Research Article ISSN: 2836-3647

American Journal of Pathology & Research

Histopathological Nature of the Tissue that Develops in the Plantar Pad of BALB/c Strain Mice When Inoculated with *Leishmania mexicana* and its Relevance in the Life Cycle of the Parasite

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Received: 10 Apr 2026; **Accepted:** 15 May 2026; **Published:** 24 May 2026

Citation: Ofelia Pérez-Olvera, Luz Graciela Cervantes-Pérez, Ricardo Eloi Arellano Pérez, et al. Histopathological Nature of the Tissue that Develops in the Plantar Pad of BALB/c Strain Mice When Inoculated with *Leishmania mexicana* and its Relevance in the Life Cycle of the Parasite. American J Pathol Res. 2026; 5(3): 1-6.

ABSTRACT

Leishmaniasis is one of the most common tropical diseases worldwide. There are three main forms of leishmaniasis: visceral, the most severe; cutaneous, the most common, which generally causes skin ulcers; and mucocutaneous. We reproduced the disease by inoculating the plantar pad of BALB/c mice with the species Leishmania mexicana. No morphological studies have defined or identified the histopathological nature of this murine model; therefore, we decided to study and describe it using histological, immunohistochemical, and immunofluorescence techniques. We found that it is a lipoma in which adipose tissue is an important histological component, and in this case, it is populated by Leishmania mexicana in its amastigote form. Generally, we know that adipose tissue performs metabolic functions such as energy storage, which leads us to recognize the role it plays in the reproduction and migration of the parasite. To reinforce these histopathological studies, we also inoculated the parasite at the base of the tail of these mice, which strengthened our results, as it turned out to be the same morphological description as that of the plantar pad model, where adipose tissue is a visibly important element. With these results, we suggest that adipose tissue be added to the Leishmania spp life cycle.

Keywords

Leishmaniasis, Cutaneous Leishmaniasis, *Leishmania mexicana*, BALB/c mouse, Histopathology.

Introduction

Leishmaniasis is a disease transmitted by the female sandfly of the genus *Phlebotomus* in Europe and *Lutzomyia* in the Americas. An estimated 12 million people are infected worldwide, with 2 million new cases occurring annually. In Mexico, the disease is found in 13 states, primarily in coastal areas [1-4]. There are three types of leishmaniasis: cutaneous, mucocutaneous, and visceral. The tissue that develops from the sand fly bite in mammals is called a lesion or ulcer. Cell culture studies with *Leishmania infantum*, reported the presence of *Leishmania* in adipose tissue [5,6]; however, there are no morphological studies that define and identify the nature of the tissue affected by *Leishmania* in murine models. In this work, we aimed to define the morphology and histopathology of this experimental lesions *in situ* using two murine models. We found

that six weeks after inoculating mice with *Leishmania mexicana* in both the plantar pad and the base of the tail, and using histological techniques (hematoxylin and eosin), immunohistochemistry, and immunofluorescence, the tissue that grows is adipose tissue inhabited by the parasite in its amastigote form, which contrasts with the histology of the healthy leg. In general, adipose tissue has been considered an energy reservoir; however, it is now considered a highly complex tissue because it houses multiple cell types such as: mature adipocytes, preadipocytes, fibroblasts, smooth muscle, endothelial cells, monocytes, macrophages, and lymphocytes [7,8]. It is known that preadipocytes in an environment similar to that of macrophages acquire the ability to phagocytize and not only that, but they also acquire the phenotype of macrophages (4). These results lead us to propose a life cycle of the parasite in which the presence of adipose tissue is integrated as an important element that, in addition to determining the growth and replication of the parasite, can also acquire the ability to phagocytize.

Objectives

Make the histopathological description of the resulting lesion when the parasite *Leishmania mexicana is inoculated* in mice of the BALB/c strain, both in the plantar pad and at the base of the tail, whose macroscopic appearance of the latter is similar to the lesion in humans. Relate histopathological results to the life cycle of the parasite.

