rK39: A Cloned Antigen of Leishmania chagasi that Predicts Active Visceral Leishmaniasis


The diagnosis of visceral leishmaniasis (VL), a serious and often fatal parasitic disease caused by members of the Leishmania donovani complex, remains problematic. Current methods rely on clinical criteria, parasite identification in aspirate material, and serology. The latter methods use crude antigen preparations lacking in specificity. A previously described cloned antigen, rK39, of Leishmania specific for all members of the L. donovani complex (L. chagasi, L. donovani, L. infantum) was very useful in the serodiagnosis by ELISA of both human and canine VL. The present study demonstrated that rK39 seroreactivity correlated with active disease. The sera from early or self-healing infected subjects reacted with leishmanial lysate and were generally nonreactive with rK39. These data demonstrate the utility of rK39 in the serodiagnosis of VL and as an indicator of active disease.

Visceral leishmaniasis (VL) is a widely distributed disease with high morbidity and mortality [1]. In areas in which it is endemic, its prevalence has been recorded at 1–10 per 1000 persons yearly [1–3]. The tools available for the diagnosis of VL include visualization of the parasite in bone marrow or splenic aspiration [4], demonstration of specific antibodies in sera of infected subjects [5–11], and isolation of the parasite by in vitro culture or by hamster inoculation [1]. However, none of these procedures is sensitive enough for identifying all infected subjects within the spectrum of leishmanial infection, which ranges from asymptomatic to acute. Current serologic tests using crude antigen preparations are severely limited in terms of both specificity and assay reproducibility [5, 7, 9].

Patients with asymptomatic or subclinical (or both) VL usually have relatively low antibody titers that fall into the gray zone, in which cross-reactivity is very high in serologic tests that use whole parasites (e.g., immunofluorescent antigen test, IFAT) or parasite lysate [10]. In addition, the demonstration of parasites in these patient groups is extremely difficult [10, 11].

Recently we reported the cloning of an antigen gene coding for a repeat antigen (rK39) of Leishmania chagasi and evaluation of that antigen in detecting specific antileishmanial antibody in sera from patients with VL [12]. The initial characterization revealed that this rK39 was highly sensitive and specific for VL. Here we report the sensitivity and specificity of this rK39 in comparison with crude leishmanial lysate antigen for the detection of specific antileishmanial antibody and hoped to show that seroreactivity to rK39 is an early surrogate marker for disease progression in VL.

Materials and Methods

Study population. Sera from 2162 persons were tested using a Falcon assay screening test (FAST)–ELISA (Becton Dickinson,
Mountain View, CA) to detect specific antileishmanial antibodies from the following groups, defined according to the visceral leishmaniasis spectrum: Acute VL was diagnosed when the patient had fever, hepatosplenomegaly, anemia, and leukopenia and when a bone marrow or splenic aspirate revealed leishmanial amastigotes in Giemsa-stained smears [3].

Children with subclinical VL were characterized by mild constitutional symptoms such as malaise, diarrhea, cough, poor work-play tolerance, and intermittent hepatomegaly. These patients typically have specific circulating antileishmanial antibodies, and frequently the bone marrow aspirate direct smear is negative [10]. Liver biopsy may demonstrate histologic abnormalities and leishmanial amastigotes [11]. In general, 60%–75% of patients with subclinical VL will resolve their illness after a prolonged period (mean, 35 months) without specific therapy. These are grouped in a category named subclinical self-healing. The remaining subclinical patients, initially indistinguishable from the self-healing group, will progress to full blown kala-azar syndrome in 5–12 months. These patients are grouped in a category known as subclinical progressing to full blown VL.

Asymptomatic L. chagasi infection represents a group of subjects with positive antileishmanial serology, yet who remain asymptomatic. The majority of these patients (80%) have a strong positive delayed hypersensitivity to leishmanial antigens for 1–3 years following seroconversion [3].

Acute VL. During 1987–1993, 135 sera from patients with clinically and parasitologically confirmed VL were collected and stored at –20°C. All sera were obtained before the initiation of specific antimonial therapy.

Subclinical VL. During the last 10 years, >100 cases of subclinical VL patients were identified in the town of Jacobina, Brazil, by an annual epidemiologic survey for case detection. The index sera from 45 patients were selected for this study because the patients had been carefully followed over the previous 10 years. Fifteen of them progressed from subclinical VL to acute VL (subclinical progressing to VL group) within 12 months of serologic evaluation. The remaining 30 subclinical cases self-healed during the follow-up observation period (subclinical self-healing group).

