptoms range from self-healing skin lesions to diffuse cutaneous and mucosal manifestations, or severe visceral involvement of the spleen, liver, and lymph nodes. Visceral leishmaniasis (VL) is generally caused by *Leishmania donovani* in Africa, India, and southern Europe or *Leishmania chagasi* in Latin America. In VL, high antibody levels are observed prior to the detection of parasite-specific T-cell responses (1). This antibody response has been exploited for the diagnosis of *L. chagasi* and *L. donovani* infection (2-5). The current World Health Organization's estimate of 12 million cases of leishmaniasis and recent epidemics of VL in Sudan and India (6, 7) highlight the need for more effective control measures. Diagnostic tests use whole or lysed *Leishmania*, and a few studies have begun to examine patient antibody responses to specific antigens (2, 8-10). Such studies may improve diagnostic assays and help to evaluate B-cell responses during disease progression.

We report the cloning and expression of an *L. chagasi* gene sequence and an evaluation of patient antibody responses to the recombinant product, rK39. The sequence encodes an immunodominant protein with a repetitive epitope closely conserved between *L. chagasi* and *L. donovani*. This repeat is part of a large kinesin-related protein expressed predominantly by amastigotes. More than 98% of Brazilian and 100% of Sudanese VL patients had readily detectable serum antibody responses to the K39 repeat.

**MATERIALS AND METHODS**

**Parasites.** *L. chagasi* (MHOM/BR/82/BA-2,CI), *L. chagasi* (MHOM/BR/84/Jonas), *Leishmania amazonensis* (IFLA/BR/67/PH8), *Leishmania braziliensis* (MHOM/BR/75/M2903; obtained from Diane McMahon-Pratt, Yale University), *Leishmania guyanensis* (MHOM/BR/75/M4147), *L. donovani* (MHOM/ET/67/HU3), *Leishmania infantum* ( IPT-1; obtained from Lee Schnur, The Hebrew University-Hadassah Medical School, Jerusalem), *Leishmania major* (LTM p-2, obtained from David Moser, Temple University, Philadelphia), and *Trypanosoma cruzi* (MHOM/CH/00/ Tulahuen C2) were used. Promastigotes and epimastigotes were cultured in axenic media. *L. chagasi* and *L. amazonensis* amastigotes were obtained from spleens of Syrian hamsters and footpads of BALB/cByJ mice, respectively, and purified as described (11).

**Patient Sera.** Brazilian leishmaniasis sera and *T. cruzi* infection sera were from well-characterized patients in Bahia, Brazil; Sudanese leishmaniasis sera were from *Leishmania*-positive patients; normal sera were from healthy individuals in Sudan or the United States (2, 4, 12).

**Identification and Purification of rK39.** A genomic library was constructed with sheared DNA of *L. chagasi* (MHOM/BR/82/BA-2,CI) in Lambda ZAPII (Stratagene) and screened with polyclonal serum (13) of a *L. donovani* patient. The 39-kDa recombinant antigen of clone K39 (K39) was purified from a 25-40% ammonium sulfate fraction of bacterial lysate by preparative isoelectric focusing (IEF) with the Rotofor IEF cell and 1% 3/10 amphotolys (pH range 3.5-9.5; Bio-Rad) in the presence of 8 M urea and 10 mM dithiothreitol. Peak fractions were concentrated by ammonium sulfate precipitation and dialyzed against 25 mM Tris-HCl, pH 8/150 mM NaCl (TBS). Protein concentrations were determined by using the Pierce BCA assay, and purity was assessed by Coomassie blue-staining following SDS/PAGE (14).

**Immunoblot Analysis.** Immunoblots of parasite lysates or purified rK39 were prepared (11). Filters were blocked with TBS containing 5% nonfat dried milk and probed with patient sera (1:250 dilution) or rabbit sera (1:400 dilution) diluted in TBS containing 0.1% Tween-20 and 1% bovine serum albumin.

