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Real-Time PCR Assay for Clinical Management of Human Immunodeficiency Virus-Infected Patients with Visceral Leishmaniasis

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To evaluate the usefulness of a real-time PCR for Leishmania DNA in the diagnosis and follow-up of patients with human immunodeficiency virus type 1 (HIV-1) and Leishmania coinfection, Leishmania DNA levels were measured in whole peripheral blood from 25 HIV-infected patients with clinical features suggestive of visceral leishmaniasis. Leishmania DNA was detected in 10 of 25 patients with microscopically confirmed visceral leishmaniasis and in none of those without this disease. Following treatment with liposomal amphotericin B, a clinical response was observed in 9 of 10 patients, in association with significantly decreased parasite loads. Seven patients relapsed clinically a median of 110 days after the end of treatment, in association with substantial increases in Leishmania DNA levels. Leishmania DNA levels correlated with the clinical course of visceral leishmaniasis, and their measurement at diagnosis and during and after treatment seems to be useful in the clinical management of HIV-infected patients with this disease.

Visceral leishmaniasis is a frequent and severe complication of advanced human immunodeficiency virus (HIV) infection in patients living in the Mediterranean basin. In this area, 25 to 75% of visceral leishmaniasis cases occur in HIV-infected patients, and 2 to 9% of HIV-infected patients acquire this disease (4, 11). Clinical manifestations include fever, hepatomegaly and/or splenomegaly, and pancytopenia. Bone marrow aspirate or biopsy followed by demonstration of Leishmania parasites by microscopic and/or cultural examination is the most common diagnostic procedure. Although microscopic examination of bone marrow aspirate has been reported to be 62 to 93% sensitive, it is an invasive procedure and the diagnostic yield may be low in HIV-infected patients, because of a hypoplastic bone marrow (7). Serology has limited diagnostic value in HIV-infected patients, because only 43 to 78% of leishmaniasis patients show detectable levels of anti-Leishmania antibodies (12, 14). Recently, PCR for Leishmania DNA in peripheral blood has been shown to be sensitive and specific for diagnosis and follow-up of patients with visceral leishmaniasis (2, 3, 8, 10, 13).

The response to antileishmanial treatment is lower in HIV-infected patients than in immunocompetent patients (10, 12, 14). HIV-infected patients show high rates of visceral leishmaniasis relapse (between 25 and 61%). Highly active antiretroviral therapy (HAART) has significantly reduced the incidence of visceral leishmaniasis (6, 9). However, both relapse and de novo cases of visceral leishmaniasis are commonly observed under HAART (5). Optimal treatment for visceral leishmaniasis should both target the pathogen by specific drugs and restore host immunity by the use of potent anti-HIV combinations. In order to monitor the response to this treatment strategy, markers of Leishmania infection are required, to be assessed in parallel with markers of HIV infection. In this study, a quantitative real-time PCR assay was used to measure Leishmania DNA levels in peripheral blood both at the time of diagnosis and during follow-up, in order to establish the value of this method for monitoring of HIV-Leishmania coinfection.

MATERIALS AND METHODS

Patients. Twenty-five HIV-infected patients with clinical features suggestive of visceral leishmaniasis underwent bone marrow aspiration. Visceral leishmaniasis was diagnosed in 10 of 25 patients (40%) by demonstration of Leishmania amastigotes in Giemsa-stained bone marrow smears. A first diagnosis was made for six patients, while four patients had a relapse. Leukopenia was observed in all of the patients (median, 2,200 cells/mm3; range, 1,300 to 3,200); fever, splenomegaly or hepatomegaly, and anemia (8.9 g/dl; range, 7.8 to 10.9) each were observed in nine patients; and thrombocytopenia was observed in eight patients (64,000 cells/mm3). CD4 cell counts, HIV type 1 (HIV-1) RNA levels, and anti-Leishmania antibody titers at the time of diagnosis are shown in Table 1. No other opportunistic disorders were identified in these patients. Of the 15 remaining patients, 3 had non-Hodgkin lymphoma, 1 had atypical mycobacteriosis, and 11 had no opportunistic and/or neoplastic diseases identified. None of these 15 patients had anti-Leishmania antibodies. Their mean CD4+ cell count and HIV RNA load were 107 cells/ml (range, 48 to 367) and 5.02 log copies/ml (range, 3.14 to 6.11), respectively.

