rK39 Enzyme-Linked Immunosorbent Assay for Diagnosis of *Leishmania donovani* Infection

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The rK39 enzyme-linked immunosorbent assay (ELISA) was compared with the direct agglutination test (DAT) for *Leishmania donovani* infection in the Sudan. rK39 ELISA proved more sensitive than DAT in diagnosis of kala-azar (93 and 80%, respectively); both tests may remain positive up to 24 months after treatment. For patients with post-kala-azar dermal leishmaniasis and individuals with subclinical infection, rK39 ELISA performed as well as DAT but could detect infection 6 months earlier in ~40% of patients. Conversion in DAT and rK39 ELISA also occurred in leishmanin skin test (LST)-positive individuals, suggesting active parasite replication (rK39 is an amastigote antigen) in these presumably immune individuals. In contrast to DAT, rK39 ELISA also detected infection in randomly selected LST-positive individuals (in four of six) and endemicity (LST-negative) controls (in one of five). rK39 ELISA appears more sensitive than DAT and may prove an important tool in epidemiological studies.

*Leishmania donovani* infection presents with a spectrum of clinical entities. Infection may lead to clinical visceral disease (kala-azar) which runs a fatal course if left untreated. After cure, 80% of individuals develop a positive leishmanin skin test (LST) result 6 months after treatment, which is thought to be associated with protective immunity (5). Recently, we demonstrated that more than 50% of kala-azar patients in one area of endemicity in the Sudan develop post-kala-azar dermal leishmaniasis (PKDL), which causes morbidity and has possible implications for transmission (10). In other individuals infection does not lead to clinical disease, and they develop subclinical infection as evidenced by conversion in a serological test or LST without clinical symptoms or signs.

The diagnosis of kala-azar is classically made by demonstration of *Leishmania* parasites in various tissues. Of the procedures currently used, lymph node aspiration is easy to perform but has a sensitivity of only 58%. Bone marrow aspiration is more sensitive (70%) but is resented by patients. Splenic aspiration has excellent sensitivity (96%); however, it carries some risk and should preferably be done in a hospital (9). Clearly, there is a need for simple serological tests that can be used under field conditions; among the available tests, the direct agglutination test (DAT) performed best, with high sensitivity and specificity (3, 4); proved cheap and reliable; and can be used under field conditions. However, it cannot differentiate among past kala-azar, subclinical infection, and active disease (8). As these tests use whole or lysed promastigote antigens, test performance may be improved by using more specific antigens. rK39 is the cloned antigen of 39 amino acid repeats of a kinases-like gene found in *L. chagasi*. Antibodies to rK39 antigen could be detected by enzyme-linked immunosorbent assay (ELISA) in nearly 98% of sera from patients with visceral leishmaniasis in Brazil and Sudan (2) and China and Pakistan (6) but were virtually absent in South American patients with mucocutaneous leishmaniasis and cutaneous leishmaniasis (CL) (2) as well as in patients with CL in Turkey (6). In addition, antibody titers were found to correlate with parasite burden and may be useful in evaluating the result of chemotherapy (7). Recently, it was suggested that the presence or absence of antibodies to rK39 may predict progression to VL or self-healing, respectively (1).

In this study we investigated the value of the rK39 ELISA in diagnosis of various clinical conditions along the spectrum of *L. donovani* infection in an area in the Sudan in which kala-azar is endemic.

**MATERIALS AND METHODS**

Data for this study were collected between 1991 and 1995 in an ongoing longitudinal field study in the village of Um-Salala (population, 1,430) in the area in eastern Sudan in which kala-azar is endemic. Details on the methodology of this study have been previously described (10). In brief, the village was visited twice yearly at approximately 6-month intervals. During each visit, all individuals were screened for evidence of *Leishmania* infection. A detailed history was taken, and a clinical examination was conducted. A drop of blood was collected on filter paper for the DAT (4). A titer ≥1:6,400 was considered positive. The same filter papers were used for rK39 ELISA (see below). An LST was performed by intradermal injection of 0.1 ml of *L. infantum* antigen (Istituto Superiore di Sanità, Rome, Italy), and the result was read after 48 h. A reaction of ≥5 mm was considered positive. For subjects with a negative result the test was repeated in the next survey; those who were tested and proved positive were not retested subsequently.