*Abbreviation:* VL, visceral leishmaniasis.

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*The sequence reported in this paper has been deposited in the GenBank database (accession no. L07879).*
Fig. 1. Expression and purification of rK39. Coomassie blue-stained SDS/12% polyacrylamide gel of molecular mass markers (lane 1), Escherichia coli lysates from uninduced (lane 2) and induced (lane 3) cultures of clone K39, and purified rK39 (2 µg) (lane 4). Sizes are shown in kDa.

RESULTS

Identification of L. chagasi Antigen Genes. To characterize Leishmania antigens recognized by VL patients, a L. chagasi expression library was screened with sera from a L. donovani patient. From ~32,000 recombinants, 7 clones were selected that contained inserts of 0.9–2.6 kb and produced immunoreactive recombinant proteins of 35–100 kDa. The recombinant antigen of clone K39 (rK39) was exceptionally reactive with test serum. rK39 migrated as a 39-kDa protein in induced bacterial extracts (Fig. 1, lane 3) and was purified (Fig. 1, lane 4) with a yield of 25–30 mg per liter.

Both L. chagasi and L. donovani VL sera Recognize rK39. The reactivity of patient sera with rK39 was evaluated by immunoblot. Both rK39 and L. chagasi promastigote lysate were strongly recognized by L. chagasi (Fig. 2, lanes A–C) and L. donovani (Fig. 2, lanes D–F) infection sera but not by pools of sera from mucosal (Fig. 2, lanes G and H) or cutaneous (Fig. 2, lanes J) leishmaniasis patients or with Chagas disease patient sera (Fig. 2, lanes I). All sera reacted strongly with promastigote lysates. These results indicate that K39 may be specific to L. chagasi and L. donovani and/or K39 induces a strong antibody response only in VL patients.

Sequence Analysis of the K39 Gene. The DNA and deduced amino acid sequences of clone K39 were determined (Fig. 3A, nucleotides 2426–3319) revealing a single open reading frame encoding 298 amino acids with a predicted molecular mass of 32.7 kDa and pl of 4.4. rK39 contains an additional 6.2 kDa of plasmid fusion sequences. Of particular interest were 6.5 copies of a tandemly arrayed 39 amino acid repeat (Fig. 3B).

Clones containing sequences flanking the K39 gene fragment were isolated from the L. chagasi library. Sequence analysis of one overlapping clone, LcK39 (Fig. 3A, nucleotides 1–3109), showed that the open reading frame extended 1971 bp in the 5’ direction, encoding 657 nonrepetitive amino acids; 5’ to the putative ATG initiation codon, 454 bp of sequence were obtained with multiple termination codons in each reading frame. Partial characterization of clones containing 5’ flanking sequences indicated that the repeat domain extended ~3–4 kb.

Data bank searches revealed sequence similarity between LcK39 and members of the superfamily of kinesin-related proteins, particularly in the N-terminal motor domain. Fig. 4
shows a comparison between the N-terminal domain of LeaKin from the motor domain of the *Caenorhabditis elegans* kinase (22), with 43.8% identity over 349 amino acids. Strong sequence conservation was observed in the putative ATP and microtubule binding domains (23). The remaining 500 residues showed limited similarity to the tail regions of kinases and myosin. Secondary structure analyses predicted this portion of LeaKin (amino acids 426–955) to contain >90% helical structure characteristic of the coiled-coil tail regions of several motor proteins. Thus, the repetitive K39 epitope appears to present in *L. chagasi* as part of the tail region of a *Leishmania* kinin-related protein.

