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Diffuse cutaneous leishmaniasis responds to miltefosine but then relapses

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Summary

Background Diffuse cutaneous leishmaniasis (DCL), although rare, is profoundly incapacitating. At present there is no successful treatment for this progressive protozoan infection, which is associated with the absence of specific cell-mediated immunity (CMI) to *Leishmania*. This disease shares features with visceral leishmaniasis (VL), including specific CMI inactivity during active disease and a heavy parasitic burden, but VL responds well to treatment. Miltefosine is the first orally administered drug which has shown efficacy in the treatment of VL; it has not been adequately evaluated in the treatment of DCL.

Objectives To evaluate the efficacy of miltefosine in the treatment of DCL, using clinical, parasitological, histopathological and immunological criteria.

Methods Sixteen patients with DCL were treated with miltefosine, 2·0–2·5 mg kg^{-1} daily, for variable periods of time (75–218 days). Patients were hospitalized for the first month and evaluated every 2 weeks until the termination of treatment with routine laboratory chemistry, percentage clinical improvement, presence of parasites in skin smears, growth of parasites in culture medium and in hamsters, histopathological characteristics of the granulomas, adverse side-effects, and reactivity to leishmanin skin test antigen. Further cycles of treatment were given in some of these patients, particularly after suspension of treatment was followed by relapse.

Results Patients showed dramatic clinical improvement and reduction in the parasite burden by day 15 after the initiation of treatment, which continued while treatment was maintained. By day 45, 15 patients showed 80–90% clinical improvement. Nevertheless, suspension of treatment was followed by the development of new lesions in all but one patient. Inoculation in hamsters was observed to be the most sensitive technique to detect persisting parasites. Adverse events were very mild.

Conclusions Miltefosine produced a dramatic clinical and parasitological response in patients with DCL and improvement continued during drug administration, but with a single exception all patients presented new lesions after suspension of treatment. There was no histological or skin test evidence to suggest the development of CMI during treatment, which may be an indispensable criterion for the evaluation of potentially effective drugs against DCL.

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Conflicts of interest
None declared.

Leishmaniasis is an infectious disease caused by flagellated protozoa belonging to the genus *Leishmania*, transmitted to humans by a phlebotomine insect vector bite. The disease is characterized by a clinical, histopathological and immunological spectrum that depends on the immunological capacity of the host, the species and virulence of the parasite and poorly

defined environmental factors. Clinical manifestations of leishmaniasis include cutaneous, mucosal and visceral lesions.

Diffuse cutaneous leishmaniasis (DCL) is a rare form of the disease that is associated with a defective cell-mediated immune response to the *Leishmania* parasite,[1] allowing its uncontrolled multiplication. This form of leishmaniasis was described in Venezuela by Convit and Lapenta in 1948.[2] It occurs as isolated cases in Venezuela, the Dominican Republic, Brazil, Mexico, Bolivia, Colombia, Peru and Africa.[3] In America, DCL is usually caused by parasites of the *Leishmania* (*Leishmania*) subgenus, most frequently *L.* (*L.*) *amazonensis*,[3] although cases of DCL produced by *L.* (*Viannia*) *braziliensis* have been reported in immunocompromised individuals. In the Old World, the most frequent cause of DCL is *L. aethiopica*.

DCL usually begins in infancy and is characterized clinically by the appearance of erythematous or skin-coloured papules, plaques or nodules, generally in localized areas. These may be asymmetrical lesions affecting a single limb or symmetrical but limited to upper or lower limbs.[4,5] The lesions usually do not ulcerate unless they are traumatized. When the mucosa is affected, the inflammatory reaction is not severe. The lesions can remain localized for a long period of time with little modification and subsequently disseminate over the body surface in a symmetrical manner.[5]

