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In Vitro and In Vivo Resistance of *Leishmania infantum* to Meglumine Antimoniate: a Study of 37 Strains Collected from Patients with Visceral Leishmaniasis

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Primary and secondary unresponsiveness to meglumine has long been described in human visceral leishmaniasis. However, no studies have been performed to elucidate if these therapeutic failures were due to strain variability in meglumine sensitivity or were related to host factors. We have studied the in vitro sensitivity of 37 strains of *Leishmania infantum* isolated from 23 patients (11 human immunodeficiency virus-infected and 12 immunocompetent patients) with visceral leishmaniasis. Sensitivity tests were performed by infecting murine macrophages with *Leishmania* parasites and culturing them in medium containing different concentrations of meglumine. For each test we calculated a 50% effective dose (ED_{50}) corresponding to the meglumine concentration at which 50% of the *Leishmania* parasites survived. In vitro results were strongly correlated to immediate clinical outcome. All strains requiring an ED_{50} of >70 μg/ml were related to therapeutic failures, whereas all strains requiring an ED_{50} of <40 μg/ml corresponded to an initial efficiency of meglumine. Among those patients who were initially improved, relapses occurred in all immunocompromised patients and in most immunocompetent patients who had a short duration of treatment (15 days). Finally, we found that in vitro sensitivity of strains decreased progressively in relapsing patients treated with meglumine. Consequently, the physician may be encouraged to alternate meglumine with other treatments such as amphotericin B or pentamidine, especially in the case of relapsing patients.

Although pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) represent the first-line drugs for treatment of visceral leishmaniasis, numerous treatment failures have been reported. These failures are classified as primary unresponsiveness, when they occur from the beginning of the treatment, and secondary unresponsiveness, when they happen during a relapse (4). Cases of secondary unresponsiveness were mainly described in immunocompromised patients and have been generally attributed to immunodepression (14). However, secondary unresponsiveness was also reported in immunocompetent subjects who relapsed due to insufficient antimonial treatment during the initial stage of the disease. Even in immunocompetent hosts, the clinical efficacy of pentavalent antimonials seems to decrease during relapses (4, 5), suggesting that *Leishmania* strains may become resistant to meglumine treatment. This phenomenon, which has also been described in dogs infected with *Leishmania infantum* (9), has not been demonstrated by in vitro studies with strains isolated from cases of human visceral leishmaniasis.

In order to examine whether the inefficacy of meglumine treatment was dependent upon acquisition of resistance by *Leishmania* strains, we studied the sensitivity of strains of *L. infantum* isolated from patients with visceral leishmaniasis during the course of the disease. In vitro tests were performed on *Leishmania* amastigotes cultured in murine macrophages (12). The evolution of strain susceptibility was then studied by comparing in vitro sensitivities of strains isolated from each patient throughout the course of infection.

MATERIALS AND METHODS

Patients and samples. Twenty-one strains were isolated from 11 human immunodeficiency virus (HIV)-infected adults with less than 100 $CD4^+$ cells/ml. Sixteen other strains were collected from patients (four adults and eight children) who were neither immunodeficient nor receiving immunosuppressive treatment. One to three samples were collected from each patient during the course of the disease.

In 31 instances, patients were given meglumine for at least 15 days after strain isolation. In three cases meglumine treatment was stopped before 15 days, and in the three remaining cases patients were not treated with meglumine. Patients treated with meglumine were classified as improved if they become afebrile and had a decrease in splenomegaly before the 15th day of treatment.

For immunocompetent patients, meglumine, alternative drugs, or splenectomy was used in cases of failure or relapse (see Table 1). For HIV patients, maintenance therapy with meglumine or alternative drugs was always applied (see Table 2).

Leishmania parasites were isolated from bone marrow aspirates (32 cases), blood (2 cases), cutaneous biopsies (2 cases), and a splenic biopsy (1 case). Parasites were isolated and cultivated in RPMI medium supplemented with 15% fetal calf serum.

