Leishmania infantum: Lack of Parasite Resistance to Amphotericin B in a Clinically Resistant Visceral Leishmaniasis

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Amphotericin B (Amb) has been used as a second-line treatment of visceral leishmaniasis, particularly in human immunodeficiency virus-positive patients. Amb median effective doses (ED50s) were determined on an isolate obtained before any treatment and on a second isolate obtained 4 years later from the same Amb-treated patient. ED50s were similar (0.059 and 0.067 mg/kg of body weight, respectively), demonstrating the first evidence of Amb ED50 stability of Leishmania infantum after a long-term drug exposure. An isoenzymatic study was performed in order to verify that the second isolate originated from the same parasite as the first isolate. The present case report showed that treatment failure was not due to parasite resistance in spite of a prolonged exposure to the drug.

In Leishmania-human immunodeficiency virus (HIV)-coinfected patients, the first course of antimonial pentavalent compound is unsuccessful in more than 50% of patients (1). Relapses after apparent clinical recovery occur after a mean time of 4.5 months (8). Amphotericin B (Amb), an antifungal agent which has proved to be active also against Leishmania spp., is currently proposed as an alternative first-line treatment (12). Amb was first used with the deoxycholate formulation (Fun-gizone), but acute and chronic toxic side effects following Amb deoxycholate administration have promoted the use of lipid formulations which are much better tolerated (16). The liposomal formulation of Amb (Ambisome) represented progress in the fight against visceral leishmaniasis (VL). However, most immunocompromised Amb-treated patients, initially considered cured, relapsed clinically and parasitologically after 3 to 22 months (3). Secondary cures by Ambisome were also followed by relapses (4). These relapses, probably related to the immune status of patients, might also reflect ineffective drug levels in contact with some Leishmania parasites which could be in unusual locations in HIV patients (6). In some cases, primary or secondary resistance to Amb could be involved in treatment failures as was described for antimonal compounds. A previous study showed that different Leishmania infantum strains may require different Amb levels to be cleared (7). Thus, the median effective dose (ED50) determined in BALB/c mice was 0.17 mg/kg of body weight for an isolate obtained from an untreated patient, whereas the ED50 was 0.41 mg/kg for an isolate obtained from a patient who had received 12.4 g of Amb over 3 years (7). A study conducted on isolates obtained from the same patient before and after treatment was essential in order to assess the occurrence of secondary resistance to Amb. The aim of the present work was to determine ED50s on parasites obtained from a Leishmania-infantum-HIV-coinfected patient before and after Amb therapeutic exposure, in a model associating L. infantum and BALB/c mice. As the patient lived in an area of endemicity during the treatment period, we have ensured by isoenzymatic study that the isolate obtained after 4 years of therapeutic exposure had the same profile as that of the initial isolate.

The first isolate was obtained in 1992 from an HIV-positive drug addict patient. The strain was identified as L. infantum; its zymodeme was close to zymodeme MON-1 but with a difference on one electromorph. During November 1992, the patient presented the first episode of typical VL: febrile pancytopenia and hepatosplenomegaly. This first episode was treated successfully with two courses of Glucantime (antimoniate meglumine). The first relapse occurred 10 months later, and subsequent relapses were treated either with Amb (Fungizone or Ambisome) or pentavalent antimony (SbV). Itraconazole and allopurinol were introduced occasionally as secondary prophylaxis. By December 1996, the patient had received a total dosage of SbV in excess of 325 g and a total dosage of Amb (whatever the formulation) exceeding 14 g. During the 4-year course, 27 Leishmania isolates were obtained from this patient, either on Novy-MacNeal-Nicolle medium or after inoculation into a golden hamster. All the isolates were stocked in nitrogen liquid. The first isolate was obtained before any treatment. Five isolates, randomly selected among the 27 isolates, were characterized by isoenzyme analysis (three isolates obtained from bone marrow in November 1992, September 1993, and September 1994 and two isolates obtained from blood in September 1994 and November 1996).