For this study, patients were assigned to one of the following clinical categories.

**Kala-azar.** Patients parasitologically confirmed, by demonstration of *Leishmania* parasites in a lymph node or bone marrow aspirate, were categorized as having kala-azar.

**PKDL.** Patients diagnosed on clinical grounds as described elsewhere (11) were categorized as having PKDL.

**Subclinical infection.** Individuals with a negative LST result who converted in DAT at any time point during the study, without evidence of kala-azar, and who remained healthy during follow-up were categorized as having subclinical infection.

**Positive LST result and DAT conversion.** Some individuals were LST positive at the start of the study, converted in the DAT later in the study, without evidence of kala-azar, and remained healthy during follow-up. These individuals...
differ from those with subclinical infection, as they have evidence of previous exposure to CL or kala-azar, as indicated by the positive LST result. Fifty-three percent of the population in the village is LST positive, and the majority are adult individuals who have migrated from their homeland in western Sudan, where CL is common and kala-azar is absent. A high percentage of these individuals (91% in those 16 years of age and older) are LST positive, most likely as a result of previous CL (10).

**Positive LST result without DAT conversion.** Some individuals were LST positive at the start of the study but remained negative by the DAT throughout the observation period, without history of kala-azar before and during the study. Unlike individuals in the four clinical categories above, where patients were selected because an event occurred (clinical disease or seroconversion), these individuals were randomly selected.

**Endemicity controls.** Randomly selected individuals from the area of endemicity who remained negative by the DAT and LST throughout the study and were healthy served as endemic-area controls.

**Nonendemicity controls.** Individuals from an area where kala-azar is not endemic who had no previous history of kala-azar or CL served as nonendemicity controls. They were tested by rK39 ELISA, not by the DAT.

rK39 ELISA. rK39 was prepared as previously described, and a standard ELISA procedure was followed (2). Briefly, the Falcon plates were coated with 50 ng of rK39 per well, overnight at 4°C. The plates were blocked with 1% Tween in phosphate-buffered saline for 1 h. Serum eluted from the filter paper was added to the plate at a final serum concentration of 1/100 in phosphate-buffered saline–0.1% Tween, and the plates were incubated for 1/2 h at room temperature. Excess serum was washed five times, and bound antibodies were detected with horseradish peroxidase-protein A (Sigma), used at a concentration of 1/2,000, for 1/2 h at room temperature. The bound enzyme was detected with 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)-H2O2 in citrate buffer. The optical density (OD) was read at 405 nm. Positive and negative sera were run in each plate to standardize the readings and plate and day variations. The cutoff point between negative and positive readings was calculated as the mean of the negative controls plus 3 standard deviations.

To assess differences in decreases in DAT titers and rK39 ELISA OD values in kala-azar patients during follow-up, the following statistical methods were used. The DAT titers and rK39 ELISA OD values were logarithmically (ln) transformed to reduce skewness, and for observations not influenced by disease, means and standard deviations were calculated. Observations subsequent to diagnosis were Z transformed by subtracting these means and by dividing these differences by the standard deviations. Differences and ratios of the DAT and rK39 Z scores were then calculated and regressed on time after diagnosis to explore a trend in their differences or ratio.

**RESULTS**

The results for the first four clinical categories are shown in Table 1.

**Kala-azar** (n = 15; mean age, 6.9 years [range, 3 to 17]). In the kala-azar group the rK39 ELISA gave a positive result in all but one case (sensitivity, 93%; mean OD, 1.43 ± 0.76), whereas the DAT showed a sensitivity of 80% (12 of 15). For all those who were tested, the DAT remained positive in follow-ups for up to 24 months. The patient who was negative by rK39 ELISA at diagnosis was not available for follow-up; all others who were positive at diagnosis remained positive during follow-up, except one patient who converted to negative after 6 months. There was no statistically significant difference in trend for the OD values of the rK39 ELISA and the DAT titers during follow-up (at diagnosis: ln mean OD, −1.69 ± 1.39; ln mean DAT, 0.76 ± 2.31; during follow-up: ln mean OD, 0.89 ± 0.98; ln mean DAT, 1.59 ± 0.70).