Thirty-one strains were analyzed using an isoenzymatic method (18), and 6 strains were analyzed by sequencing a PCR product (15). All were identified as *L. infantum.*

Determination of in vitro sensitivity. The in vitro sensitivity of *L. infantum* strains was studied after phagocytosis of 3×10^5 *Leishmania* cells by unstimulated BALB/c mouse macrophages (8×10^4 cells) in 200 μl of RPMI medium. The macrophages were recovered from peritoneal washings with RPMI medium and cultivated (4×10^5/ml) in a Lab Tek tissue culture chamber by using macrophage adherence to glass (2). Parasites and macrophages were cultured in RPMI medium containing 15% heat-inactivated fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). Meglumine antimoniate solutions at concentrations of 0 (control cultures), 7.5, 15, 30, 45, and 60 μg/ml were added to the cultures. Different meglumine concentrations (none toxic for peritoneal macro-

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TABLE 1. Results of in vitro tests of strains isolated from immunocompetent patients

Patient[a]	Age (yr)	Circumstances of sampling	Previous meglumine treatment	Meglumine treatment after strain isolation		Clinical outcome after meglumine treatment	Alternative therapy	ED_{50} (μg/ml)
				Dose (mg/kg)	Duration (days)			
1	3	1st episode	No	60	15	Definitive cure		17
2	3	1st episode	No	60	28	Definitive cure		21
3	34	1st episode	No	60	15	Definitive cure		23
4	1.5	1st episode	No	60	15	Definitive cure		28
5	3	1st episode	No	60	28	Definitive cure		49
6	2	1st episode	No	60	28	Definitive cure		64
7A	35	1st episode	No	60	15	Improvement and relapse	Meglumine	12
8	2	1st episode	No	60	15	Improvement and relapse	Liposomal amphotericin B	14
7B	35	1st relapse	Yes	60	15	Improvement and relapse	Amphotericin B	28
9A	25	1st episode	No	60	15	Improvement and relapse	Meglumine	36
10A	35	1st episode	No	60	15	Improvement and relapse	Gamma interferon	36
10B	35	1st relapse	Yes	60	15	Treatment failure	Splenectomy	61
9B	25	1st relapse	Yes	60	15	Treatment failure	Splenectomy	63
11	2	1st episode	No	60	15	Treatment failure	Liposomal amphotericin B	207
12A	1	1st episode	No	60	28	Treatment failure	Liposomal amphotericin B	223
12B	1	1st relapse	Yes	No meglumine			Liposomal amphotericin B	234

[a] For those patients sampled more than once, isolates are indicated by a letter following the patient number (A for the first isolate and B for the second).

phages) were determined according to their peak serum levels (20). Because the drug is not stable for as long as a week, fresh meglumine-containing RPMI medium was added on the second and fifth days. After 7 days of incubation at 37°C, cultures were washed and surviving *Leishmania* cells were counted for each meglumine concentration by examining 100 macrophages after Giemsa staining (3).

Determination of effective dose of the strains. Statistical analysis was performed according to the procedure described by Daniel and Woods (6).

For each test, the number of the *Leishmania* cells counted at various meglumine concentrations (0, 7.5, 15, 30, 45, and 60 μg/ml) was plated on a semilogarithmic scale to obtain a better alignment of the points. The 50% effective dose (ED_{50}) was calculated as the meglumine concentration which decreased the survival of leishmania cells by half.

Statistical analyses were done using the Mann-Whitney U test to compare independent variables (decimal logarithm of number of surviving *Leishmania* cells and meglumine concentration) and the Wilcoxon test to compare double variables (ED_{50} for two isolates from the same patient before and after treatment).

RESULTS

In vitro sensitivity of *Leishmania* strains and the relationship between sensitivity and clinical outcome after meglumine treatment. The results of the in vitro tests and the main clinical data are shown in Tables 1 and 2.