The isolates were characterized by starch gel electrophoresis with 15 enzymatic systems (11). The same isoenzymatic profile was identified each time and was identical to that of the reference strain L. infantum MON-1, except for one electromorph (malic enzyme) which was absent in every characterization. Amb ED50 were determined in a murine model on the first naive stock (stock A, November 1992) and on the 19th stock (stock B, November 1996), as previously described (7). Experiments were conducted with BALB/c male mice (5 weeks old, 20 ± 2 g) purchased from IFFA CREDO (l’Arbresle, France). Cryopreserved promastigotes of the patient stocks were thawed and cultivated on Novy-MacNeal-Nicolle medium...
and then on RPMI medium. On day 0, mice were inoculated via the tail vein with 2 × 10⁷ infective Leishmania promastigotes in a 0.1-mL volume. This procedure induced a liver parasite burden of 10.4 × 10⁷ amastigotes per mg of liver weight for the first isolate (stock A) and 0.52 × 10⁷ for the second (stock B). The stocks required high inoculum and 3 weeks to visceralize, reflecting a relatively low virulence in BALB/c mice for the two stocks studied in this work, in comparison with previous studies using other strains (7).

Mice infected with stock A and mice infected with stock B were randomly divided into five groups. On days 21, 23, and 25, control groups (10 mice) received isotonic sodium chloride, and four groups of five mice received 0.05, 0.1, 0.5, and 0.8 mg of AmB deoxycholate, respectively, per kg. On day 28, the animals were killed by cervical dislocation and autopsied. The Guiding Principles for Biomedical Research involving animals, published in 1986 by the Council for International Organizations of Medical Sciences, were followed during all procedures.

The drug efficacy was determined by evaluating the liver parasite burden and the measure of efficacy doses. The liver parasite burden was evaluated after Giemsa staining of the smears. The number of amastigotes per 500 hepatocytes was calculated and related to liver weight (in milligrams) from the formula of Staub et al. (13). The percentage of parasite suppression was calculated as [1 – (mean Staub count of the treated group/mean Staub count of the control group)] × 100. ED₅₀ (doses of the drug calculated to eliminate 50% of parasites compared to controls) were determined by the Michaelis-Menten model.

Statistical analysis. Results were expressed as means ± standard errors of the means. A one-way analysis of variance or a U test was performed to compare the influences of various parameters. A P value lower than 0.05 was considered statistically significant.

Results were as follows.

Control experiments. In group A, mice showed a mean of 0.40 L. infantum amastigotes per liver cell nucleus at the end of the 28-day period of experimentation (range, 0.1 to 0.84; n = 10). In group B, mice showed a mean of 0.02 L. infantum amastigotes per liver cell nucleus at the end of the same period (range, 0.004 to 0.028; n = 10). The average liver parasite burdens obtained for each group were significantly different (10.4 × 10⁷ in group A versus 0.52 × 10⁷ in group B).

Treated groups. Maximal parasite suppression obtained with AmB deoxycholate was 96.8% ± 0.1% (Table 1) in group A and 100% ± 0% in group B (Table 1). These maximal parasite suppressions obtained at the same dose in the two groups (0.8 mg/kg) were not significantly different. The ED₅₀ were 0.053 ± 0.010 mg/kg in group A and 0.067 ± 0.016 mg/kg in group B. These results were not significantly different (P = 0.26).

Results showed unchanged AmB ED₅₀ for the identity-controlled Leishmania stocks. This is the first evidence of AmB ED₅₀ stability after a long-term drug exposure in a parasite obtained after a clinical course. Mice infected with stock A showed a liver parasite burden 20 times higher than that of mice infected with stock B. The relatively low liver parasite burden observed in mice infected with stock B reflected a decrease of parasite virulence. A comparison between ED₅₀ determined in mice having similar parasite burdens would be ideal. However, it was not possible to obtain higher inocula of stock B without performing subcultures, which could modify the parasite. The studied stocks were separated by 4 years. The very long general course of the VL was probably due to the low pathogenicity of the parasite and to the treatment failure, which may be attributed to the immunocompromised patient status. It is known experimentally that pathogenicity may differ from one isolate to another (14).

