Evaluation of Enzyme-Linked Immunosorbent Assay for Diagnosis of Post-Kala-Azar Dermal Leishmaniasis with Crude or Recombinant k39 Antigen

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The diagnosis of post-kala-azar dermal leishmaniasis (PKDL), a dermatosis that provides the only known reservoir for the parasite Leishmania donovani in India, remains a problem. Timely recognition and treatment of PKDL would contribute significantly to the control of kala-azar. We evaluated here the potential of the enzyme-linked immunosorbent assay (ELISA) as a diagnostic tool for PKDL. Antigen prepared from promastigotes and axenic amastigotes with parasite isolates that were derived from skin lesions of a PKDL patient gave sensitivities of 86.36 and 92%, respectively, in the 88 PKDL cases examined. The specificity of the ELISA test was examined by testing groups of patients with other skin disorders (leprosy and vitiligo) or coendemic infections (malaria and tuberculosis), as well as healthy controls from areas where this disease is endemic or is not endemic. A false-positive reaction was obtained in 14 of 144 (9.8%) of the controls with the promastigote antigen and in 14 of 145 (9.7%) of the controls with the amastigote antigen. Evaluation of the serodiagnostic potential of recombinant k39 by ELISA revealed a higher sensitivity (94.5%) and specificity (93.7%) compared to the other two antigens used. The data demonstrate that ELISA with crude or recombinant antigen k39 provides a relatively simple and less-invasive test for the reliable diagnosis of PKDL.

Materials and Methods

Patients. Blood samples were collected by venipuncture for sera from individuals in the following clinical categories.

PKDL. A group of 88 patients from Bihar, where PKDL is endemic, and reporting to Safdarjung Hospital, New Delhi, India, over a period of 4 years were included in this category. PKDL was diagnosed clinically and confirmed by the demonstration of parasites in skin lesions or by histopathologic findings (18). All patients included in this category were found to respond to therapy with sodium antimony gluconate.

Tuberculosis and Malaria. A total of 22 patients with confirmed pulmonary tuberculosis and another 19 with malaria (peripheral blood smear positive) were included in this group.

Leprosy and Vitiligo. This group included 30 patients confirmed to have lepromatous leprosy and 20 vitiligo patients (confirmed by histopathology) who reported to the Department of Dermatology, Safdarjung Hospital.

Healthy Controls. Healthy controls (n = 32) were subjects living in Delhi, India, an area where KA is not endemic.

Endemic Controls. “Endemic controls” (n = 22) were the first-degree healthy relatives of patients living in Muzafarpur, Bihar, an area known for its KA endemicity.

Parasite Cultures. Parasites isolated from lesions of PKDL patients propagated as promastigotes in M199 supplemented with 25 mM HEPES (pH 7.5) and 10% fetal calf serum as described earlier (19). Axenically grown amastigotes were cultured by the gradual adaptation of promastigotes to growth at pH 5.5 and 37°C in a 6 to 7% CO2 atmosphere as described by Joshi et al. (11).
Antigens. rk39, prepared as described previously (4), was a kind gift from Steve Reed, Corixa Corp., Seattle, Wash. Promastigotes and amastigotes of L. donovani isolated from the dermal lesions of a PKDL patient. Sera were used in a 1:200 dilution with rk39 and a 1:100 dilution for promastigote and amastigote antigens in all of the samples. The mean OD values at 492 nm as determined by ELISA for patient and control sera were plotted.

RESULTS

A total of 263 serum samples were collected and tested by ELISA using three different antigens: the promastigote antigen, the amastigote antigen, and the rk39 antigen. Initially, we compared results with antigen derived from promastigotes and amastigotes of L. donovani isolated from PKDL lesions as described above were harvested in late log phase and lysed in buffer containing 3% sodium dodecyl sulfate, 50 mM Tris-Cl (pH 7.0), and 1 mM phenylmethylsulfonyl fluoride. The amount of protein in the lysate was estimated by using a DC Protein Assay Kit (Bio-Rad).

Serum samples were tested by ELISA according to a standard method described elsewhere (28). In brief, polystyrene 96-well microtiter plates (Corning, N.Y.) were coated overnight with 10 ng of rk39 or 200 ng of promastigote or amastigote antigen in 100 μl of 0.1 M bicarbonate buffer (pH 9.0). The plates were blocked with 5% nonfat milk for 1 h at 37°C washed three times with phosphate-buffered saline (PBS)-Tween 20, and incubated for 2 h with 100 μl of patient serum at various dilutions as specified. Wells were washed three times with the same buffer and incubated with 100 μl of goat anti-human IgG conjugated with horseradish peroxidase for 2 h. This step was followed by three rinses with PBS-Tween 20 and the addition of ortho-phenylenediamine substrate with hydrogen peroxide. The optical density (OD) of each well was measured at 492 nm in an ELISA reader (Titertek Multiskan Plus). Each sample was assayed in triplicate or more, along with appropriate controls. The ELISA reader was set to subtract the reading of a blank control from the test samples. The cutoff value was made among PKDL patients. The mean OD was found to be statistically significant (P < 0.05) only with the crude antigens and not with the rk39 antigen.

