PARASITIC LOAD AND HISTOPATHOLOGY OF CUTANEOUS LESIONS, LYMPH NODE, SPLEEN, AND LIVER FROM BALB/c AND C57BL/6 MICE INFECTED WITH LEISHMANIA MEXICANA

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Abstract. The course of infection, parasitic loads, and histopathology of cutaneous lesions, draining lymph node, spleen, and liver were compared in BALB/c and C57BL/6 mice over a period of 34 weeks after inoculation in footpad with promastigotes of a Leishmania mexicana reference strain. The results show that the primary footpad lesions first present a 12-week phase that develops similarly in both strains of mice. Thereafter, a cutaneous and visceral dissemination of L. mexicana parasites occurs in BALB/c mice; the latter experience an extensive breakdown of the lymphoid organ microarchitecture, whereas C57BL/6 mice succeed in eliminating the parasite infection from the lymph nodes but not from the primary cutaneous lesion, which does not heal. These results highlight marked differences between responses of key anatomical compartments controlling L. mexicana infection in BALB/c and C57BL/6 mice.

INTRODUCTION

Leishmania are protozoa parasites that cause a spectrum of cutaneous, mucocutaneous, or visceral clinical manifestations in humans, depending on the parasite species and the host’s immune response and genetics. The agents of American cutaneous leishmaniasis belong to the L. braziliensis and L. mexicana species complexes. The species of the former complex are associated with mucocutaneous diseases, rarely seen in L. mexicana human infections. Infection with the species L. mexicana, found in patients from Central and South America, as well as from the southern United States, may be subclinical or induce cutaneous lesions (frequently located on the ear and named “chiclero’s ulcer”), which may persist for months or years. More rarely, diffuse cutaneous lesions resistant to chemotherapy or, as recently shown, visceral leishmaniasis may also be induced by L. mexicana.

Inbred mouse strains are proving to be valuable models to study the genetic and immunological factors involved in resistance and susceptibility to Leishmania infections; attention has been primarily focused on L. major disease. Indeed, CBA, C3H, C57BL/6, and B10.D2 mice infected with L. major develop cutaneous lesions that spontaneously resolve, whereas BALB/c, SWR/J, DBA/2, and A/Jax mice exhibit nonhealing primary cutaneous lesions susceptible to dissemination. Far fewer studies have been performed on L. mexicana experimental infection, though various works suggest important differences in the factors involved in the resistance and susceptibility of L. major and L. mexicana infections.

The course of L. mexicana cutaneous infection is known to be different in BALB/c and C57BL/6 mice. However, the parasite spreading and the related lesions in other organs than the skin have not been previously explored in these mouse strains, which are used the most to study L. major infection. Because organ-specific responses to parasite invasion seem decisive in disease progression, we aim to compare the course of infection, as well the parasitic loads and histopathology of key anatomical compartments controlling the parasite spreading, namely the cutaneous lesions, draining lymph nodes (LN), and spleen or liver in BALB/c and C57BL/6 mice infected with a reference strain of L. mexicana during 34 weeks.

MATERIAL AND METHODS

Parasites. Promastigotes of L. mexicana (strain MHOM/BZ/82/BEL21) were cultured in RPMI 1640 medium (Life Technologies, Merelbeke, Belgium), supplemented with 10% fetal calf serum (Life Technologies), penicillin G (100 U/mL), and streptomycin (100 μg/mL). Parasites harvested in stationary phase after 8–10 days of culture were centrifuged (2,500 × g, 10 min, 4°C) and washed 3 times in RPMI 1640 before being counted and used for inoculation to animals.

Mice, Leishmania infection, lesion monitoring, and tissue processing. Male BALB/c and C57BL/6 mice were purchased from Banting and Kingman (Hull, UK). The maintenance and care of mice complied with the guidelines of the Free University of Brussels Ethic Committee for the humane use of laboratory animals. Mice (8–10 weeks old) were infected subcutaneously into the rear left hind footpad with 10⁷ stationary phase promastigotes of L. mexicana a final volume of 25 μL (in RPMI 1640 medium). The contralateral right footpad received an identical volume of RPMI 1640 medium without parasites as internal control. The thickness of infected and uninfected footpads was regularly measured with a vernier caliper, and the difference between both measurements corresponded to the size of the lesion. Mice were also regularly examined to detect cutaneous ulcers and secondary lesions.

At selected time points, some mice of both groups were killed by ether inhalation. Footpad lesions (or normal tissue in controls) cut tangentially to the bone ground, popliteal homolateral draining LN, spleens, and livers were collected to determine their parasitic loads, to perform histological studies, or both (see below). Lesions, LN, and spleens were weighed before being processed.