Treatment. Following diagnosis, all of the patients received liposomal amphotericin B (Ambisome; Gilead, San Diego, Calif.) on days 1 to 5 and once weekly thereafter, at a dosage of 3 mg/kg of body weight, except for one patient (patient 10 [Table 1]) who received 1.5 mg/kg because of renal failure. One patient (patient 4 [Table 1]) refused to continue the treatment after day 10. For the other patients, treatment duration was established by the physician caring for the patient, based on clinical response, and varied between 17 and 66 days. Following the end of therapy, five patients received secondary prophylaxis, consisting of...
At the end of therapy, patients, except for patient 4, who stopped treatment at day 10, decreased significantly from the baseline (median, 14 versus 0.63 (66) 3 755 parasites/ml, P = 0.004). During subsequent liposomal amphotericin B administration on days 1 to 5, parasite levels decreased significantly from the baseline, with a median of 1,610 parasites/ml at day 10 (range, 110 to 41,000) (Table 1), and in none of the 15 patients without this disease. Both the diagnostic sensitivity and the specificity of the method were estimated to be 0.625 parasites/ml. The statistical significance of the changes was assessed using the Mann-Whitney and paired sign tests. A clinical response was observed in all of the 25 HIV-infected patients with clinical features suggestive of visceral leishmaniasis. For patients with visceral leishmaniasis, samples were also drawn. During secondary prophylaxis, and at the time of relapse. Informed consent was always obtained before samples were drawn. A clinical response was observed in all of the 25 HIV-infected patients with clinical features suggestive of visceral leishmaniasis. For patients with visceral leishmaniasis, samples were also drawn. Informed consent was always obtained before samples were drawn.

## RESULTS

### Lactemania DNA load at the time of diagnosis of visceral leishmaniasis

Lactemania DNA was found in all of the 10 patients with visceral leishmaniasis. Lactemania DNA was found in all of the 10 patients with visceral leishmaniasis. Lactemania DNA was found in all of the 10 patients with visceral leishmaniasis. Lactemania DNA was found in all of the 10 patients with visceral leishmaniasis. Lactemania DNA was found in all of the 10 patients with visceral leishmaniasis. Lactemania DNA was found in all of the 10 patients with visceral leishmaniasis.

### Table 1: Clinical and laboratory parameters of HIV-infected patients with visceral leishmaniasis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Risk factor</th>
<th>CD4 cell count (cell/μL)</th>
<th>HIV RNA viral load (log copies/ml)</th>
<th>Anti-Leishmania antibodies</th>
<th>HAART</th>
<th>DNA load (log copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>M</td>
<td>IDU</td>
<td>450</td>
<td>4.5</td>
<td>negative</td>
<td>therapy</td>
<td>3.7</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>F</td>
<td>Hetero</td>
<td>400</td>
<td>3.5</td>
<td>negative</td>
<td>therapy</td>
<td>3.2</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>M</td>
<td>Hetero</td>
<td>350</td>
<td>3.0</td>
<td>negative</td>
<td>therapy</td>
<td>2.8</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>M</td>
<td>Hetero</td>
<td>300</td>
<td>2.5</td>
<td>negative</td>
<td>therapy</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>M</td>
<td>Hetero</td>
<td>250</td>
<td>2.0</td>
<td>negative</td>
<td>therapy</td>
<td>1.9</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>M</td>
<td>Hetero</td>
<td>200</td>
<td>1.5</td>
<td>negative</td>
<td>therapy</td>
<td>1.4</td>
</tr>
<tr>
<td>7</td>
<td>55</td>
<td>M</td>
<td>Hetero</td>
<td>150</td>
<td>1.0</td>
<td>negative</td>
<td>therapy</td>
<td>1.0</td>
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<tr>
<td>8</td>
<td>60</td>
<td>M</td>
<td>Hetero</td>
<td>100</td>
<td>0.5</td>
<td>negative</td>
<td>therapy</td>
<td>0.6</td>
</tr>
<tr>
<td>9</td>
<td>65</td>
<td>M</td>
<td>Hetero</td>
<td>50</td>
<td>0.0</td>
<td>negative</td>
<td>therapy</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>M</td>
<td>Hetero</td>
<td>0</td>
<td>-1.0</td>
<td>negative</td>
<td>therapy</td>
<td>0.0</td>
</tr>
</tbody>
</table>