From the immunological point of view, deficiencies in cytokines and accessory signals are associated with parasite-specific anergy. In the epidermis of patients with DCL, Tapia *et al.*[6] found few CD1a+, CD83+ Langerhans cells, and defective response of keratinocytes in the expression of HLA-DR or intercellular adhesion molecule-1. There is also a deficiency in the production of interleukin (IL)-6 by epidermal cells, which indicates defective production of monokines by antigen-presenting cells.[6,7]

The granulomas present in patients with DCL are characterized by a predominantly T-helper 2 response (IL-4, IL-5, IL-10), with a high percentage of naïve T lymphocytes that do not react to the parasite. The leishmanin skin test is negative except in rare cases where it is positive initially and subsequently becomes negative.[4,6,7] The production of antibodies against *Leishmania* in DCL is elevated, but they do not play a significant protective role. Ulrich *et al.* have demonstrated that the predominant isotype in the majority of these patients is IgG4, which does not activate the complement cascade.[8,9]

There is no effective treatment for DCL. Anti-*Leishmania* drugs often produce temporary improvement in the clinical manifestations, but this initial activity is inexorably followed by relapse.[3,4] The unremitting course of this disease is often accompanied by severe psychological and social repercussions in those affected.

In recent years, success has been reported in the treatment of visceral and cutaneous leishmaniasis with miltefosine, an orally administered medication that was originally developed as an antineoplastic drug.[10] Miltefosine is an alkylphospholipid, an analogue of phosphocholine. Its chemical similarity to the natural phospholipids of cellular membranes suggests that miltefosine probably inhibits transmembrane signals and the synthesis of the cellular membrane.

Miltefosine, administered by the oral route at a dose of 100 mg daily for 4 weeks in adults and 2·5 mg kg^{-1} daily in children, has been used in the treatment of visceral leishmaniasis (VL), with a cure rate of 95% and good tolerance in the patients.[10] In American cutaneous leishmaniasis varying rates of cure from 53% to 91% have been reported using a dose of 2·5 mg kg^{-1} daily, depending upon the species of *Leishmania* causing the infection.[11]

Based on previous reports of the efficacy of miltefosine in the treatment of other forms of leishmaniasis, a previous case report showing encouraging results in the treatment of a single case of DCL,[12] and the absence of any definitive treatment for this disease, in this study we have evaluated the therapeutic efficacy of miltefosine in the treatment of DCL in a significantly larger group of patients.

Patients and methods

Patients

Sixteen patients from rural Venezuela, with a diagnosis of DCL confirmed in the Leishmaniasis Clinic of the Institute of Biomedicine, were included in the study. All had been followed for years and had received multiple cycles of treatment with anti-*Leishmania* drugs and immunotherapy with killed *Leishmania* promastigotes and bacille Calmette-Guérin. The clinical protocol was approved by the Ethical Committee of the Institute. The patients or legal representatives of minors signed informed consent forms before their admission into the study.

Treatment

A detailed clinical history of each patient was compiled. They were clinically examined, and photographs of lesions were taken, as were biopsies of skin lesions for histopathological study, cultivation of parasites in artificial media and inoculation into hamsters. Skin smears also were taken for parasitological evaluation. Laboratory examinations included full blood count, blood glucose, urea, creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total and fractionated bilirubin, human immunodeficiency virus and Venereal Disease Research Laboratory test.

All of the patients were treated with miltefosine in a dose of 2–2·5 mg kg^{-1} daily and were hospitalized during the first month of treatment. They were evaluated clinically and had weekly laboratory examinations and photographs taken of their lesions. One month after completing treatment in the hospital the patients were released, after verifying that there were no severe secondary effects. Biweekly monitoring until the treatment was terminated included clinical evaluation, laboratory examinations (full blood count, hepatic and renal profiles), skin biopsies for culture, animal inoculation and histopathology, and skin smears. A standard leishmanin test was performed after completion of the first cycle of treatment.

Histopathological study

Biopsies were processed and stained with haematoxylin and eosin, to verify the presence or absence of parasites and to evaluate the structure of the granulomas. Skin smears were stained with Giemsa to detect parasites.