**PKDL patients** (n = 7; mean age, 7 years [range, 3 to 19]). At diagnosis, the DAT was positive for all PKDL patients and the rK39-ELISA was positive for all but one patient (mean OD, 1.82 ± 0.53). All PKDL patients had a history of recent local treatment for kala-azar (therefore not confirmed by us) in the interval after the previous survey 6 months before. In that survey, all were negative by DAT, and clinical symptoms or signs were absent; however, in three patients, rK39 ELISA was already positive. In one patient the rK39 ELISA was positive as early as 12 months before.

**Subclinical infection** (n = 12; mean age, 11 years [range, 3 to 25]). For all individuals with subclinical infection, rK39 ELISA results (mean OD, 1.81 ± 0.86) were positive at the time of diagnosis, as were the DAT results (by definition). At −6 months, however, three of the eight patients tested (38%) already had a positive result by the rK39 ELISA, whereas all DAT results were negative (by definition). Following conversion, the majority of patients remained positive by both tests at +6 months (7 of 10 [70%] for DAT and 8 of 10 [80%] for rK39 ELISA) and at +12 months (5 of 7 [71%] for DAT and 6 of 7 [86%] for rK39 ELISA).

**Positive LST and DAT conversion** (n = 10). For subjects who were LST positive at the start of the study and then converted in the DAT, the following results were obtained. At the time of DAT conversion, one individual (age, 6 years) who was born in the village did not convert to a positive rK39 ELISA and remained negative during follow-up. The other nine (mean age, 35 years [range, 28 to 55]) were positive by rK39 ELISA (mean OD, 1.26 ± 0.96). All were immigrants from the west with previous exposure to L. major; five of these had typical scars of CL. Six of eight individuals tested (75%) were already positive at −6 months before the study, when the DAT result was negative (by definition). At +6 months, four of six (67%) and three of six (50%) subjects were positive by rK39 ELISA and DAT, respectively; at +12 months, five of seven (71%) and four of seven (57%) subjects were still positive by both tests.

**Positive LST result without DAT conversion** (n = 6; mean age, 37 years [range, 22 to 64]). For subjects who were LST positive at the start of the study without subsequent DAT conversion, the following results were obtained. Two individuals converted in rK39 ELISA during the study period and re-

| TABLE 1. Results of DAT and rK39 ELISA for four clinical conditions caused by L. donovani infection |
| Test result | No. of subjects at the following time point* |
|-------------|------------------------------------------|
| Kala-azar   |                                          |
| DAT         | + 0 0 12 10 5 6 3                        |
|            | − 7 10 3 0 0 0 −                        |
| rK39 ELISA  | + 0 0 14 9 4 5 2                       |
|            | − 8 10 1 1 1 1 1                       |
| PKDL        |                                          |
| DAT         | + 0 0 7 4 2 1 1                       |
|            | − 5 7 0 1 1 0 0                       |
| rK39 ELISA  | + 1 3 6 5 3 1 1                       |
|            | − 4 4 1 1 0 0 0                       |
| Subclinical infection |                                 |
| DAT         | + 0 0 12 7 5                           |
|            | − 11 0 3 2                            |
| rK39 ELISA  | + 3 12 8 6                            |
|            | − 5 0 2 1                            |
| LST positivity with DAT conversion |                                |
| DAT         | + 0 0 10 3 4                           |
|            | − 8 0 3 3                            |
| rK39 ELISA  | + 6 9 4 5                            |
|            | − 2 1 2 2                            |

* 0, time of diagnosis; other numbers refer to the number of months before (−) or after (+) diagnosis.
mained positive on three and four subsequent occasions, respectively. Two others were found positive in the first survey; one of these converted to negative subsequently, whereas the other remained positive. In those with repeated positive rK39 ELISA results, OD values were consistently low (mean OD, 0.17 ± 0.05).

Endemicity controls (n = 4; mean age, 4.6 [range, 3 to 6]). In the endemicity control group, one individual tested converted in the rK39 ELISA and remained positive for 2 years (OD average for five observations, 0.17), while his DAT results remained negative throughout.

