BEHAVIOR IN A MOUSE MODEL OF ISOLATES OF *LEISHMANIA DONOVANI* SENSU LATO CULTURED FROM THE BLOOD OF PATIENTS WITH CHRONIC CUTANEOUS LESIONS

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**Abstract.** Our objective was to characterize biologically in an animal model two isolates of *Leishmania* parasites unexpectedly encountered in the circulating blood of two patients with chronic cutaneous leishmaniasis. Both isolates were classified by cellulose acetate electrophoresis as belonging to the *L. donovani* sensu lato complex. We elected to use BALB/c mice, an inbred strain that has been proven to be very sensitive to the dermotropic parasite *L. major*. This study demonstrated that for the same number of parasites, the course of infection with the *L. donovani* strain was different from that developed in similar animals that received the *L. major* strain. After a protracted incubation period, *L. donovani* produced scanty cutaneous lesions and mainly a systemic disease. This is in contrast to the rapidly spreading skin lesion that kills *L. major*-infected animals within a few months. It is concluded that BALB/c mice are an adequate animal model for the *L. donovani* strain, which had an unusual clinical presentation in humans. The prolonged incubation period, such as observed here, may lead to erroneous conclusions of host resistance if the experiment were terminated based on clinical manifestations caused by the Old World protozoan parasites of the genus *Leishmania*. The most common and economic animal model is undoubtedly the mouse. The best defined system for leishmaniasis is *L. major* in the BALB/c syngeneic strain of mice. It is a stable and highly reproducible model.

The present work is concerned with two isolates of *Leishmania* obtained from blood culture from two patients with chronic cutaneous lesions that were described elsewhere. Since the beginning of this study, several additional isolates of apparently the same strain have been made. Isoenzyme patterns determined by cellulose acetate electrophoresis showed that these parasites belonged to the *L. donovani* complex. The behavior of the two initial isolates in BALB/c mice was compared with the pathology caused by a reference stabilate of *L. major* in the same strain of mice.

The importance of animal models in the study of human disease problems cannot be overemphasized. Several animal models have been developed for the different disease manifestations caused by the Old World protozoan parasites of the genus *Leishmania*. The most common and economic animal model is undoubtedly the mouse. The best defined system for leishmaniasis is *L. major* in the BALB/c syngeneic strain of mice. It is a stable and highly reproducible model.

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**MATERIALS AND METHODS**

**Mice.** Male BALB/c mice (4–6 weeks old) were used in all experiments. All experiments were performed twice. The initial stock of mice was obtained from Zentral-Institut fur Versuchtiere Lettow (Hannover, Germany) and bred and maintained in the Animal Facility of the American University of Beirut in an insect-free room.

Experimental animals were divided into four groups of 20 mice each. For each group, the experiment was done twice, giving a total of 40 mice per group. Group A received the FL isolate of *Leishmania*, group B received the UKh isolate, group C received *L. major*, and group D were maintained as controls. The control group was injected with 0.1 ml of an excipient composed of Hanks' balanced salt solution, pH 7.2–7.4, adjusted with HEPES buffer (both obtained from Gibco/BRL Life Technologies, Gaithersburg, MD). In the experimental groups, all inoculations consisted of 7 × 10^6 promastigotes suspended in 0.1 ml of Hanks' balanced salt solution, pH 7.2–7.4. Mice were injected subcutaneously in the dorsum at the base of the tail. The animals were examined weekly and clinical signs of disease were noted. Once a lesion formed, its size was monitored by measuring its widest diameter. Moribund or sick animals were killed with ether. All dead animals were necropsied and the livers were checked for the presence of parasites using Giemsa-stained impression smears.