Cultures and inoculation in experimental animals

After washing the skin biopsy with phosphate-buffered saline (PBS) containing penicillin 2000 IU mL^{-1} and gentle maceration in a tissue grinder, 0·1 mL of the suspension was seeded in tubes of blood agar base containing 10% defibrinated rabbit blood with a liquid phase of 1% glucose in PBS. Two hamsters were inoculated in the footpad with 0·1 mL of each suspension. The cultures and hamsters were observed weekly for the presence of promastigotes and the appearance and persistence of infiltrated lesions, respectively.

Identification of *Leishmania* isolates

Identification was based on isoenzymatic and molecular approaches. The isoenzymatic technique was based on starch-gel electrophoresis using 13 isoenzyme systems, according to Rioux *et al.*[13] The 13 enzyme systems investigated were malic enzyme (ME; EC 1.1.1.40), isocitrate dehydrogenase (ICD; EC 1.1.1.42), 6-phosphogluconate dehydrogenase (PGD; EC 1.1.1.44), glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), diaphorase NADH (DIA; EC 1.6.2.2), nucleoside purine phosphorylases 1 (NP_1; EC 2.4.2.1) and 2 (NP_2; EC 2.4.2.*), glutamate-oxaloacetate transaminases 1 (GOT_1; EC 2.6.1.1) and 2 (GOT_2; EC 2.6.1.1), phosphoglucomutase (PGM; EC 5.4.2.2), fumarate hydratase (FH; EC 4.2.1.2), mannose-phosphate isomerase (MPI; EC 5.3.1.8) and glucosephosphate isomerase (GPI; EC 5.3.1.9). The molecular approach was based on the sequencing of both strands of a 567-bp region of the RNA polymerase II large subunit gene using primers RP720-D 5′-AAGTACCAGCAGTCCCTCATC-3′and RPOII-FOR 5′-GCAGCCGCACAATGCGCT-3′. Sequences were compared with a personal data bank of various *Leishmania* species.[13]

Results

The 16 patients comprised 13 males and three females, with a mean age of 27 years. Their disease duration ranged from 1·5 to 30 years. All of the patients had received multiple cycles of treatment with pentavalent antimonials, immunotherapy and, in two cases, amphotericin B, with limited improvement and subsequent relapse. The length of treatment with miltefosine was variable, as shown in Table 1. *Leishmania amazonensis* was identified in 11 cultures, *L. mexicana* in two, and three were not definitively identified.

Before beginning therapy with miltefosine, 13 patients presented papules, plaques and erythematous nodules, symmetrically distributed over the entire body surface (Fig. 1). Three patients presented erythematous plaques asymmetrically located on the face, arms and legs. The leishmanin reaction was negative in all of the patients and the histological sections showed a vacuolated macrophagic granuloma invaded by

Table 1 Miltefosine in the treatment of diffuse cutaneous leishmaniasis, first cycle of treatment

Patient	Age (years)	Weight (kg)	Daily dose (mg)	Day of suspension of treatment	Molecular and isoenzymatic identification of *Leishmania*, MON
1	32	60	150	120	*L. amazonensis*, MON – 41
2	15	49	150	75	*L. amazonensis*, MON – 41
3	45	66	150	101	*L. amazonensis*, MON – 41
4	20	62	150	70	*L. amazonensis*, MON – 41
5	19	75	150	75	*L. amazonensis*, MON – 41
6	33	59	150	111	*L. amazonensis*, MON – 41
7	14	30	100/50	84	*L. mexicana*, MON – 40
8	48	65	100	111	*L. amazonensis* (contaminated culture of the strain)
9	51	70	150	218	*L. mexicana*, MON – 40
10	22	70	150	190	*L. amazonensis*, MON – 41
11	38	58	150	190	*L. amazonensis* (contaminated culture of the strain)
12	40	80	150	190	*L. amazonensis*, MON – 41
13	33	51	150	140	*L. amazonensis*, MON – 41
14	10	30	50 × 40 days; 50/100[a]	190	*L. amazonensis*, MON – 41
15	3	19	50	114	*L. mexicana* (contaminated culture of the strain)
16	8	20	50	73	*L. amazonensis*, MON – 41

[a]Patient received 50 mg daily for 40 days, then 50 or 100 mg on alternating days.