Leishmania strains required ED_{50} of greater than 70 μg/ml in 10 cases. Meglumine treatment was used in seven of these cases and always failed. Eleven strains required ED_{50} of be-

TABLE 2. Results of in vitro tests of strains isolated from immunocomprised patients

Patient[a]	Age (yr)	Circumstances of sampling	Previous meglumine treatment	Meglumine treatment after strain isolation		Clinical outcome after meglumine treatment	Alternative therapy	ED_{50} (μg/ml)
				Dose (mg/kg)	Duration (days)			
13	30	1st episode	No	60	25	Improvement	Pentamidine/amphotericin B[b]	19
14A	25	1st episode	No	60	28	Improvement and relapse	Meglumine	19
15A	39	1st episode	No	60	20	Improvement and relapse	Meglumine	32
16A	25	1st episode	No	60	30	Improvement and relapse	Meglumine	34
17A	29	1st episode	No	60	28	Improvement and relapse	Meglumine	39
16B	25	1st relapse	Yes	60	30	Improvement and relapse	Data not known	43
18A	35	1st episode	No	60	15	Improvement and relapse	Pentamidine	43
14B	25	1st relapse	Yes		Stopped before day 15	Death		19
19A	35	1st episode	No		Stopped before day 15	Undetermined	No treatment	27
19B	35	1st relapse	Yes		Stopped before day 15	Undetermined	No treatment	42
20	45	5th relapse	No	60	15	Treatment failure	Pentamidine	44
21A	26	1st relapse	Yes	60	15	Treatment failure	Meglumine	44
18B	35	1st relapse	Yes	60	15	Treatment failure	No treatment	45
21B	26	2nd relapse	Yes	25	20	Treatment failure	Meglumine	65
15B	39	1st relapse	Yes	60	20	Treatment failure	Amphotericin B	73
22	41	4th relapse	Yes	60	28	Treatment failure	Gamma interferon	73
17B	29	1st relapse	Yes	60	28	Treatment failure	Data not known	81
21C	26	3rd relapse	Yes	25	20	Treatment failure	Gamma interferon/allopurinol	127
23A	30	1st relapse	Yes	60	28	Treatment failure	Pentamidine/amphotericin B	129
17C	29	2nd relapse	Yes	No meglumine			Amphotericin B	113
23B	30	2nd relapse	Yes	No meglumine			No treatment (death)	151

[a] For those patients sampled more than once, isolates are indicated by a letter following the patient number (A for the first isolate, B for the second, and C for the third).

[b] Administered as maintenance therapy.

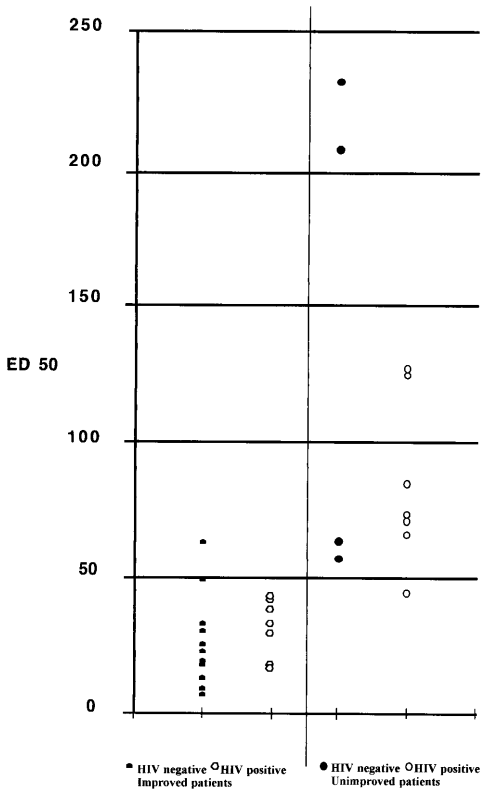

FIG. 1. Distribution of ED_{50} for strains isolated before meglumine treatment, according to the clinical outcome of the patient.