### Table 2: DNA load (titer) during treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>DNA load (titer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1024</td>
</tr>
<tr>
<td>2</td>
<td>1:4096</td>
</tr>
<tr>
<td>3</td>
<td>1:16384</td>
</tr>
<tr>
<td>4</td>
<td>1:65536</td>
</tr>
<tr>
<td>5</td>
<td>1:262144</td>
</tr>
<tr>
<td>6</td>
<td>1:1048576</td>
</tr>
<tr>
<td>7</td>
<td>1:4194304</td>
</tr>
<tr>
<td>8</td>
<td>1:16777216</td>
</tr>
<tr>
<td>9</td>
<td>1:67108864</td>
</tr>
<tr>
<td>10</td>
<td>1:268435456</td>
</tr>
</tbody>
</table>

The number of days from the end of treatment is given in parentheses. ND, not done. A clinical response was observed in all of the 25 HIV-infected patients with clinical features suggestive of visceral leishmaniasis. For patients with visceral leishmaniasis, samples were also drawn. Informed consent was always obtained before samples were drawn.
FIG. 1. (A) *Leishmania* DNA levels in peripheral blood of patients with visceral leishmaniasis in the course of treatment. Blood samples were drawn prior to weekly drug administration. The horizontal line indicates the detection limit of the PCR assay. Dashed horizontal bars indicate median parasite levels. (B) *Leishmania* DNA levels in three patients (patient 6, patient 7, and patient 8 according to the numbering in Table 1) with visceral leishmaniasis receiving secondary prophylaxis. Blood samples were drawn prior to monthly drug administration. Open symbols indicate the time of clinical relapse.
**Leishmania** DNA load during posttreatment follow-up and clinical relapse. Following treatment, five patients received secondary prophylaxis (Table 1). The patient with undetectable *Leishmania* DNA in blood after therapy (patient 10) discontinued prophylaxis after 3 months, never relapsed, and had no detectable *Leishmania* DNA for 5 years of follow-up. One patient (patient 9) was clinically stable at the last follow-up after 2 months of prophylaxis, with decreased *Leishmania* DNA levels (median, 5 parasites/ml). Three patients had a clinical relapse of visceral leishmaniasis a median of 268 days after the end of treatment. Their *Leishmania* DNA levels were low at the end of treatment (median, 3 parasites/ml) but increased progressively during prophylaxis, reaching a median of 364 parasites/ml at the time of clinical relapse (Table 1; Fig. 1B). In these patients, an increase in *Leishmania* load above 10 parasites/ml preceded clinical relapse by 50 to 120 days (Fig. 1B).

Four patients did not receive secondary prophylaxis. All of them relapsed clinically a median of 78 days after the end of the treatment, with a median of 365 parasites/ml (Table 1). Blood samples from the time between the end of treatment and relapse were not available. Patient 4, who refused to continue treatment after day 10, died 250 days after the diagnosis of leishmaniasis.

Upon HAART, two of seven patients showed a virological response (Table 1). For one patient (patient 10), the CD4 count increased to >500 cells/μL, *Leishmania* DNA remained undetectable, and there was no relapse. The CD4 cell count for the other patient (patient 8) remained below 100 cells/μL, the *Leishmania* DNA load increased progressively, and the patient relapsed. No significant response to HAART was observed in the six remaining patients.