Quantification of L. mexicana amastigotes in tissues. The parasite burdens in footpad lesions and LN of infected animals were determined after tissue homogenization by staining released amastigotes with acridine orange (Sigma, Brussels, Belgium). This dye renders them easily detectable by fluorescence microscopy by means of the high contrast between the
green nucleus and kinetoplast on the dark background, as previously reported for Plasmodium and Leishmania promastigotes.21,22

Briefly, tissues were cut into small pieces, mechanically teased in a mortar after addition of 5 or 10 mL of RPMI 1640 medium, and homogenized by means of a potter on an ice bath (3 × 2 min at 3,500 rpm). The tissue suspensions were then centrifuged at 280 × g for 5 min at 4°C to precipitate most of the debris and to collect amastigotes in the supernatant. Acridine orange stain was added into the amastigote suspension, either undiluted or diluted 1/5 to 1/10 according to the parasite density (1 μg of stain per 100 μL of suspension) and incubated at room temperature in the dark for 10 min. After centrifugation at 2,400 × g for 5 min at 4°C, the parasites were washed with 5 mL of phosphate-buffered saline, fixed with 100 μL of parformaldehyde (2%;TAAB Laboratories Equipment, Aldermaston, Berkshire, UK), and counted in a Thoma chamber by fluorescence microscopy (Leica, Heerbrugg, Switzerland). Such quantification of acridine orange-stained amastigotes was shown to be reproducible (variation coefficient < 5%) and accurate for parasite loads up to 16 × 10⁴ amastigotes/mL. In order to facilitate comparisons of data, the results were expressed per milligram of tissue.

**Histological studies.** Footpad tissues, LN, spleens, and livers were fixed in 10% formalin and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin-eosin-saffron to study their microarchitecture by light microscopy. The number (in spleens) and location (in footpads, LN, spleens, and livers) of intracellular parasites (amastigotes) was estimated in 4 sections per organ. Because the size of spleen sections differed considerably during the course of L. mexicana infection, the surface area of each individual section was measured and the quantitative results were expressed per square millimeter of tissue. Photomicrographs were taken with a Leica Orthoplan microscope.

**RESULTS**

The course of L. mexicana infection in BALB/c and C57BL/6 mice. As shown in Figure 1, during roughly the first 3 months of infection, both strains of mice developed primary footpad lesions of similar sizes. Although such lesions in C57BL/6 mice remained stable ~1.5–2.0 mm and did not resolve until 34 weeks postinfection (wpi), those of BALB/c mice continued growing strongly after wpi 12 to reach 10–11 mm (i.e., a mean diameter ~5-fold higher than in C57BL/6 animals) on wpi 34. The cutaneous lesions induced in BALB/c mice became ulcerated, and single or multiple secondary lesions appeared in the contralateral posterior or anterior footpads, the eyelids, the nostrils, the ears, and the base of the tail in most mice from wpi 18 onward (Figure 2A, B). This was never observed in C57BL/6 animals. All C57BL/6 survived L. mexicana infection, whereas 11% of infected BALB/c mice died at wpi 30.

A small increase of LN and spleen weights was apparent on wpi 6 in both strains, whereas considerable popliteal lymphadenopathy and splenomegaly were observed in BALB/c mice on wpi 24 and 34 (Figure 3A, B). At these time points, LN reached 40–45-fold their initial weights in L. mexicana-infected BALB/c, against only 10–13-fold in C57BL/6. At the same time, the spleens underwent a 7–10-fold weight increase in BALB/c, whereas only a slight increase of 1.5- to 2-fold occurred in C57BL/6 mice.

Parasitic load in cutaneous lesions, LN, and spleens of BALB/c and C57BL/6 mice infected with L. mexicana. Amastigote counts were performed in tissues of mice infected during 2, 6, 10, 14, 24, and 34 weeks. As shown in Figure 4A, a regularly progressive multiplication of parasites occurred in the footpad lesions of BALB/c mice, harboring 12.5 × 10⁶ parasites per milligram of tissue at wpi 34 (i.e., roughly 28 × 10⁹ per lesion and 2,800-fold the inoculum size). If similar parasitic loads were observed for the C57BL/6 mice until wpi 14, a significant control of parasite replication was clearly occurring at wpi 24 and 34. However, although the parasite burden was stabilized around 0.51 × 10⁶ parasites/mg (i.e., roughly 1.1 × 10⁶ per lesion and 254-fold less than in BALB/c), resolution of the footpad lesion did not occur. There was a positive significant correlation between parasite burdens and lesion sizes in BALB/c mice (r = 0.95; P < 0.05; data not shown).

As shown in Figure 4B, parasites were more abundant in LN of BALB/c than C57BL/6 mice at wpi 6 (at 7–10-fold lower levels than in respective footpads). At wpi 24, the parasitic load in C57BL/6 was strongly reduced in comparison to BALB/c, and amastigotes were no longer detected in LN at wpi 34. Such a favorable evolution was not observed in BALB/c LN, which remained massively infected at wpi 24 and 34, concomitant with extensive hyperplasia (Figure 3A).