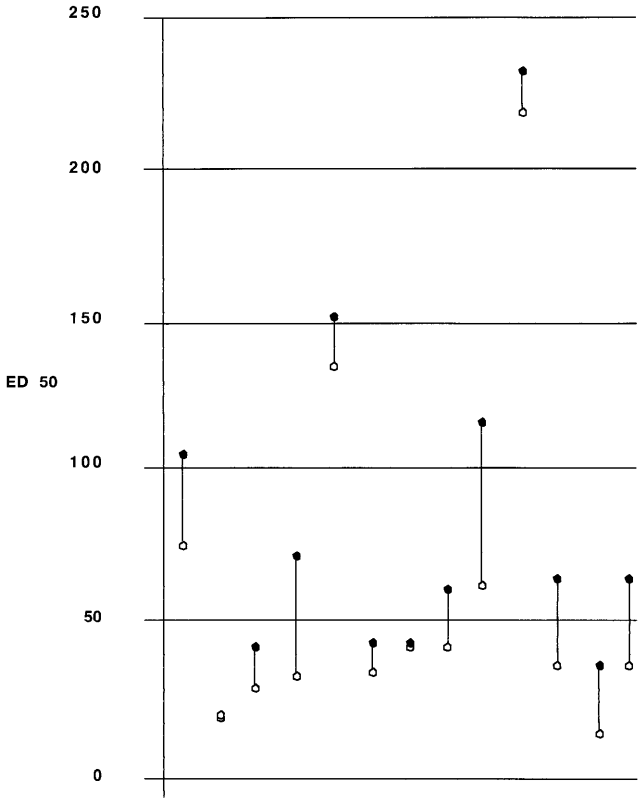

FIG. 2. ED_{50} for strains isolated from patients before (open symbols) and after (closed symbols) meglumine treatment.

tween 40 and 70 μg/ml. Meglumine treatment was used with all these patients except one and led to clinical failure in five cases, to improvement followed by relapse in four cases, and to a cure in only one case. Of the 13 treated patients with strains requiring ED_{50} of less than 40 μg/ml, 5 were cured and 8 improved but later relapsed. The relationship between clinical outcome after treatment and ED_{50} for the isolated strains is shown in Fig. 1.

Among the HIV subjects, the difference between the mean ED_{50} for strains isolated from improved and not improved patients was significant ($P = 0.0015$, Mann-Whitney). The mean ED_{50} was also significantly higher for strains isolated from immunocompetent patients who did not improve compared with that for strains isolated from immunocompetent patients who did improve ($P = 0.01$, Mann-Whitney).

After an initial improvement, the long-term effect of the meglumine treatment was not related to ED_{50}. There was no significant difference between mean ED_{50} for strains from cured patients (mean, 34 μg/ml; range, 17 to 64 μg/ml) and that for isolates from patients who experienced a relapse after an initial improvement (mean, 32 μg/ml; range, 14 to 45 μg/ml).

In contrast, the long-term effect of therapy appeared to depend on the duration of treatment and on coinfection with HIV. All the HIV-infected patients who initially improved experienced a relapse, whereas most (6 of 12) of the immunocompetent patients were cured. All immunocompetent patients who relapsed after an initial improvement had a short duration of meglumine treatment (15 days).

Role of previous meglumine courses on strain sensitivity. Seventeen *Leishmania* strains were collected from patients previously treated with meglumine (relapsing patients), and 20 others were collected from patients who had never been treated with meglumine. The ED_{50} was significantly higher for strains in previously treated patients (mean, 82 μg/ml; range, 19 to 234 μg/ml) compared to that for strains obtained from patients who were not previously treated (mean, 49 μg/ml; range, 12 to 223 μg/ml) ($P < 0.001$, Mann-Whitney).

Among the 20 strains isolated from patients who were not previously treated with meglumine, 3 corresponded to patients who later failed to respond to this treatment. Of these, two strains required a very high ED_{50} (206 and 222 μg/ml) and were collected from immunocompetent patients. The third one required a lower ED_{50} (44 μg/ml) and was isolated from an HIV-infected patient. These three cases were classified as primary unresponsiveness.