**Southern Biot Analysis of LeaKin Gene Sequences.** Genomic DNAs from several *Leishmania* species were analyzed by Southern blot by using probes of LeaKin derived from the kinesin homology domain (probe A, Fig. 5A) or from the K39 repetitive domain (probe B, Fig. 5B). Probe A hybridized strongly to multiple Pst I restriction fragments of all *Leishmania* spp. tested (Fig. 5A, lanes 1–3), indicating conservation in the kinesin homology domain. Polymorphisms in the size and number of hybridizing fragments were noted. Less conservation in the repetitive domain of the LeaKin gene was observed as probe B hybridized with varying intensity to Pst I restriction fragments of *L. chagasi*, *L. amazonensis*, *L. brasiliensis*, *L. donovani*, *L. infantum*, and *L. major* but not to *L. guyanensis* (Fig. 5B, lanes 3–9). The K39 repeat appeared most closely related between *L. chagasi* and *L. donovani* (Fig. 5B, lanes 3 and 7). No hybridization with either probe was observed with *T. cruzi* DNA (Fig. 5, lane 10).

When using *L. chagasi* digested DNA, two Pst I fragments were detected, with probe B indicating the presence of a second copy of the LeaKin gene or polymorphism in restriction sites present in the 3′ repetitive sequences (Fig. 5B, lane 3). Probe A hybridized to three fragments in each of the *BamHI*, *HindIII*, and Pst I digests of *L. chagasi* DNA (Fig. 5A, lanes 1–3). One *BamHI* fragment and no other fragments are present within the probe A sequence. Taken together, the Southern blot data show that the LeaKin gene is present in a minimum of two or three copies in the *L. chagasi* genome and that related sequences are present in seven species of *Leishmania* examined.

**Identification of the Native LeaKin Antigen.** Rabbit anti-rK39 serum was used to probe SDS/PAGE blots of *L. chagasi* promastigotes and tissue amastigote lysates. The antiserum bound specifically to purified rK39 (Fig. 6A, lane 2) and to an n230-kDa L. chagasi antigen present in amastigotes (Fig. 6B, lane 6) and to a lesser degree in promastigotes (Fig. 6B, lane 5). No reactivity with this serum was detected in promastigotes and amastigote lysates of *L. amazonensis* (Fig. 6B, lanes 7–8). Comparable amounts of lysate were loaded in all lanes.
as shown by the reactivity of a rabbit antiserum raised against a constitutively expressed *L. chagasi* ribosomal phosphoprotein, LcP0 (Fig. 6C) (24).

**Reactivity of Patient Sera with rK39.** The reactivity of patient sera with rK39 was evaluated by ELISA. Among VL patients, 98.2% of the Brazilian sera (56 of 57) and 100% of the Sudanese sera (52 of 52) were positive on rK39 (absorbance values were >3 standard deviations above the mean of normal controls). Values ranged from 0.05 to 2.0 (mean = 1.38) among Brazilian VL sera and from 0.094 to 2.0 (mean = 1.60) among Sudanese VL sera (Fig. 7B). Detectable antibody to rK39 was restricted to VL patients, as little or no anti-rK39 response was observed in mucosal or cutaneous leishmaniasis sera or with *L. cruzi* infection sera, despite reactivity with crude *L. chagasi* lysate (Fig. 7A).

**DISCUSSION**

We have identified a 230-kDa antigen of *L. chagasi*, LcK, with homology to the kinesin superfamily of motor proteins. LcK is predominant in amastigotes, is present in diverse species of *Leishmania*, and contains an extensive repetitive domain. Southern analyses showed the repeat of LcK to be variable among species, but closely related in *L. chagasi* and *L. donovani*. We demonstrated high antibody titers in Brazilian and Sudanese VL patients to rK39, which contains several amino acid repeats, indicative of the conservation of the repeat between *L. chagasi* and *L. donovani*.