Materials and Methods

Inoculation of *Leishmania mexicana* (6 million) in mice of the BALB/c strain: one group in the plantar pad, and the other group at the base of the tail. Hematoxylin and Eosin (HE): 2 micron sections were deparaffinized for 30 min at 60°C; 2 steps of 10 min in xylol; graded alcohols, running water, Hematoxylin (3 min.); wash in running water; acid alcohol (quick bath); running water; ammonia water (bath); running water; 96% alcohol (bath); Eosin, (3 min); dehydration, graded alcohols (baths); xylol (2 baths); mount with entellan resin.

Immunohistochemistry: the infected tissue was processed after being embedded in paraffin and sectioned at 2 microns. The sections were hydrated and immersed in PBS 1X. For antigen recovery, they were incubated for 10 minutes at 95°C in PBS 1X. Endogenous peroxidase was blocked with 0.9% hydrogen peroxide for 5 minutes. Non-specific staining was blocked with bovine serum diluted in PBS 1X and 0.1% Triton for one hour at room temperature. After washing three times with distilled water, the sections were incubated for 45 minutes with the primary antibody (anti-rabbit), PP2C in PBS, diluted 1:100. The secondary antibody (BIOCARE #DB537, Lot N/A) was incubated for 30 minutes, diluted 1:100 in PBS. After washing with PBS for 4 minutes, the slides were developed with diaminobenzidine (BIOCARE #DB538, Lot N/A), and the reaction was stopped by washing with PBS. The slides were counterstained with hematoxylin and mounted with entellan resin for microscopic observation.

Immunofluorescence: the infected tissue with Leishmania was cryopreserved and sliced into slices 10 µm thick with a cryostat Leica brand, model CM150 (Wetzlar, Germany). The cuts were mounted about microscope slides covered with gelatin and frozen at -70°C until moment of his use. The cuts were fixed with methanol absolute for 10 minutes, then they were incubated with 1 % Triton -X for 5 minutes. Then the cuts were washed 3 times for 5 minutes with PBS and became blocked for an hour with a 1% albumin solution. The next step was incubated with the following combinations: a) PP2c-fitc + Adiponectin -Alexa 647; b) PP2c-fitc + Macrophage -Alexa 594 and c) Adiponectin -Alexa 647+macrophage-alexa 488. The primary antibodies were incubated overnight at 4 degrees Celsius in a humid chamber, at a 1:100 dilution. Secondary antibodies were incubated for 30 min at room temperature, in a 1:300 dilution. Once the incubation was complete, the histological sections were washed 3 times for 5 minutes with PBS 1X. The sections were mounted on fluoromont and immunofluorescence was checked using a Leica TCS-SP8 inverted confocal microscope, Heidelberg, Germany.

