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Antigenemia in Patients with Mediterranean Visceral Leishmaniasis▽

Bernard Ferrua,[1,2]* Nicolas Rol,[1] Grégory Michel,[1,2] and Pierre Marty[1,2,3]

Université de Nice Sophia Antipolis, Faculté de Médecine, Nice F-06107 Cedex 2, France[1]; INSERM, U895, Centre Méditerranéen de Médecine Moléculaire (C3M), Equipe 6, Toxines Microbiennes dans la Relation Hôte-Pathogène, Nice F-06204 Cedex 3, France[2]; and Centre Hospitalier Universitaire de Nice, Laboratoire de Parasitologie-Mycologie, Nice F-06202 Cedex 3, France[3]

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Antigenemia in patients with Mediterranean visceral leishmaniasis (MVL) due to *Leishmania infantum* was retrospectively assessed by sandwich enzyme-linked immunosorbent assay (ELISA). Circulating *Leishmania* antigens, partially in free form, were in evidence in 53% of serum samples from immunocompetent individuals with MVL. Following successful therapy, antigenemia decline as measured by ELISA was more pronounced than antibody decrease.

Although the definitive diagnosis of Mediterranean visceral leishmaniasis (MVL) due to *Leishmania infantum* is established upon the detection of parasites by microscopic examination, culture, or PCR, generally performed with bone marrow aspirates or blood samples, the detection of specific anti-*L. infantum* antibodies is generally concomitantly performed as an aid for diagnosis. Nevertheless, anti-*L. infantum* antibodies persist for months in cured patients and occur in some asymptomatic individuals living in areas of endemicity (7), therefore somewhat limiting the diagnostic and prognostic value of antibody detection. In addition, the low antibody responses occurring in some human immunodeficiency virus (HIV)-*L. infantum*-coinfected patients remain undetectable by enzyme-linked immunosorbent assays (ELISA) (9). A few previous studies (1, 2, 8) using ELISA and Western blot analyses of polyethylene glycol-precipitated immune complexes have been devoted to the detection of circulating *Leishmania* antigens (CLAs) in patients with MVL or leishmaniasis due to other parasite species. However, these methods are not quantitative and do not detect circulating, nonprecipitable free antigens. Therefore, in this study we assessed the presence of *Leishmania* antigens in MVL patients by using a sandwich-type ELISA (3) previously developed for measuring parasite burdens in BALB/c mice experimentally infected with *L. infantum*. The assay was performed as follows. High-binding microtiter plates (Greiner Bio-One) were coated for 4 h with an anti-*L. infantum* human immunoglobulin G fraction (5 μg in 100 μl of 0.1 M phosphate buffer, pH 7.2) prepared from high-titer MVL human serum (3) by protein A-Sepharose chromatography. The plates were saturated for 30 min with phosphate buffer containing 1% skim milk and 0.12% Triton X-100 (assay buffer). The assay (detection range, 0 to 2 μg/ml *Leishmania* antigens) was calibrated with a soluble Nonidet P-40 extract of *L. infantum* promastigotes (a sample of 10^6 promastigotes corresponds to 4 μg of a bovine serum albumin equivalent of *Leishmania* proteins) diluted in pooled human sera originating from an area where *Leishmania* is not endemic (Reims, France). Duplicate 0.1-ml aliquots of standards or undiluted, untested samples were delivered into the wells, and the plates were incubated for 18 h at room temperature. After the plates were repeatedly washed, a peroxidase-labeled anti-*L. infantum* F(ab)′ fragment (500 ng in 0.1 ml of assay buffer) was dispensed into the wells and the plates were incubated for 2 h. Bound-enzyme activity was revealed with a chromogenic substrate as described previously (3). The threshold assay sensitivity was 0.02 μg/ml of *Leishmania* antigens, corresponding to 5,000 parasites/ml. The method was validated with a panel of cryoconserved serum samples obtained from the collection of the parasitology-mycology department of the Centre Hospitalier Universitaire de Nice. The analyzed samples included (i) control samples from an area where *Leishmania* is not endemic (Reims, France), (ii) samples from asymptomatic contacts of infected patients, diagnosed on the basis of positive results from Western blotting against 14- and 18-kDa *Leishmania* antigens and/or positive skin tests (6, 7), from an area of endemicity (Nice, France), (iii) samples from immunocompetent or HIV-coinfected patients (23 males aged 22 to 75 years and 26 females aged 18 to 81 years) from an area of endemicity (Nice, France) with patent MVL diagnosed on the basis of parasite detection by PCR or direct examination, and (iv) samples from patients with African trypanosomiasis or acute malaria (these samples were a gift from B. Bouteille, Limoges, France). All samples were previously tested at a 1/500 dilution for the presence of anti-*L. infantum* antibodies by classical ELISA using *Leishmania* antigen-coated plates (4). CLAs (Fig. 1) were undetectable in 13 control serum samples from an area where *Leishmania* is not endemic, as well as in samples from 19 healthy contacts from an area of endemicity, 2 (10.5%) in the latter group being antibody positive by ELISA using crude *Leishmania* antigens. In contrast, at the time of diagnosis, CLAs (range, 0.03 to 4 μg/ml) were detected in 23 (53%) of 44 immunocompetent patients with MVL and higher levels (range, 0.2 to 20 μg/ml) were detected in 4 (80%) of 5 patients coinfected with HIV (Fig. 1). Interestingly, two of these four coinfected patients with detectable CLAs (Fig. 1) were negative by antibody ELISA. In addition (Fig. 1), serum samples from acute malaria patients or individuals with Afri-

* Corresponding author. Mailing address: Bâtiment Universitaire Archimed, INSERM U895, C3M, Equipe 6, 151 Route St Antoine de Ginestière, BP 2 3194, 06204 Nice Cedex 3, France. Phone: 04 89 06 42 45. Fax: 33 4 89 06 42 21. E-mail: ferrua@unice.fr.