**Parasites.** *Leishmania major*, classified as MHOM/IL/67/Jericho II, was obtained from the Liverpool School of Tropical Medicine. The parasite isolates are designated FL and UKh and were obtained from blood cultures of two patients who were the subjects of an earlier clinical report. Briefly, the FL isolate was obtained from a 60-year-old woman referred to us because of activity associated with a 20-year-old dry, hyperemic, hyperkeratotic skin lesion. The lesion occupied the center of her forehead and measured 8 × 4 cm and covered the glabella and extended down to the lateral aspects of the nasal bridge. The patient was worried because she noticed some wetness at the edges of the lesion near the curuncles. The UKh isolate was obtained from a 49-year-old woman who came to us because of three-year-old skin lesions over the forehead and the right cheek. Both lesions started as small papules that grew gradually to 2 × 3.5 cm and 1 × 1 cm over the forehead and cheek, respectively. Both were brownish-red and covered by an atrophic skin. In both patients, the remainder of the physical examination results were negative. Both patients failed to improve on a course of antibiotics and antifungal therapy. The findings of a skin biopsy substantiated the presumptive diagnosis of chronic cutaneous leishmaniasis. *Leishmania* sp. parasites were obtained from the blood cultures but not from cultures of skin biopsies. The isolates were classified as *L. donovani sensu lato* by isoenzyme patterns obtained by electrophoresis of parasite samples on cellulose acetate strips. In an earlier investigation using only 12 enzymes, these isolates were designated Salti 5 and Salti 6 MHOM/LB/84/FL and MHOM/LB/84/UK, respectively, and were found to be iden-
Behavior of three different isolates of *Leishmania* parasites in three experimental groups and one control group of 40 mice each*  

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* A = FL; B = UKh; C = *L. major*; D = healthy controls; f = facial alopecia; t = total alopecia.

The parasites have been maintained in the laboratory on modified Nicolle-Novy-McNeal (NNN) medium overlaid with Lock’s solution supplemented with antibiotics and heat-inactivated 10–15% fetal bovine serum. Promastigotes are periodically injected into hamsters (Charles River SPA, Cel- co Como, Italy) and alternately cryopreserved. Both the UKh and FL isolates required 5–8 months to become dense enough in a primary culture to allow subculturing (a few of the isolates behaved this way). Subsequently, passage onto fresh media had to be carried out every 18–24 days because of their sluggish growth. Animal inoculation into hamsters was followed by cryopreservation from in vitro cultures that did not exceed seven passages. The UKh and FL isolates were obtained after cryopreservation at −84°C for periods not exceeding six months when the parasites were recultured from the cryogenic medium on NNN medium slants. After six or seven passages in vitro (4–5 months) and to safeguard their virulence, these organisms were inoculated into hamsters. Sick animals were killed and the parasites were isolated from their spleens in vitro for another round of propagation. Once cultures reached a high density, the cycle of cryopreservation and inoculation into animals was repeated as necessary, especially since cryopreservation at −84°C has a limited longevity.

**RESULTS**

Table 1 summarizes the findings on the four groups of animals that have been combined in duplicate experiments. Our findings are expressed as absolute numbers.

The control group (group D) injected with excipient alone had a normal life span; five animals died (two at 20 months and three at 30 months). Ten of the remaining 35 control animals had mild facial alopecia at week 36 after injection with the excipient. None of the animals in this group exhibited any abnormality at necropsy in the gross and microscopic examination of the abdominal viscera.

The illness in the group (C) infected with *L. major* followed a dramatic course starting with a skin lesion appearing at the injection site between 16 and 28 days after injection. Most (30 of 40) had detectable lesions three weeks after infection. The lesions started as small nodules that soon ulcerated and began to spread over the caudal part of the back and extended posteriorly over the perianal skin and often ventrally up to the urethra (in 37 of 40 animals). The tail soon became involved and its entire skin replaced by a scab (Figure 1). Between the eighth and sixteenth weeks postinfection, the rest of the integument became infected as the fur became sparse and the animal appeared wasted with a hunched back. The eyes became involved, with either the eyeballs beginning to bulge or the eyelids becoming inflamed and producing a smaller aperture. Within 16 weeks, the animal was so moribund that it either died or was killed. Examination of impressions of either liver or spleen samples from these animals demonstrated amastigote forms of parasites both intracellularly and extra cellularly. Fusiform-shaped organisms reminiscent of the promastigote forms were observed extracellularly.