Results

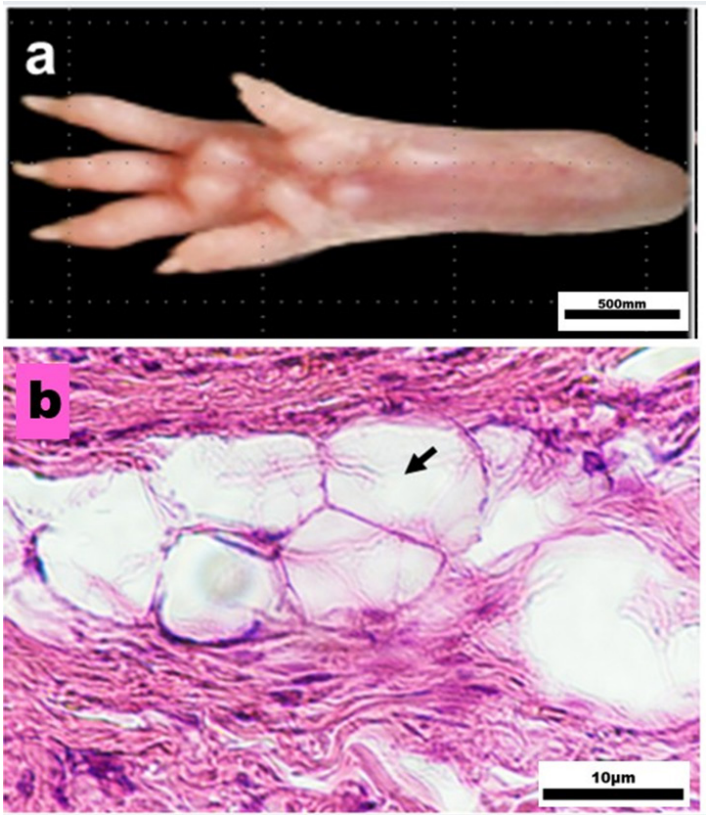

Figure 1: a) Healthy leg, **b)** Histology of the healthy plantar pad, the arrow points to adipose tissue.

The results of the Hematoxylin and Eosin staining in the healthy plantar pad showed healthy epithelial tissue and some adipose tissue, as can be seen in Figure 1a and 1b; meanwhile, in the infected plantar pad, a rounded tumor of approximately 2 cm in diameter grew figure 2a, where upon sectioning abundant adipose tissue inhabited by amastigotes of *Leishmania mexicana* was

observed, Figure 2b. In Figure 3a and 3b, the cutaneous lesion at the base of the tail is observed, very similar to that seen in humans; the histology was the same as that found in the murine model of the plantar pad, abundant adipose tissue within which the amastigotes of the parasite are found.

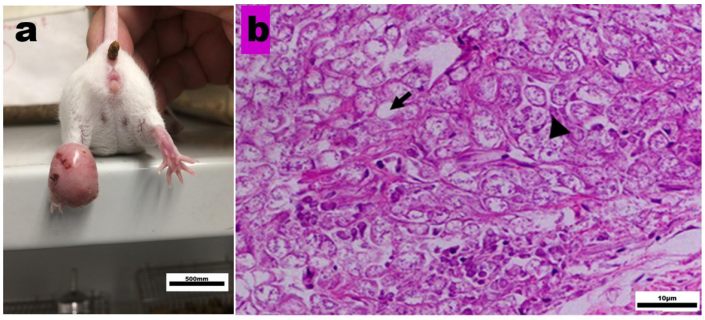

Figure 2: a) Leg six weeks after infection and **b)** Histology of the infected leg, the arrow points to parasites and the arrowhead points to adipocytes.