Amastigotes were also detected in histological sections of spleen (Figure 4C) and liver of BALB/c mice at wpi 24 and 34, but never in C57BL/6 mice. Parasites were also abundantly found in the secondary lesions, attesting to the extensive parasite spreading and visceralization of L. mexicana infection occurring in BALB/c, but not in C57BL/6 mice.

**Histopathological studies of cutaneous lesions, LN, spleens, and livers from BALB/c and C57BL/6 mice infected with L. mexicana.** Histopathological studies were performed at 0 (control), 2, 6, 10, 14, 24, and 34 wpi. In the footpad, an
Inflammatory infiltrate consisting mainly of lymphocytes, plasma cells, and eosinophils was present in the dermis of both strains 2 weeks after parasite inoculation. Vacuolated histiocytes containing parasites were seen in the dermal inflammatory infiltrate at 2 wpi, and their number increased to infiltrate the dermis, hypodermis, and the muscle layers at 6 and 10 wpi in C57BL/6 and BALB/c, respectively. A lesion consisting of a large papule formed by vacuolated histiocytes full of parasites and covered by a thin epithelium was seen at wpi 10 in C57BL/6 and from wpi 14 in BALB/c mice. Granuloma-like structures were visible deep in the 14-wpi lesions of C57BL/6, but rarely at wpi 24 and 34 in BALB/c mice (Figure 5A-C). At wpi 24, the C57BL/6 inflammatory infiltrate consisted of lymphocytes, plasma cells, and eosinophils, but slightly fewer infected macrophages contained a reduced number of amastigotes, and fibrosis was visible at wpi 34. Such evolution was never seen in BALB/c mice.

In LN, a follicular hyperplasia appeared at wpi 6 in BALB/c mice, but only at wpi 14 for C57BL/6 mice, with germinal centers larger and more numerous than normal and containing large, pale staining lymphocytes and numerous apoptotic bodies. Plasma cells were increased in the sinuses and in the cortical area of both strains of mice. Vacuolated histiocytes containing parasites were more easily detected around wpi 10–14 and overall at wpi 24 in BALB/c than in C57BL/6 mice. A marked hyperplasia of sinuses and a massive infiltration of LN by infected macrophages with a destruction of germinal centers and a rupture of the LN capsule was seen in BALB/c at 24 and 34 wpi. Interestingly, in some cases, parasite-infected cells were found in postcapillary venules, indicating their presence in the bloodstream. Parasites were mainly found in the cortex of BALB/c LN (64.7% of LN parasites) and less in the medulla (20.8% of LN parasites) and sinus (14.5% of LN parasites) at wpi 24. Parasitized histiocytes appeared mainly in the para cortical LN area of C57BL/6 mice at wpi 24 and no more at wpi 34 (Figure 5D-F).

In spleen, a follicular hyperplasia of the white pulp with increased number and size of lymphatic nodules appeared at wpi 10 and 24 in BALB/c and C57BL/6 mice, respectively. The germinal centers were easily detectable and were sur-
rounded by a large ring of small lymphocytes. Vacuolated histiocytes containing parasites were seen in the red pulp in BALB/c at wpi 24 (Figure 5G) and widely infiltrated the portal tract and the sinusoids of BALB/c mice at wpi 34. No vacuolated histiocytes containing parasites were seen at any step of infection in C57BL/6 mice.

In liver, a portal inflammatory infiltrate with sinusoid dilation was seen as early as wpi 10 in BALB/c mice. Vacuolated histiocytes containing parasites appeared at wpi 24 (Figure 5H) and widely infiltrated the portal tract and the sinusoids of BALB/c mice at wpi 34. No vacuolated histiocytes containing parasites were seen at any step of infection in C57BL/6 mice.

DISCUSSION

Our results show that the infection with *L. mexicana* promastigotes results in a similar development of primary footpad lesions in BALB/c and C57BL/6 mice during the first 3 months of infection. The parasite dissemination occurring later in BALB/c mice, resulting in secondary cutaneous lesions and visceralization of the disease, is associated with hyperplasia and extensive destruction of lymphoid organs that are massively infected. So BALB/c are fully susceptible to *L. mexicana* infection, in agreement with the conclusions of previous studies on the cutaneous lesions induced by other strains of *L. mexicana*.16–19 In contrast, C57BL/6 mice succeed in eliminating parasite infection from LN but not from the primary cutaneous lesions that do not heal. In our experimental conditions, this mouse strain seems therefore less susceptible than BALB/c but not resistant to *L. mexicana* infection, in contrast to most previous reports.16–18