The course of illness in groups A and B (injected with isolates FL and UKh, respectively) exhibited little difference among the animals in either group. The earliest signs
of disease (7–8 months after inoculation) were loss of fur and dermatitis at the inoculation site. These signs developed in a minority of the animals in groups A and B (an average of six in the two groups in the first experiment and eight in the same groups in the duplicate experiment). A smaller number of mammals (six in groups A and B) in the first experiment and three in both groups in the duplicate experiment showed a small ulcer at the site of inoculation. In two of these six animals, the ulcers were ectopic (over the lateral aspect of the shoulder or on the flank) in two animals (Figure 2), but such lesions never exceeded 0.5 cm in diameter. The skin around the lesion was injected, and the rest of the integument in these animals and in all experimental groups (except for group C) was normal 7–8 months after inoculation. The rest of the experimental animals started to develop signs of sickness 13–18 months after the inoculation, irrespective of whether they received isolate FL or UKh. They began to lose their fur, which became more sparse all over the animal (starting with the area around the face and muzzle).

Of the 40 experimental animals in group A, seven died spontaneously without any sign of morbidity at 20–30 weeks after injection. In group B, four died similarly at 21–28 weeks after injection. The rest survived until they were emaciated, lost a good deal of fur, and acquired a hunched back, and finally died at 26–28 weeks after injection (Figure 3). In the duplicate experiment, two animals in group A died without any signs of morbidity at about the 30th week and one from group B died at about 24 weeks postinjection with no apparent pathology.
DISCUSSION

In a region that has been endemic for visceral leishmaniasis for centuries and with sporadic cases reported from different areas in the country, the isolation of *L. donovani* from cases with only skin lesions was not expected, although the number of reports in the literature on *L. donovani* and *L. infantum* causing cutaneous disease is increasing.3±14 However, post-kala-azar dermal leishmaniasis (PKDL) cannot be ruled out. Although both of our patients had no history of systemic involvement and PKDL has not been reported in this geographic area, it is possible that both patients may have contracted a subclinical systemic disease resulting in PKDL. This situation is not uncommon in endemic regions.11, 13, 15±19 In one patient, the parasites were not detected by either histopathology or culture of the skin lesions, a situation similar to that of recent reports from this country of *L. infantum* sensu lato causing cutaneous leishmaniasis, with the source of the parasite being the blood stream.1, 2 The source of the parasites in both cases described here was the blood stream. Unfortunately, blood is rarely if ever tested for parasite growth in PKDL or in any other form of cutaneous leishmaniasis.

Our strains were classified as *L. donovani sensu lato* consistent with the fact that their zymodeme patterns were not identical to any of the established strains. Their isoenzyme profiles were similar to, although not identical with, those of *L. donovani donovani* or *L. donovani chagasi*. This feature was reflected clinically by their unusual presentation in both patients.

Although we have not studied the behavior of these two isolates in dogs, the disease pattern cause by each of these isolates in the murine model mimics the behavior of similar parasites in dogs.20 It also closely resembles clinical syndromes associated with these parasite strains in humans, i.e., a prolonged incubation period.18, 19 Therefore, the advantages offered by the BALB/c mouse model are several. First, it is a familiar model to most researchers in the field. Second, being a well-established model in the literature for *L. major* and *L. donovani*, our results can be compared with those obtained by other investigators for strains that cause well-defined pathology in humans (either cutaneous or systemic disease). Finally, this model offers an opportunity to characterize a strain that may be the cause of subclinical systemic disease in humans. The occurrence of this parasite is not a rare event because we subsequently isolated the same strain from skin lesions and/or the blood stream of a number of patients.2

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