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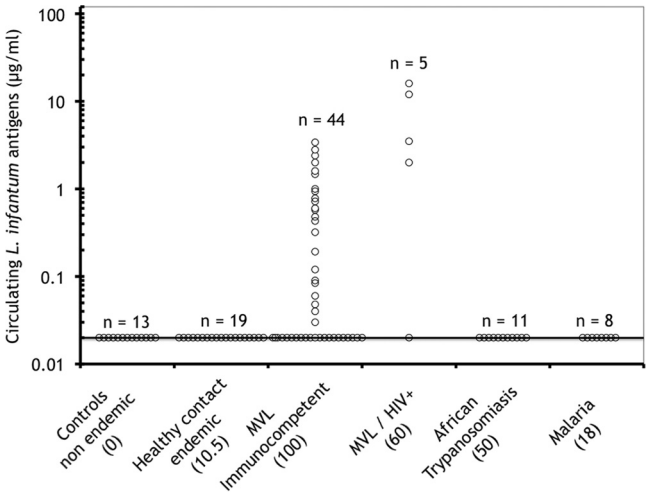

FIG. 1. Sensitivity and specificity of CLA level measurement by ELISA for MVL diagnosis. Numbers in parentheses indicate the percentages of samples positive for anti-*L. infantum* antibodies by classical ELISA using promastigote lysate.

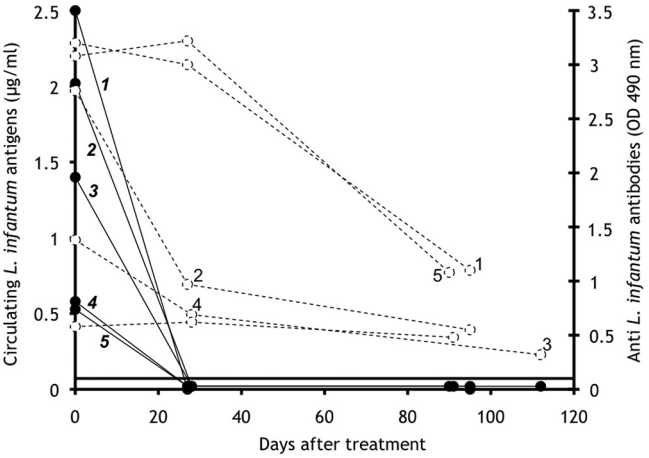

FIG. 2. Follow-up evaluation showing decreases in CLAs (solid lines with numbers in bold) and the corresponding anti-*L. infantum* antibodies (dotted lines) in five immunocompetent MVL patients receiving successful liposomal amphotericin B therapy. The horizontal line indicates the optical density cutoff value for antibody positivity. OD 490 nm, optical density at 490 nm.

can trypanosomiasis, which showed cross-reacting anti-*L. infantum* antibodies upon ELISA analysis in 18 and 50% of cases, respectively, gave CLA values close to background levels. Therefore, for the panel of sera studied, direct detection of CLAs by ELISA exhibited overall sensitivity of 55.1% and specificity of 100% for the diagnosis of MVL. Furthermore, monitoring of antigenemia in immunocompetent MVL patients receiving successful liposomal amphotericin B (Ambisome) chemotherapy (Fig. 2) indicated that in all studied cases, CLAs were completely cleared from circulation by day 25 but that antibody levels decreased only slowly during this period. Consequently, antigenemia decline measured by direct ELISA following chemotherapy is a more sensitive indicator of therapeutic efficacy than antibody decrease, at least in immunocompetent MVL patients. Finally, molecular sieving of serum samples from immunocompetent MVL patients by using a Sephacryl S-200 column (data not shown) indicated that a large proportion of assayable circulating *L. infantum* antigens eluted in the 68- to 46-kDa range and consequently circulated in the free form. The other portion (>150 kDa) is likely to correspond to free high-molecular-mass CLAs and/or immune complexes of various sizes formed in the context of antigen excess.

In this study, using a direct sandwich ELISA, we evaluated the minimal frequency of CLAs in a serum panel from MVL patients at the time of diagnosis and showed for the first time that a great proportion of CLAs occurred in the free form in serum. The method was less sensitive than antibody serology performed with crude promastigote extracts but showed 100% specificity for MVL diagnosis. Nevertheless, the specificity of antibody serology could be improved by selecting serodiagnostic methods based on a single *Leishmania* antigen such as rK39 or rK26 protein (10, 11). As a portion of CLAs circulate in the form of immune complexes, false-negative results may be reduced by previous dissociation of antigen-antibody complexes, as described elsewhere for improving antibody or antigen detection in samples from patients with various infectious diseases (5, 12, 13). In addition, the detection of parasite antigens in urine samples from patients may increase the assay sensitivity for MVL diagnosis. Finally, the test proved to be a more sensitive indicator than antibody detection to monitor the recovery of MVL patients receiving chemotherapy.

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