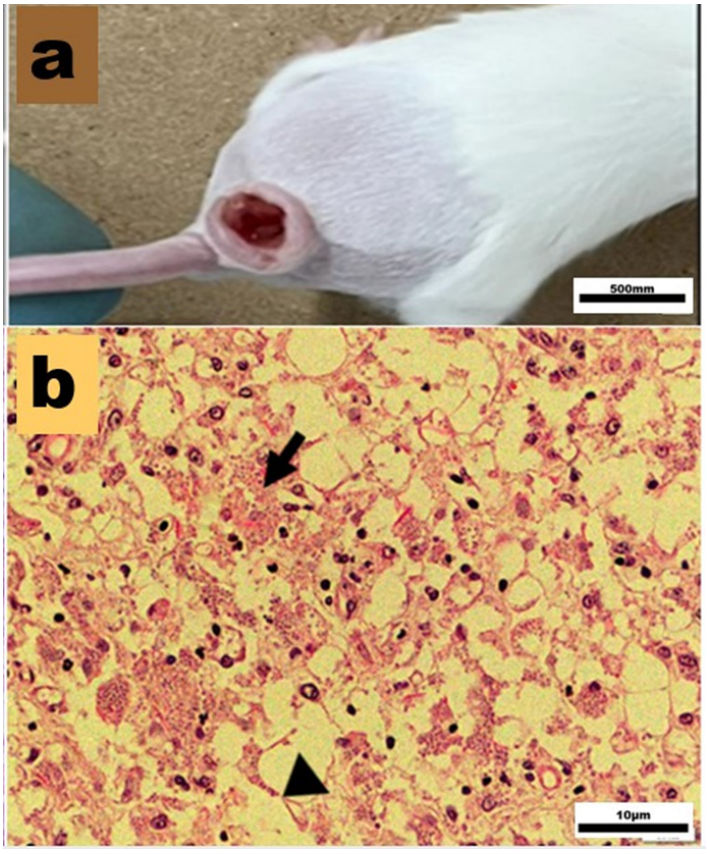

Figure 3: a) Base of the tail after six weeks of inoculation; **b)** Histology of the infected tail base, the arrow points to parasites and the arrowhead points to adipocytes.

The immunohistochemical results (Figures 4a and 4b) in both models were positive for the PP2C antibody, clearly indicating *Leishmania mexicana*. Figures 5a and 5b show a detailed view of the adipose tissue with abundant parasite content, both with hematoxylin and eosin (H&E) staining and with immunohistochemistry positive for *Leishmania mexicana*. Using phase contrast technique and an orange filter, we demonstrated the presence of adipocytes forming infected adipose tissue (brown fat). The histopathological nature is that of a tumor classified as a lipoma, which in this case harbors the parasites, Figure 6. The positivity to adiponectin with immunofluorescence, Figure 7, confirmed the histopathological nature of lipoma. Figure 8 shows, using immunofluorescence, the abundant presence of macrophages filled with the parasite. Figure 9 is an immunofluorescence image summarizing our results. Figure 10 demonstrates that there is migration of the parasites, in this case, towards the heart, as has been reported in the current life cycle of the parasite.

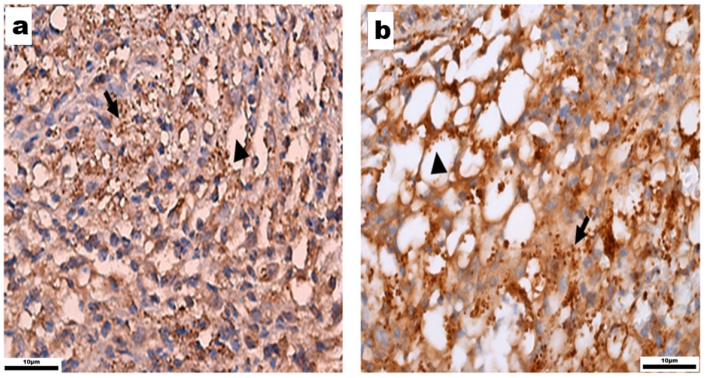

Figure 4: Immunohistochemistry, general view: **a)** infected plantar pad, **b)** infected tail base. Positivity, in both murine models, for the primary antibody PP2C, which marks the parasite in brown. The arrows point to the parasites and the arrowheads to adipose tissue.

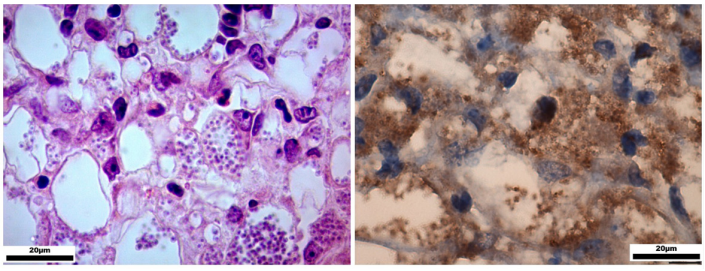

Figure 5: Detailed view of the infected plantar pad. Left: H&E staining, highlighting the adipose tissue inhabited by *Leishmania mexicana.* Right: immunohistochemistry, parasites positive for PP2C within the adipose tissue.