As seen in Table 1 the ELISAs sensitivities for the detection of PKDL cases were 84 of 88 (94.5%), 81 of 88 (92%), and 76 of 88 (86.36%), respectively, for the rk39, amastigote, and promastigote antigens. Among the controls, the use of rk39 led to a correct diagnosis in 135 of 144 (93.7%) cases. The use of amastigote and promastigote antigens gave correct diagnoses in 131 of 145 (90.3%) and 130 of 144 (90.2%) of the control samples. The estimated sensitivity of the ELISA test at a 95% confidence interval ranged from 98.7 to 92.5% for the rk39 antigen and from 96.1 to 88% and from 91.3 to 83.7% for the amastigote and promastigote antigens, respectively.

Analysis of the mean OD as a function of a history of KA was made among PKDL patients. The mean OD was found to be independent of a history of KA (Table 2). A majority (62 of 88) of the PKDL patients had recovered from KA more than 5 years previously, whereas 9 of 88 were not aware of any history of KA.
DISCUSSION

In India, PKDL occurs in 10 to 20% of KA cases months to as many as 35 years after patients are cured of KA. This is quite different from the situation with PKDL in Sudan, since PKDL occurs in >50% of cured KA cases usually weeks or months after recovery from the visceral disease (30). Diagnosis of PKDL is a problem since it is often confused with other dermatological conditions such as leprosy. It is important to identify and treat PKDL patients, since they constitute the only known reservoir for L. donovani in India (27). We recently described a sensitive and species-specific PCR assay for the diagnosis of PKDL (21); however, PCR is expensive and requires sophisticated facilities and trained personnel. Each of the ELISA tests described in the present study, although not as sensitive or as specific as a PCR assay, would provide a more economical and practical assay for the diagnosis of PKDL.

The serodiagnostic potential of rk39 for VL has been shown with subjects from various parts of the world, including the Indian subcontinent, Brazil, and Sudan, establishing conservation of the k39 epitope among visceralizing species of Leishmania (2, 16, 22, 29). In a study with a limited number of PKDL patients in Sudan, an rk39 ELISA was found to be sensitive and specific as a diagnostic test (29). In the present study we used a large number of serum samples to demonstrate that PKDL patients in India have high titers of anti-k39 IgG and that ELISA with rk39 antigen provides a highly sensitive (95.45%) and specific (93.5%) tool for diagnosing PKDL.

Several studies have shown serological tests to be useful in the diagnosis of KA; however, methods for the diagnosis of KA often lack sensitivity for the diagnosis of PKDL. In order to improve the sensitivity of the ELISA, we used promastigote antigen prepared with parasite isolates from dermal lesions of a PKDL patient. The indigenous PKDL antigen was found to give generally higher titers than those obtained with the reference strain (AG83), which is similar to the observation made during a direct agglutination test for PKDL in Sudan (9). Since Leishmania parasites are present in the amastigote form in the human host, the humoral immune response would be directed against antigens of the amastigote form. Antigen prepared from amastigotes has indeed been shown to be superior to the promastigote-derived antigen (25); however, amastigotes are generally difficult to isolate in large quantity and in pure form.

To overcome this problem, we set up an axenic amastigote culture from parasites isolated from dermal lesions and used it for antigen preparation. The amastigote antigen was found to give significantly higher absorbance values and better sensitivity compared to the promastigote antigen, although the specificity was similar in both the cases. Hence, the amastigote antigen is recommended for use when recombinant K39 is not available or affordable. Use of crude antigen is known to lead to false-positive results with samples of other diseases due to certain common antigenic epitopes. Sera from patients suffering from other common skin disorders in India, namely, leprosy and vitiligo, have been examined, since PKDL is most frequently misdiagnosed as one of these. Among this group use of the promastigote and amastigote antigens gave false-positive results in only 1 of 50 cases. False-positive results were more frequent in patients with malaria (2 of 19) and tuberculosis (4 of 22). The overall specificity of detection was ca. 90% with either promastigote or amastigote antigens.

The causative organism in PKDL has been characterized as L. donovani by methods such as isoenzyme typing (5), reaction with monoclonal antibodies (P. Salotra et al., unpublished data), and species-specific PCR (21). It has also been demonstrated that there are indeed molecular differences between PKDL isolates and KA isolates of L. donovani (7). Gene expression in the parasite is also expected to be different in PKDL since not only the site of infection but also the host humoral immune response is distinct compared to KA (20). The antibody titer to leishmanial antigens is known to be much lower in PKDL patients than in KA patients (8). We observed here high anti-k39 titers of up to $10^{-5}$ in PKDL patients in a large number of serum samples, indicating that k39 is abundantly expressed in parasites causing PKDL.

Antileishmanial antibody titers measured by direct agglutination test have been reported to remain positive for up to 5 years after recovery in >50% of VL patients examined (14). In our study only 17 of 88 (19.3%) patients had a history of KA of <5 years. The remaining 80.7% had either no history of KA or a history of KA exceeding 5 years for periods of even up to 15 years. Therefore, it would be reasonable to conclude that the antibodies detected in ELISA were largely due to PKDL and not those persisting due to a history of KA.

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