Nonendemicity controls. All nonendemicity controls (n = 25) had negative rK39 ELISA results (mean OD, 0.05 ± 0.03).

For subjects found positive by rK39 ELISA, there was no statistically significant difference (P = 0.32) in mean OD values between the first four clinical categories; the mean ODs of both control groups were found to differ significantly from those of the four clinical groups (P < 0.05).

DISCUSSION

Infection with L. donovani in the Sudan may cause a variety of clinical conditions that differ in morbidity and management. Differentiation between these conditions is important for our understanding of the epidemiology and the development of control strategies. Serological tests may be useful for differentiating between these clinical conditions. Unlike the antigens in currently used serological tests, rK39 antigen is specific for members of the L. donovani complex (2), and, as it occurs predominantly on amastigotes, it reflects infection by L. donovani followed by active parasite replication.

In the diagnosis of kala-azar, rK39 ELISA showed higher sensitivity than the DAT (93 versus 80%). rK39 ELISA titers remain positive for up to 2 years after treatment; no difference in trend could be detected in the declines of OD values and DAT titers in this (limited) study. Thus, rK39 ELISA does not allow discrimination between active infection and past infection.

rK39 ELISA and DAT were both sensitive and specific for diagnosis of PKDL. The PKDL subjects in this study developed the rash following kala-azar that became clinically apparent after the previous survey 6 months before, when neither clinical signs nor the DAT alerted us to possible ongoing infection. However, for three of these patients, rK39 ELISA was already positive at that time point, indicating that infection had already taken place. It is unclear whether these patients have a more protracted, milder disease or have a higher risk of developing PKDL than those who develop overt kala-azar shortly after becoming infected. These findings should be substantiated in larger studies.

A similar capability to detect infection earlier was shown for subjects with subclinical infection who were diagnosed by seroconversion. Almost half of these individuals had already converted in rK39 ELISA 6 months before, showing that rK39 ELISA is superior to DAT in detecting infection at an earlier stage. Our findings are in contrast to a study in Brazil in which antibodies to rK39 were absent in children who had subclinical disease and self-healed without specific therapy (1). Our findings indicate that K39-positive individuals may self-heal or proceed to clinical disease.

Interestingly, conversion in DAT and rK39 ELISA also occurred in people who are LST positive and who are considered to have immunity, as shown by their skin test, as a result of previous infection with Leishmania. The majority of this group are adults (mean age, 35 years) with previous exposure to L. major in their homeland. Although L. major infection may protect from disease (10), these LST-positive individuals had rK39 reactivity and thus active parasite replication. K39 is abundant in L. donovani complex strains and scarce in L. major, which may indicate that this reactivity is in fact caused by L. donovani. This is consistent with our 5-year observation that no CL occurs in the area. A member of this group, however, has as much exposure as any other individual in the same area to sand fly bites and infection with L. donovani. Our data show that although no clinical disease develops, infection actually occurs, as shown in the conversion in DAT and rK39 ELISA. Moreover, infection is followed by active parasite replication, as rK39 is an amastigote antigen. OD values were not found to differ from those of subjects with kala-azar, PKDL, and subclinical infection. Apparently, they are protected by cell-mediated immunity, as shown by the positive LST results, from developing overt disease.

Also, in the LST-positive group without DAT conversion, which was randomly selected because clinical and serological observations did not point to current infection, some individuals had (conversion to) positive rK39 ELISA results, albeit with lower OD values than in the four clinical groups. Possibly, these people have low-grade infection that could not be detected by the DAT. This shows, in addition to what was found in the other study groups, that infection and parasite replication as evidenced by rK39 ELISA may be much more frequent than suspected on the basis of the use of the DAT only. The reservoir of individuals in a community harboring Leishmania parasites for a certain period may therefore be much greater than previously assumed, which may have implications for transmission dynamics. This study shows that our understanding of infection characteristics and clinical conditions caused by L. donovani is limited by the diagnostic tools that are available.

rK39 ELISA is a sensitive test for detection of early infection and an important improvement in our diagnostic potential. However, as with the DAT, a positive test result may point to a current (sub)clinical infection or may stem from earlier exposure and should be interpreted with caution.

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