Asymptomatic L. chagasi infections. One hundred sera of asymptomatic seropositive persons were evaluated for this study. None of these persons had developed clinical signs or symptoms of VL over a 10-year period.

Epidemiologic surveys. In total, 1125 human sera from the population surveyed in Jacobina were retested by ELISA using both leishmanial lysate and rK39. From all subjects, we routinely obtained basic demographic data, epidemiologic information about leishmaniasis and other tropical diseases, and physical examination for hepatosplenomegaly.

Other diseases. One hundred sera were analyzed from human clinically and parasitologically confirmed diseases including cutaneous leishmaniasis, Chagas’ disease, and schistosomiasis (Schistosoma mansoni) plus bacteriologically confirmed tuberculosis and leprosy. All sera were obtained from the Infectious Disease Research Unit at Hospital Universitário Professor Edgard Santos (Bahia, Brazil).

Healthy subjects. One hundred sera from persons living in areas of Bahia where leishmaniasis and Chagas’ disease are not endemic were also evaluated.

Canine leishmaniasis. A prospective study of the epidemiology of canine VL was initiated in 1989 in Jacobina [13]. The dogs (300–500/year) have been evaluated annually for the presence of L. chagasi infection. During this time, 90 dogs were found to have acute VL, with high serologic titers of antileishmanial antibodies and demonstration of leishmaniae in liver, bone marrow, or splenic aspirates. The sera were collected and stored before the dogs were sacrificed by the Brazilian National Leishmaniasis Control Program.

In December 1992, dogs were serologically surveyed in Monte Gordo, 80 km from the capital city of Salvador (Bahia, Brazil). The survey was done as part of an evaluation of an outbreak of human VL identified during the previous 5 years [14]. In a single day, blood was collected from 467 dogs in this small village. Serologic evaluation was done simultaneously with both antigens (lysate and rK39). All dogs were held at home until the serologic results were known. After the results were available, the serologically positive dogs were sacrificed and negatives were released. From each sacrificed dog, an imprint was prepared from spleen tissue for direct examination for Leishmania organisms.

Serology was done using both leishmanial promastigote lysate and rK39. Leishmanial promastigote lysate from a clone of L. chagasi (MHOM/BR/82/Ba-2) was prepared as described [15]. rK39 was the 298-amino-acid sequence with a predicted molecular mass of 32.7 kDa and an isoelectric point of 4.4. rK39 contained an additional 6.2 kDa of plasmic fusion sequences that we recently cloned from the L. chagasi clone as described [12].

FAST-ELISA. Microassay plates (Probind; Falcon, Becton Dickinson, Mountain View, CA) or the lid with beads were sensitized overnight at 4°C with rK39 (50 ng/well) or promastigote lysate (1–2 μg/bead-well), followed by blocking with PBS containing 1% Tween 20 for 1 h at room temperature. The assays were done as described [13].

Statistical analysis. Confidence intervals (95%) were calculated using the standard normal distribution formula for proportions. The P values stated for comparisons regarding sensitivity are two-sided and are derived from the Mc Nemar test.

Results

Figure 1 presents ELISA results obtained with a panel of 425 sera from the study populations. The numbers of sera falling within different ranges of absorbance values are shown according to their reactivities with lysate and rK39. The rK39 antigen gave significantly higher absorbance values between the two antigens tested (P < .05). In addition, rK39 had no cross-reactivity with sera from other parasitic diseases, while the crude lysate had a specificity of 68%. Most of the cross-reactive sera were from subjects with cutaneous leishmaniasis or Chagas’ disease. False-positive reactions did not occur with sera from healthy controls from areas without endemic leishmaniasis or Chagas’ disease with any of the antigens tested.

The sensitivities of the two antigen preparations for detecting specific antileishmanial antibodies were compared (table 1). Both lysate and rK39 were highly sensitive (98% and 99%, respectively) using human or dog VL sera. Seroreactivity to lysate was observed in all infection categories, including sub-
clinical and asymptomatic, as this is a criterion for characterizing leishmanial infection. However, none of the sera from the subclinical self-healing patients and only 4 of 100 sera from asymptomatic children had antibodies to rK39. On the other hand, rK39 detected antibodies in 13 (87%) of 15 index sera from children with subclinical VL who progressed to full-blown disease within 5–10 months.