The isoenzymatic study accurately demonstrated that the ED₅₀ were determined on the same parasite obtained before and after drug exposure. The isoenzymatic characterization of isolates obtained from VL patients who had relapsed in a zone of endemicity had been already reported in a previous study (9). New episodes of VL in all patients (n = 10) were always caused by the same zymodeme, indicating relapse of the infection with the original parasites rather than reinfection. However, these identities were assessed over relatively short periods (mean = 4 months) and did not formally exclude reinfection by a parasite of the same zymodeme, especially when a zymodeme is as geographically predominant as zymodeme MON-1 in the Mediterranean basin. In the present work, the particularly uncommon profile of the studied parasite ensured that both stocks came from the same initial infection. Because in areas of endemicity subjects may be infected, and reinfected, by different zymodemes (10), having such a marker was essential.

Primary and secondary unresponsiveness to pentavalent antimonial compounds has been reported for Leishmania donovani in immunocompetent patients. Secondary unresponsiveness to Sb⁶⁺ has also been reported for L. infantum (2). This secondary unresponsiveness was correlated with a decrease of parasite susceptibility. Conversely, no primary or secondary unresponsiveness to AmB has been reported to date for immunocompetent patients (15). The present study did not show an emergence of a lower susceptibility of the parasite despite long-term treatment. The results emphasized in this case the value of AmB secondary prophylaxis. In particular, AmB liposomal formulations, which are better tolerated, may improve the clinical outcome despite a persistent parasite burden. Similar studies conducted on more strains are required in order to confirm the stability of AmB L. infantum susceptibility and therefore the usefulness of AmB in long-term treatment of immunocompromised patients.

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### Table 1. Suppression of experimental leishmaniasis in mice treated with AmB deoxycholate

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>No. of amastigotes/500 hepatocytes</th>
<th>% Suppression (range)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>198.9 ± 35.4</td>
<td></td>
</tr>
<tr>
<td>AmB deoxycholate</td>
<td>0.05 124.2 ± 50.4</td>
<td>39.6 ± 4.4 (29.5–43.5)</td>
</tr>
<tr>
<td></td>
<td>0.1 35.2 ± 4.13</td>
<td>76.3 ± 1.3 (74.6–78.8)</td>
</tr>
<tr>
<td></td>
<td>0.5 25.4 ± 3.6</td>
<td>84.0 ± 2.3 (77.1–88.3)</td>
</tr>
<tr>
<td></td>
<td>0.8 4.6 ± 0.33</td>
<td>96.8 ± 0.4 (96.0–97.2)</td>
</tr>
</tbody>
</table>

#### Group A
- Control: 11.2 ± 3.1
- AmB deoxycholate: 0.05 6.3 ± 1.1, 0.1 1.7 ± 0.3, 0.5 0.2 ± 0.2, 0.8 0.0 ± 0.0

#### Group B
- Control: 11.2 ± 3.1
- AmB deoxycholate: 0.05 6.3 ± 1.1, 0.1 1.7 ± 0.3, 0.5 0.2 ± 0.2, 0.8 0.0 ± 0.0

#### Notes
- Mice were infected with stock A (naïve for AmB).
- Mice were infected with stock B (AmB, 14 g for 4 years).
- Calculated with the Stauber count versus untreated mice. Data are expressed as means ± standard errors for five mice per group, except the control group (n = 10).

#### References
- Michaelis-Menten model.
- Control experiments.
- Treated groups.
- ED₅₀ stability after a long-term drug exposure in a parasite obtained after a clinical course.
- Leishmania donovani in immunocompetent patients.
- L. infantum (2). This secondary unresponsiveness was correlated with a decrease of parasite susceptibility. Conversely, no primary or secondary unresponsiveness to AmB has been reported to date for immunocompetent patients (15).

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