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.

Individuals infected with the protozoan parasite *Leishmania donovani* present with the clinical disease visceral leishmaniasis (VL) or kala-azar (KA), which is fatal if left untreated. The annual incidence and prevalence levels of VL are 0.5 and 2.5 million, respectively, of which 90% of cases occur in the Indian subcontinent and Sudan (3). Post-KA dermal leishmaniasis (PKDL) is a dermatotropic form of disease caused by *L. donovani* that develops as a sequel in 10 to 20% of VL cases in India and in >50% of VL cases in Sudan (18, 30). PKDL is characterized by hypopigmented macules and erythematous eruptions leading to the formation of papules and nodules (13, 18). In India, PKDL occurs several months to as many as 35 years after KA is cured and is considered to be the main reservoir for transmission of the visceral disease in the absence of a zoonotic host (1, 27).

Definitive diagnosis of PKDL by demonstration of *L. donovani* parasites (LD bodies) in skin biopsies has a sensitivity of only 58% (23), since parasites are scanty in the lesions. The disease is therefore often misdiagnosed as leprosy, a coendemic disease that resembles PKDL both clinically and pathologically (17). Serodiagnosis has been used as an important alternative for the diagnosis of KA, although its value is often limited for specificity and reproducibility when crude parasite antigen is used (6, 12, 24, 25). The use of recombinant k39 (rk39) has been shown to overcome these limitations to a considerable extent (2, 16, 22, 26, 29). Antileishmanial antibodies of the immunoglobulin G (IgG) and IgM classes have been demonstrated in the sera from PKDL patients (8, 20); however, limited studies have been conducted to develop serological methods for the diagnosis of PKDL (9). Increased sensitivity has been reported when the immunoperoxidase technique and PCR are used (10, 15, 21). We evaluate here the utility of the enzyme-linked immunosorbent assay (ELISA) in diagnosing PKDL with total antigen extract and rk39. Antigen extracts were prepared from indigenous parasites at two different developmental stages—promastigotes and amastigotes—isolated from PKDL lesions.

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.

KA. Thirty patients reporting to the Department of Medicine, Safdarjung Hospital, with fever and splenomegaly and parasitologically confirmed to have leishmania parasites in bone marrow aspirates were categorized as having KA.

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 Muzaffarpur, Bihar, an area known for its KA endemicity.

Parasite cultures. Parasites isolated from lesions of PKDL patients propagated as promastigotes in M99 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).
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 of reference strain AG83 (MHOM/IN/83/AG83) and those derived from dermal lesions and found that the latter gave higher absorbance values upon ELISA with PKDL sera (data not shown). Subsequently, the promastigotes and amastigotes used for antigen preparation were those cultured from parasites isolated from PKDL lesions. The test samples included 88 PKDL patients and 146 controls, along with 30 KA patients to serve as positive control samples. In PKDL samples, the mean OD was highest with rk39 antigen, even though the serum dilution was double (1:200) compared to that used for amastigote and promastigote antigens (1:100; Fig. 1). The titer for amastigote antigen in 100 Å of patient serum at various dilutions as specified is shown. Subsequently, the promastigotes and amastigotes used for antigen preparation were those cultured from parasites isolated from PKDL lesions as described above. 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 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 a cutoff value of 0.45 was used as a cutoff value in all cases. Ag, antigen.

**RESULTS**

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 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.

**TABLE 1. Comparison of the results of ELISA for the three PKDL cases grouped according to KA history**

<table>
<thead>
<tr>
<th>Group</th>
<th>KA history (yr)</th>
<th>No. of cases</th>
<th>ELISA result (Mean OD ± SD) with:</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>rk39 Ag</td>
</tr>
<tr>
<td>PKDL</td>
<td></td>
<td></td>
<td>1,306 ± 0.217</td>
</tr>
<tr>
<td>KA</td>
<td>&lt;5</td>
<td>17</td>
<td>1.087 ± 0.424</td>
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<tr>
<td></td>
<td>5–10</td>
<td>43</td>
<td>1.27 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>10–15</td>
<td>19</td>
<td>1.31 ± 0.324</td>
</tr>
<tr>
<td></td>
<td>&gt;15</td>
<td>9</td>
<td>1.306 ± 0.217</td>
</tr>
</tbody>
</table>

*Ag, antigen. 

**TABLE 2. ELISA values obtained with the three antigens in 88 PKDL cases grouped according to KA history**

<table>
<thead>
<tr>
<th>Group</th>
<th>KA history (yr)</th>
<th>No. of cases</th>
<th>ELISA result (Mean OD ± SD) with:</th>
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**FIG. 1. Comparison of ELISA with rk39 antigen and antigens derived from 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.**
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 lack sensitivity for the diagnosis of KA; however, methods for the diagnosis of KA are acknowledged.

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REFERENCES