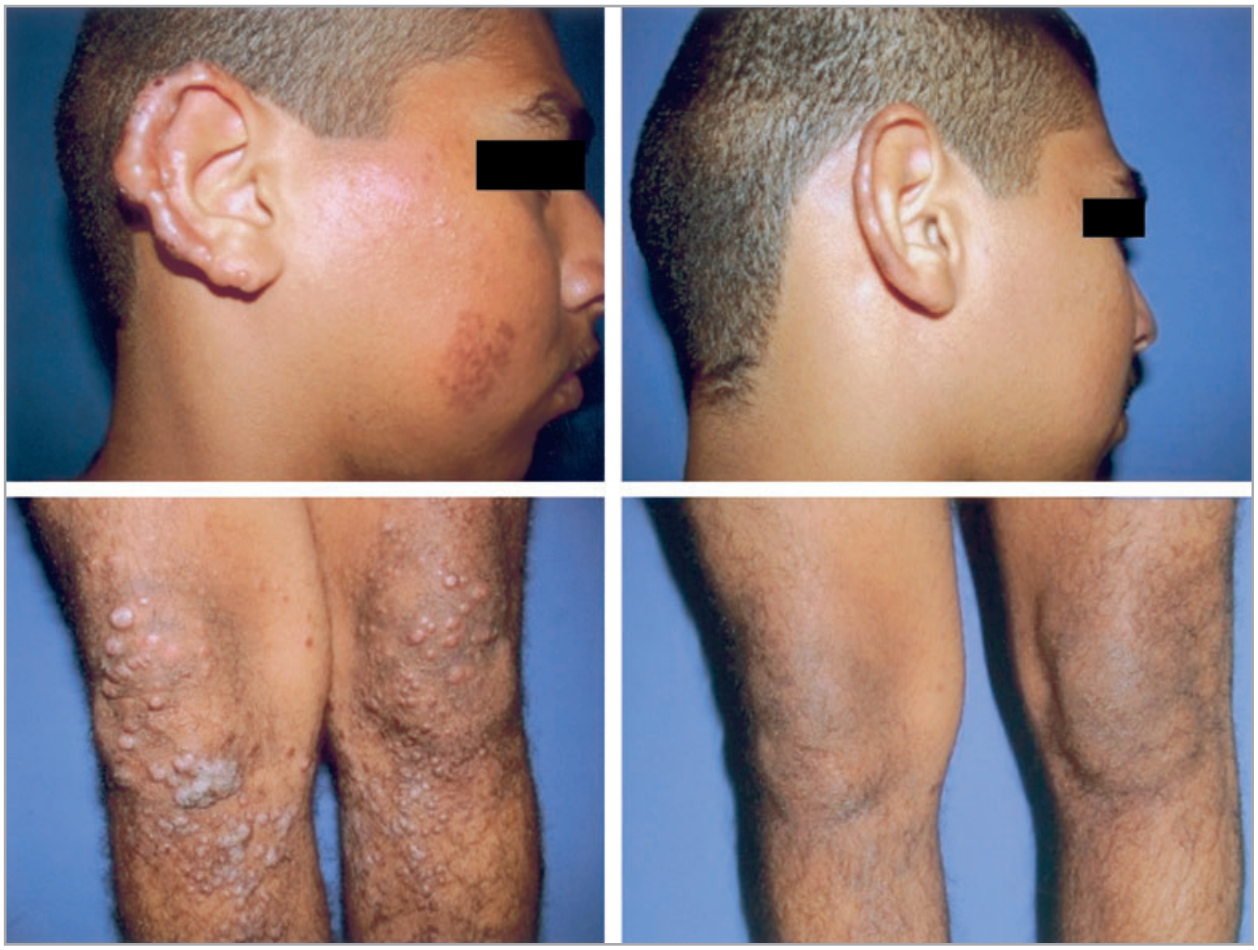

Fig 1. Patient 5 on day 0 (left) and during remission after treatment with miltefosine (right). Note prominent nodular lesions on the ears, cheeks and knees and legs before treatment, flattened and essentially without scarring after treatment.

numerous lymphoid and plasma cells, with numerous *Leishmania* amastigotes in the macrophages (Fig. 2).

Evolution

We developed a clinical scale to evaluate clinical improvement, including elements of inflammation, extension or reduction of affected areas, development of new lesions, and formation of scar tissue. Clinical improvement was estimated to be 30% in 13 patients and 50–65% in three patients after 15 days of treatment. At day 30, 15 patients presented improvement >60%, and one continued with about 30% improvement. At day 45, 15 patients showed 80–90% improvement and one continued to show modest improvement. By day 75, 10 patients showed 100% improvement and in five, improvement was estimated at 80–95% (Table 2).

In histological sections, at day 15 we observed evidence of macrophage differentiation, reduced cellularity and fewer parasites. By day 30, the granulomas showed very few parasites (Fig. 3). The granulomas disappeared in the majority of the patients by day 75 and the only observation was nonspecific perivascular inflammatory infiltration.

In skin smears, parasites were observed until day 30 in six patients, until day 45 in seven and until day 60 in two. One remained positive until day 190, when treatment was suspended in the absence of a detectable therapeutic response.

Growth of parasites in culture medium was observed in 100% of the patients before beginning treatment. Fifteen days after beginning treatment with miltefosine, only three cases gave positive growth in culture medium.

Inoculation in hamsters was positive in the 16 patients before therapy was begun and remained positive until day 45 in four. Biopsy material of six patients produced lesions until day 60, one case until day 70, one until day 120 and one until day 190, when treatment was suspended (Table 2).

Side-effects were observed in four patients: two complained of nausea and vomiting and reduced the dose without consultation, with improvement in symptoms. One patient presented dizziness on the second day of treatment. On day 20 one patient presented urticaria, that improved with systemic antihistamines. None presented alteration of laboratory parameters during the study. One patient, a 15-year-old boy, died during the course of this study with a clinical diagnosis of pneumonia. He had no haematological, hepatic or renal alterations