Among the 17 relapsing patients previously treated with meglumine, 12 were again given the same treatment after strain isolation. Nine of these patients failed to respond to a second meglumine treatment. The ED_{50} for the corresponding strains were high (mean, 80 μg/ml; range, 44 to 129 μg/ml). These cases were classified as secondary unresponsiveness, and these patients were further treated with other therapeutics such as pentamidine or amphotericin B.

Evolution of the sensitivity of the strains during meglumine treatment. In 14 cases the ED_{50} for strains isolated before meglumine treatment were compared to the ED_{50} for strains isolated from the same patients after treatment. The evolution of ED_{50} for each strain is shown in Fig. 2. The ED_{50} increased in all cases except one ($P = 0.001$, Wilcoxon test). In the latter case, the strain sensitivity was not modified (18.5 μg/ml after treatment versus 19.1 μg/ml before treatment).

DISCUSSION

One of the main objectives of the study was to determine whether resistance of *Leishmania* strains to meglumine may explain, at least partially, therapeutic failures that have been observed in both HIV-positive and HIV-negative patients. In order to exclude the role of the immune system we used an in vitro experimental system with the intracellular amastigote form of the parasites. In humans, *Leishmania* parasites are rapidly phagocytosed by macrophages. Thus, to be effective in human visceral leishmaniasis, drugs must be incorporated into macrophages and be efficient under the conditions of the intracellular environment. The meglumine concentrations used corresponded to therapeutic concentrations (30 to 40 μg/ml); the toxic concentration is >60 μg/ml.

In vitro sensitivity to meglumine correlated with the immediate in vivo response to the treatment. Drug-sensitive strains (ED_{50} of <40 μg/ml) were isolated from patients who responded quickly to the meglumine treatment, whereas all the strains which were resistant under in vitro conditions (ED_{50} of >70 μg/ml) corresponded to clinical failures. In contrast, the long-term outcome of meglumine treatment did not appear to be correlated to the results of the in vivo test, and relapses occurred even in patients harboring a sensitive strain. Numerous studies have shown the importance of T-cell-mediated immunity in the prevention of relapses (8). This may explain the high frequency of relapses observed in AIDS patients, even after effective treatment. However, we also found that some immunocompetent patients infected with sensitive strains relapsed when the duration of treatment was too brief (15 days). Such a duration of treatment has long been proposed to cure visceral leishmaniasis in children living in the Mediterranean area (17). The recent occurrence of treatment failure has led us to reevaluate this therapeutic approach (16). Our data support the opinion that Mediterranean visceral leishmaniasis in children requires a 30-day treatment, as recommended by the World Health Organization (20).

Of the three strains that corresponded to cases of primary unresponsiveness to meglumine, two were isolated from immunocompetent hosts. Cases of primary unresponsiveness were previously described in humans with visceral leishmaniasis caused by *Leishmania donovani* in India and in East Africa (4) but never in immunocompetent hosts living in the Mediterranean area.

The other main objective of the study was to determine whether the sensitivity of strains decreased in patients undergoing several courses of treatment. In vitro studies have shown that *Leishmania* parasites can develop resistance when cultured in the presence of pentavalent antimonials (10). Using a different methodology, other authors demonstrated the role of meglumine treatment in the selection of resistant strains in dogs, which are the main reservoir of the parasite in the Mediterranean area (1, 9). These strains were transmissible to man through phlebotomous sandflies.

The decrease of in vivo meglumine efficacy during the course of the disease was at least partially due to a decrease in the sensitivity of strains to the drug. This observation may lead to modifications in therapeutic strategy against visceral leishmaniasis in humans (7) and may lead physicians to alternate meglumine therapy with other treatments such as amphotericin B or pentamidine, especially in cases of relapsing patients. The progressive resistance observed in our study is compatible with a recent hypothesis regarding antimonial resistance mechanisms (13).

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