Characterization of a gene encoding a protozoan motor protein, represented here by the cloning of LcK, has been previously unreported to our knowledge. Members of the kinesin superfamily share on average 40% sequence identity in the N-terminal 350–400 amino acids composing the motor domain (25). These microtubule-based motors are involved in such varied intracellular processes as organelle and synaptic vesicle transport, chromosome segregation, and spindle pole body separation, nuclear fusion, protein sorting, and flagellar beating (25, 26). Although they share little sequence similarity outside of the motor domain (26), the nonmotor tail domain is predominantly \( \alpha \)-helical in structure and likely forms a coiled-coil interacting with different intracellular ligands. The LcK gene product is similar to members of this family in primary sequence, particularly in the putative ATP and microtubule binding domains, as well as in predicted secondary structure, although the specific cellular processes involving LcK are not yet known.

**FIG. 4.** Protein sequence comparison between LcK (amino acids 1–426) and the motor domain of the kinesin related protein of *C. elegans* (22) in single-letter code. Identical residues are indicated by letter; colons and periods represent conservative and neutral amino acid substitutions, respectively. Putative ATP and microtubule binding domains are overlaid (23).

**FIG. 5.** Southern blot analysis of LcK gene sequences. Genomic DNA (2.5 \( \mu \)g per lane) from *L. chagasi* digested with BamHI (lane 1), HindIII (lane 2), and PstI (lane 3) or Pst I-digested DNA from *L. amazonensis* (lane 4), *L. braziliensis* (lane 5), *L. guyanensis* (lane 6), *L. donovani* (lane 7), *L. infantum* (lane 8), *L. major* (lane 9), or *L. cruzi* (lane 10) were analyzed by Southern blotting. Blots were probed with a 2.4-kb HindIII fragment from the LcK kinesin homology domain (A) or with the 915-bp repetitive insert of K39 (B). Sizes are shown in kb.

**FIG. 6.** Reactivity of rabbit anti-rK39 antiserum on rK39 and *Leishmania* lysates. (A) Immunoblot of purified rK39 (50 ng) transferred from SDS/12% polyacrylamide gels and probed with premune rabbit serum (lane 1) or rabbit anti-rK39 (lane 2). (B) Immunoblot of 10 \( \mu \)g of *L. chagasi* promastigote (lanes 1 and 5) and amastigote (lanes 2 and 6) lysates or 10 \( \mu \)g of *L. amazonensis* promastigote (lanes 3 and 7) and amastigote (lanes 4 and 8) lysates transferred from SDS/7.5% polyacrylamide gels and probed with preimmune rabbit serum (lanes 1–4) or rabbit anti-rK39 (lanes 5–8). (C) Reactivity of rabbit antiserum raised against *L. chagasi* ribosomal protein PO (24) with material in lanes 1–4 of B. Sizes are shown in kDa.
A striking feature of LcKin was the high prevalence of antibody to the K39 repeat in VL patients from geographically distinct regions. This response was restricted to VL patients, reflecting the relatedness among members of the \textit{L. donovani} complex (27–29). The characterization of patient B-cell responses to defined \textit{Leishmania} antigens has been minimal (2, 8–10). Our studies are unique in showing VL-restricted antibody responses to a recombinant antigen of \textit{L. chagasi} and showing a marked restriction of this response to \textit{L. chagasi}- and \textit{L. donovani}-infected patients.

Repetitive amino acid domains have been observed in many parasitic protozoan antigens, often being immunodominant B-cell epitopes that detect high levels of antibody in infected patients (16, 30–34). It is unclear if such responses contribute to the development of protective immunity or are immunological “smokescreens” limiting the development of protective responses (30, 35). In preliminary studies, 10 of 25 asymptomatic or subclinical patients infected with \textit{L. chagasi} had elevated levels of anti-K39 antibody (unpublished data). As only 30–35% of such individuals develop acute VL (36), the fate of those with a positive anti-K39 titer may be informative. Such prospective studies should help to determine whether the anti-K39 response is associated with the progression or resolution of VL. Nevertheless, from a practical standpoint, the data presented here as well as data from field studies in epidemic regions of VL in Sudan (H.W.G., W.G.S., D.R.B., J.M.B., S.G.R., unpublished data) show that rK39 may be useful in the serological diagnosis of acute VL.

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