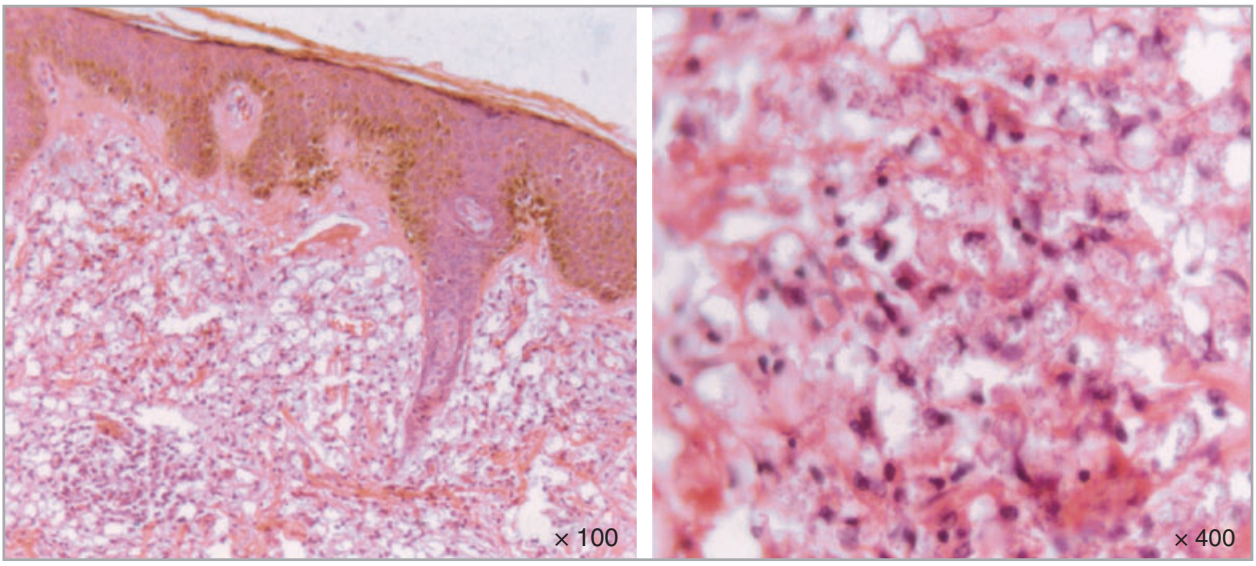

Fig 2. Histological sections before initiation of treatment with miltefosine.The granuloma is composed primarily of cells with large vacuoles containing enormous numbers of amastigotes. Haematoxylin and eosin; original magnification × 100 and × 400.

Table 2 Miltefosine in the treatment of diffuse cutaneous leishmaniasis, first cycle of treatment

Patient	100% clinical improvement (day)	Parasites detected in smears and biopsies (day)	Positive inoculation in hamsters (day)	Day of relapse after treatment	Reported side-effects
1	75	45	120	140	None
2	60	45	60	87	None
3	75	30	75	156	None
4	60	30	45	70	None
5	75	45	60	83	None
6	60	45	60	64[a]	Nausea, vomiting
7	75	60	60	30	Nausea, vomiting
8	90	45	60	95	Nausea, vomiting
9	75	45	45	156	None
10	105	60	45	70	None
11	105	30	61	No, 1 year	None
12	90	60	45	No response, 14[b]	None
13	85	45	30	Patient lost	None
14	Only 50% improvement	190	190	No improvement	None
15	65	63	15	114[c]	None
16	57	43	15	30	Nausea, vomiting

[a]New lesions appeared on day 64 of the second cycle of treatment. [b]Did not respond to a second cycle of treatment. [c]New lesions during treatment, first cycle.

when examined 15 days earlier and exhaustive review of this case has not shown any relationship of this death to DCL pathology nor to treatment with miltefosine.

Treatment was suspended in the first nine patients at 3 weeks after the disappearance of lesions and parasitological negativity in smears and histological sections. Nevertheless, new lesions appeared between 30 and 120 days after the suspension of treatment; therefore it was decided to continue treatment for a longer period, 6 months, in the other seven.

Twelve patients presented relapse after the first cycle, including those who received 6 months of treatment. A second cycle was administered and five did not respond, with persisting clinical lesions of DCL and presence of parasites in skin smears (Table 3). The period of administration in each cycle was variable; new cycles of treatment were initiated when relapse was detected. Five patients have received a third cycle, with appearance of new lesions when treatment was suspended (Table 4).