The course of *L. mexicana* infection is markedly different from that previously reported for *L. major*, both in BALB/c (slower development of cutaneous lesions) and in C57BL/6 mice (nonhealing cutaneous lesions).10,23 This likely relates to differences in both the murine genes influencing the outcome of these infections12–14 and the immune responses directed by the individual pathogen species. Interestingly, we have recently reported that the slower development of BALB/c lesions in *L. mexicana* than in *L. major* infection is likely related to the fact that the T cells, recognizing specifically the LACK (*Leishmania* homologue receptor for activated C-kinase) antigen, do not play a critical role in *L. mexicana* infection.23 Indeed, such cells are responsible for the susceptibility of BALB/c mice to *L. major* infection because they produce interleukin-4 early in infection leading to the subsequent development of a counterprotective, parasite-specific Th2 immune response.24,25 By contrast, the course of *L. mexicana* cutaneous infection is akin to that previously reported for *L. amazonensis*, a closely related species belonging to the same American *Leishmania* complex26,27 and also inducing murine immunological responses different from those generated by *L. major*.28,29

The early presence of eosinophils in cutaneous *L. mexicana* lesions of BALB/c and C57BL/6 mice agrees with previous observations in another strain of mice.30 Indeed, *Leishmania* parasites are able to produce eosinophil chemotactic factor31 and activated eosinophils can produce nitric oxide and thereby kill *Leishmania* parasites.32 So, eosinophils, besides other mechanisms depending on activated macrophages,33 might contribute to the development of primary cutaneous lesions and parasite burdens in skin during the three months of *L. mexicana* infection in both BALB/c and C57BL/6 mice.

The marked differences occurring in the development of lesions and parasite spreading in the second period of infection (after wpi 12) between BALB/c and C57BL/6 mice indi-
FIGURE 5. Histopathology of footpad (A–C), lymph nodes (LN) (D–F), spleen (G), or liver (H) from C57BL/6 (A, D) or BALB/c mice (B, C, E–H) infected with *Leishmania mexicana*. Tissues were collected at 24 weeks postinfection and the sections were stained with hematoxylin-eosin-saffron. Vacuolated macrophages containing *Leishmania* amastigotes were abundant in footpad lesions of BALB/c mice (C, circles and arrows). If the microarchitecture of C57BL/6 LN was globally preserved (D), that of BALB/c LN showed a massive infiltration with infected macrophages (F, circles and arrows). Vacuolated macrophages containing *Leishmania* amastigotes were also seen in the spleen (G, circle and arrow) and the liver (H, circles and arrows) of BALB/c mice. Bars in A and B = 150 μm; bars in D and E = 75 μm; bars in C and F = 30 μm; bars in G and H = 20 μm.
cates a late and profound change, stopping or suppressing the ongoing mechanism or mechanisms limiting the first period of infection in BALB/c mice. It remains to be elucidated whether the massive infiltration of LN and spleen, the key organs controlling the parasite dissemination,\textsuperscript{1,2,3} by *L. mexicana*-infected macrophages during the second period of BALB/c infection is the cause or the consequence of the final breakdown of the microarchitecture of T- and B-cell areas. Interestingly, similar destruction of lymphoid tissues is also observed in infection with *L. donovani*, the agent of visceral leishmaniasis.\textsuperscript{3,4,5} Moreover, the destruction of the LN capsule leading to the invasion of capillaries by *L. mexicana*-infected macrophages, as well as the high density of parasites observed in the red pulp of spleen, agree with a bloodstream dissemination of parasites to other internal organ or organs (viscerализation of the disease) and likely to other cutaneous sites (secondary lesions).

The C57BL/6 cutaneous lesions induced at the site of inoculation, although slightly decreasing in size over time, did not spontaneously heal, a finding that is in agreement with our data on parasitic loads also remaining stable over time. The occurrence of cutaneous fibrosis and granulomatous inflammation, which are generally associated with a healing process, and the total elimination of parasites in LN at wpi 34 did not exclude a later healing process that might be finalized after our observation period of 34 wpi. The mechanism or mechanisms of such persistence of parasites in C57BL/6 skin lesions also remain to be studied.

In conclusion, our long-term studies of cutaneous lesions, LN, spleens, and livers in *L. mexicana* experimental infection show that the responses of the key anatomical compartments controlling parasite spreading are markedly different in BALB/c and C57BL/6 mice. Some of these responses are different from those previously reported in *L. major* infection,\textsuperscript{10,11,12} and others are more akin to those reported in *L. donovani* infections.\textsuperscript{3,4,5} Because *L. mexicana*-infected BALB/c and C57BL/6 mice present clinical and histopathological features similar to those found in the human cases,\textsuperscript{4,8,9} these strains of mice can therefore be considered as putative models of the polarized clinical spectrum observed in *L. mexicana* human disease for further genetic and immunological studies.

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