We also evaluated the specificities of the antigen preparations using sera from healthy controls and from subjects infected with other tropical diseases (figure 1). The rK39 antigen was very specific (100%), with no cross-reactivity among 100 sera from persons with tropical diseases other than VL or 100 healthy subjects. In contrast, crude lysate had high rates of positive reactions with sera from patients with cutaneous leishmaniasis (42%, n = 10), Chagas’ disease (50%, n = 20), tuberculosis (30%, n = 20), leprosy (20%, n = 20), and schistosomiasis (20%, n = 20). Therefore, the positive predictive values of a serologic test correlating with human or dog acute VL were as follows: lysate, 64%, and rK39, 98%.

Epidemiologic survey. To determine the ability of rK39 to detect acute VL, we retrospectively analyzed human sera collected in a cross-sectional survey in the leishmania-endemic area of Jacobina. Among 1125 sera screened with the crude lysate and rK39, 110 (9.8%) were positive with crude lysate, and 11 (1%) were serologically positive with rK39. The rK39-positive sera were among the 110 lysate-positive samples. Follow-up of these 110 persons revealed that, among the 11 rK39-positive sera, 3 were from acute VL and the other 8 were from subclinical cases treated earlier with pentavalent antimony before progressing to the full-blown VL, as we previously recommended [3]. The other 99 lysate-positive sera were from asymptomatic persons who did not develop acute VL.

We also confirmed rK39 seropositivity and presence of parasites in the canine cross-sectional survey. Following the National Visceral Leishmaniasis Control Program, 467 dogs were
surveyed in a new VL focus (Monte Gordo). Specific antileishmanial antibodies were detected by FAST-ELISA using both lysate and rK39 as the solid phase. Fifty-four dog sera were positive using lysate; of these, 33 were also positive with rK39. These 33 rK39-positive dogs were sacrificed, and leishmania were found in the lymphoid tissues of all of them on direct smear examination.

Discussion

In this study, we confirmed the highly specific nature of rK39 to detect antileishmanial antibodies in acute VL sera (99% sensitivity and 100% specificity). In particular, we discovered that during the acute phase of disease, the host may produce specific antibodies against replicating leishmania, suggested by the observation that sera from patients and dogs with acute VL strongly recognized rK39, but patients with asymptomatic or self-healing infections had low or undetectable levels of anti-rK39 antibodies. On the other hand, in patients with subclinical infections who progressed to full-blown acute VL, antibodies against rK39 were detected a few months before the disease became evident. Therefore, the presence of antibodies to rK39 was 100% correlated with VL. Such a differentiation between asymptomatic and acute VL was not seen using the crude lysate as antigen.

Several methods of serodiagnosis of VL have been used to detect antileishmanial antibodies, including IFAT [5], direct agglutination [6], and ELISA [7, 9]. These tests all use whole promastigotes or lysates thereof. The use of crude parasite preparations for serologic tests presents the problem of cross-reactivity with antibodies from other diseases. Although such conditions may be distinguished clinically, past or subclinical infections with agents of these diseases can complicate diagnosis. In addition, serologic cross-reactivity between leishmania and other infectious agents, such as trypanosomes, bacteria [5–8], is a well-documented problem. These problems can be avoided in part by modifications of the assay used, as we have done [7, 15]. However, the best way to increase the specificity of a serologic assay is to use defined antigens. Because high levels of anti-K39 antibodies occur in acute VL, an improved detection system was developed. ELISA wells coated with rK39 have high epitope density compared with lysate-coated wells, resulting in a stronger signal with positive sera.

We have previously described the use of rK39, a potent amastigote antigen shared by members of the Leishmania donovani complex [12]. Use of rK39 in serologic assays increases the specificity of the ELISA for VL while retaining sensitivity. The occurrence of rK39 predominantly on amastigotes (the form that replicates and is responsible for pathology) and not in promastigotes (the form transmitted by sand flies) probably explains the high titers of anti-K39 antibody in patients with acute VL and relatively lower titers in those infected but without disease. Such persons, who are without clinical signs or symptoms, have lower numbers of replicating amastigotes in their lymphoid tissue than do patients with acute disease. We believe this to be the most likely reason that rK39 antibodies are not detected in infected persons without disease.

Our data show that rK39 is a serologic indicator of disease from L. chagasi infection. Serologic reactivity to rK39 accompanies acute disease and, as we have shown here, also exists in subclinical cases that progress to VL, preceding disease signs or symptoms. Since application of specific chemotherapy before the development of acute symptomatology can significantly improve prognosis, rK39 will prove to be a powerful tool for the management of VL as well as a valuable diagnostic antigen.

References