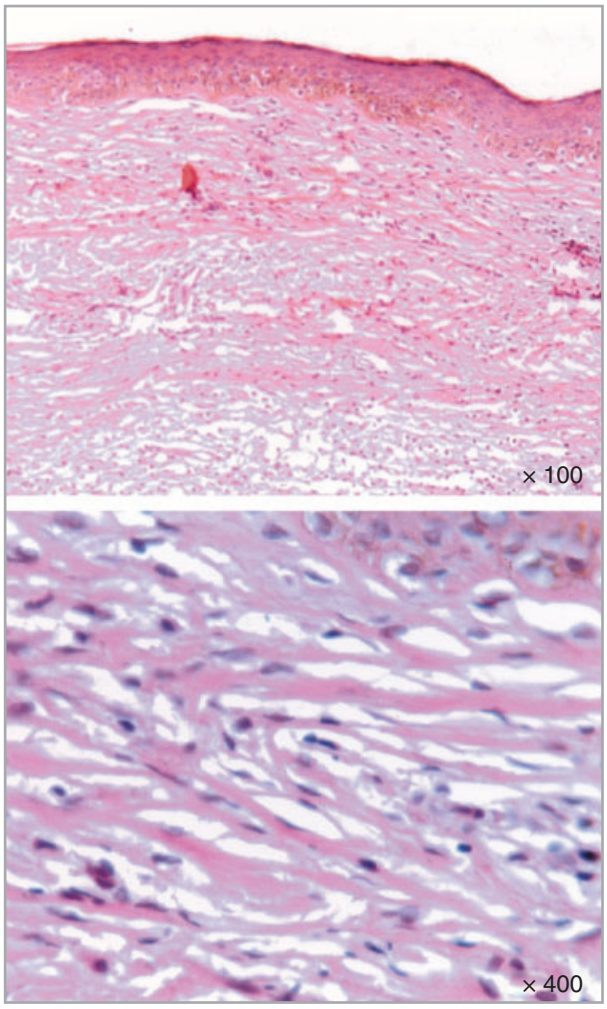

Fig 3. Histological sections at 30 days after initiation of treatment with miltefosine. The granulomatous structure is largely resolved and parasites cannot be identified. Haematoxylin and eosin; original magnification × 100 and × 400.

Table 3 Treatment of diffuse cutaneous leishmaniasis with miltefosine, second cycle

Patient	Age (years)	Weight (kg)	Daily dose	Day of suspension of treatment	Reported side-effects
1	32	60	150	141	None
2	15	49	150/100	111	Nausea
3	45	66	150	36	None
4	20	62	150	183	Nausea
5	19	75	150	50	None
6	33	59	150	103	None
7	14	30	100/50	97	Vomiting
8	48	65	100	15	Nausea, vomiting
9	51	70	150	30	None
10	22	70	150	29	None
12	40	80	150	58	None

Table 4 Treatment of diffuse cutaneous leishmaniasis with miltefosine, third cycle

Patient	Age (years)	Weight (kg)	Daily dose	Day of suspension of treatment	Reported side-effects
1	32	60	150	66	None
2[a]	15	49	150	29	None
3	45	66	150	38	None
4	20	62	150	36	Vomiting
5	19	75	150	103	None

[a]Patient 2 died during the course of the third cycle of treatment, apparently from pneumonia (under investigation). He had been evaluated by us 15 days before his death and no haematological, hepatic or renal alterations were present.

In biopsies obtained from these relapsed patients we observed the presence of granulomas formed by vacuolated macrophages containing numerous parasites. Therefore the second group received additional treatment for 6 months. These patients also presented new lesions 30 days after finishing treatment.

The leishmanin reaction remained negative in 15 patients after treatment was terminated; a single patient who has not relapsed had a leishmanin reaction of 13 mm at 1 year after treatment was completed.

Discussion

DCL is a rare form of presentation of leishmaniasis characterized by multiple nodules or nonulcerated plaques that may progress and may eventually cover much of the body surface. This disease manifestation is associated with the absence of manifestations of cell-mediated immunity to the *Leishmania* parasite.[1] The anti-*Leishmania* treatments used against DCL reduce the initial high parasite burden, but the immune response to the causal agent is not modified, and persisting parasites produce new lesions.

In this study we investigated the use of miltefosine in the treatment of DCL because of the success reported *in vitro* against *Leishmania*,[14] as well as in therapy of VL in India and of localized cutaneous leishmaniasis (LCL).[11,15] The failure of healing observed in our patients and reported frequently in the literature using conventional anti-*Leishmania* drugs also seemed to justify the trial with miltefosine.