**DISCUSSION**

*Leishmania* DNA was found in all the patients with leishmaniasis confirmed by microscopic examination of bone marrow aspirate and in none of the patients without this disease. Despite the low number of patients examined in this study, this quantitative PCR proved to be a reliable and noninvasive tool for diagnosis of visceral leishmaniasis in HIV-infected patients. Because of its high diagnostic sensitivity, this assay is also likely to aid in the diagnosis of visceral leishmaniasis in patients without HIV infection, in whom the yield of parasites may be low. Our findings confirm those obtained in previous studies with regard to the diagnostic value of PCR (2, 3, 8, 10, 13). In addition, the potential ability of the real-time PCR to accurately measure the circulating parasite load provided a valuable tool for disease control after diagnosis.

*Leishmania* DNA loads differed widely among patients. However, they were not correlated with clinical outcome or with other parameters of HIV or *Leishmania* infection. Following treatment with liposomal amphotericin B, a clinical response was observed in all of the patients. However, *Leishmania* DNA was cleared from peripheral blood in only one case. In the remaining patients, including those who received a long treatment course, PCR still revealed the presence of low levels of circulating parasite DNA. Actually, clinical relapse was observed in seven out of eight patients harboring *Leishmania* DNA in blood posttreatment, but not in the patient whose blood became PCR negative. These observations suggest that the persistence of parasites in the blood after treatment is associated with a high risk of relapse, irrespective of clinical response.

Secondary prophylaxis seemed to protect only partially against relapse. Although no difference in parasite burden was found at the time of relapse between patients who had received prophylaxis and those who had not, the latter relapsed after a shorter time than the former. However, two of four patients who did not receive prophylaxis were not on HAART, and this might have affected the clinical outcome.

It has been suggested that visceral leishmaniasis accelerates HIV replication and impairs the patient condition’s by further immunosuppression (1). In our study, HAART was administered together with specific antileishmanial therapy to seven patients. However, most of the patients showed no significant virological and/or immunological response. This might have resulted from poor adherence to HAART, but also from a synergistic immunosuppressive effect of *Leishmania* and HIV. On the other hand, the only patient who did not relapse and in whom no parasite DNA was detected showed an optimal response to HAART, in terms of both a decrease in the viral load and an increase in the number of CD4+ cells. Another patient relapsed despite a virological response to HAART, but his CD4 cell count never increased above 100 per μL. These observations support the idea that a sustained immunological response rather than a virological response is essential to cure visceral leishmaniasis in HIV-infected patients.

In conclusion, real-time PCR seems to be a reliable, rapid, and noninvasive method for the diagnosis of visceral leishmaniasis. Treatment with liposomal amphotericin B was associated with clearance or decrease of *Leishmania* DNA levels. Following the end of treatment, parasite DNA remained detectable in all patients who relapsed, including patients receiving both secondary prophylaxis and HAART. Furthermore, clinical relapse was preceded by a substantial elevation of parasite levels. Therefore, real-time PCR might also be of value in monitoring the response to antileishmanial treatment and as an early, preclinical, prognostic marker of relapse.

**REFERENCES**

ERRATUM

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Vol. 41, no. 11, p. 5080–5084, 2003. Page 5081, column 1, lines 19 to 21: “5′-AAGGTCAAAAGACAGGCCTCCG-3′ (LEIF-forward), 5′-GCATCGGAGTCGG-3′ (LEIR-reverse), and 5′-AGGAGCGTGTGCCCTTCCGAG-3′ (LEIP-probe)” should read “5′-TAGACCGCACCAAGACGAACTA-3′ (LEIF-forward), 5′-CTAATCATCTTCGATCTCCACACTTT-3′ (LEIR-reverse), and 5′-AGCGAAGGGCATCTTCAAGGAACCTCC-3′ (LEIP-probe).”