The patients treated with miltefosine showed dramatic clinical improvement beginning 15 days after treatment. Nevertheless, complete disappearance of lesions was only observed beginning on day 60, and it was decided to prolong the treatment time in these patients. This observation is in marked contrast with the report of Soto *et al.*[15] in LCL treated with miltefosine, in which clinical improvement was reported at 4 weeks of treatment. This difference probably reflects the marked difference in the granulomatous structures and components in the two forms of the disease. Cell-mediated hyper-

sensitivity, with important cellular components contributing to LCL lesions, would not be expected to respond significantly to miltefosine, which may partially explain the slower response in LCL.

In our experience the complete clinical improvement of patients with DCL treated with pentavalent antimonials is only observed after four cycles of treatment (cycles of 20 days with periods of 10 days of rest, in doses recommended by the World Health Organization). Nevertheless, these patients inevitably relapse after chemotherapy.

The dose of miltefosine used in this study was 2–2·5 mg kg^{-1} daily. Two patients took lower doses after 15 days because of side-effects. In these patients the clinical improvement was slower and more discrete. This observation coincides with that of Soto *et al.* in LCL, where the group that received 150 mg daily showed a higher percentage of cure than those who received lower doses.[11] Similar findings were reported in VL, where the group of patients treated with 100 mg daily for 4 weeks presented cure rates of 97% and the group that received 50 mg daily for 6 weeks had a cure rate of 93%.[10] These observations suggest the importance of the use of precise doses in treatment schemes, to avoid therapeutic failures and possible development of resistance to the drug.

The reduction in the parasitic burden was related to clinical improvement beginning on day 15 in skin smears and tissue sections. Nevertheless, the more sensitive method of animal inoculation showed the persistence of parasites until day 120 in a patient who showed clinical improvement of 100% at day 75 and disappearance of parasites in smears and histological sections at day 45. This observation clearly demonstrates the persistence of live parasites despite dramatic clinical and parasitological improvement.

Histopathologically, the initial granulomas formed by parasite-filled vacuolated macrophages began to show important changes beginning from day 15, with important macrophage differentiation and decrease of the parasite burden. By day 75 the granulomas had completely disappeared, demonstrating the energetic antiparasitic action of this drug.

In contrast to what we have observed with other anti-*Leishmania* drugs such as pentavalent antimonials and amphotericin B, in which severe side-effects are reported varying from local manifestations to alterations in cardiovascular, hepatic and renal function,[16–18] severe side-effects requiring the suspension of therapy were not observed with miltefosine despite the prolonged period of treatment. Laboratory values remained within normal limits during the entire study.

The leishmanin reaction remained negative after terminating the treatment, suggesting that the use of miltefosine and profound reduction in the parasite burden was not accompanied by an alteration in the immunological response of the host to *Leishmania*. This same observation has been reported subsequent to the use of other therapies in DCL.[4]

As reported with other drugs used in the treatment of DCL, the appearance of new lesions was observed after terminating treatment with miltefosine in patients treated for 70–120 days and in those treated for as long as 180 days. Some patients developed lesions during the course of treatment, which clearly suggests that parasite resistance may have developed during this prolonged treatment.

The absence of changes in the immune response and the persistence of parasites demonstrated in the inoculation in experimental animals clearly suggest the probability of relapse, and not re-infection, as the cause of new lesions.

In this study, miltefosine showed dramatic clinical and parasitological effects during the period of administration of the drug, with few side-effects. The quality of life of these patients was vastly improved and they were able to develop a normal routine of study or work. Nevertheless, apparent development of parasite resistance in some patients and other ethical considerations did not permit indefinite sustained treatment; relapse after suspension of treatment represented a very difficult situation for patients and attending physicians. The absence of immunological reactivity to *Leishmania* in these patients and those treated with other protocols clearly suggests that treatment of this very challenging pathology must be oriented toward a more intensive search for drugs, immunotherapy or even more complex genetic approaches to correct the profound specific immunological deficiency associated with DCL.

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