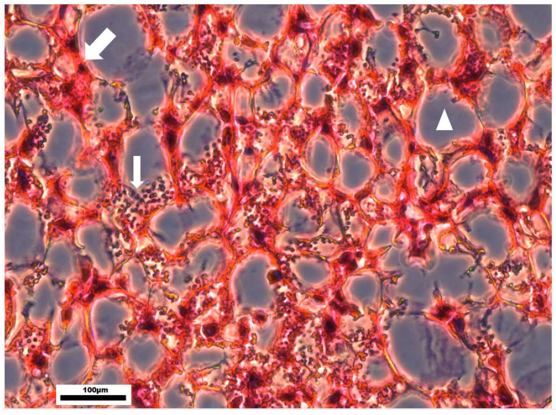

Figure 6: Section of the lipoma developed in the plantar pad. Phase

contrast, with orange filter. Arrowhead: adipocytes; thin arrow: parasites and thick arrow: macrophage with parasites.

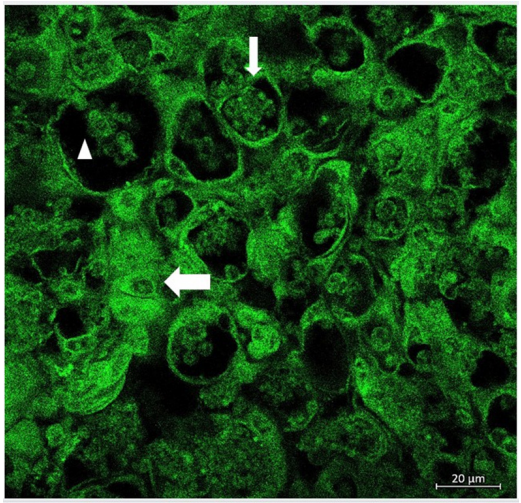

Figure 7: Immunofluorescence: Adiponectin Mouse coupled Alexa 488 1:50. Immunostained adipose tissue is observed, in green.

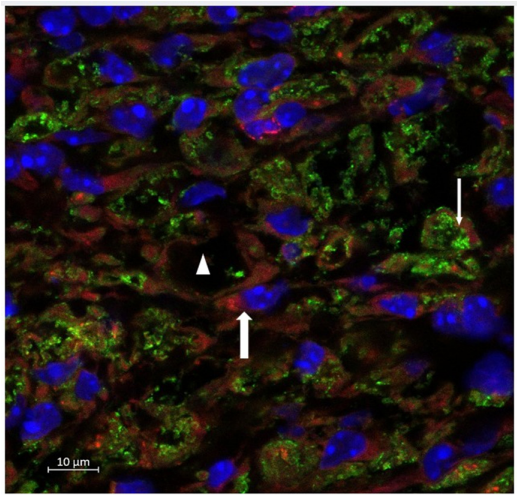

Figure 8: Immunofluorescence: Macrophage coupled to Alexa 488 mouse 1:50; adiponectin ALEXA 647, DAPI. Parasite-filled macrophages are shown in thin green arrow, preadipocytes in thick red arrow, nuclei in blue, adipocyte at the arrowhead.

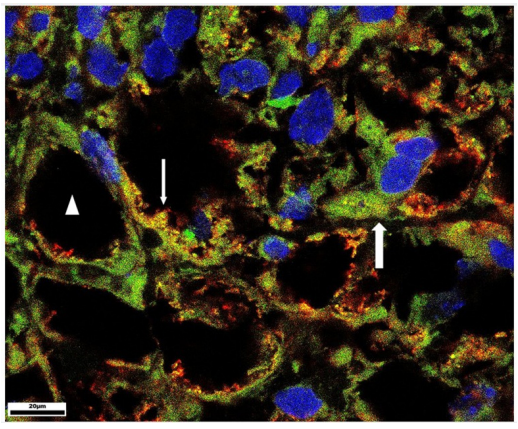

Figure 9: Immunofluorescence/ confocal microscopy, double staining: adiponectin rabbit Alexa 488/PP2C Alexa 647, nuclei stained with DAPI. Arrowhead adipocytes, thin arrow parasites and thick arrow macrophages inhabited by parasites.

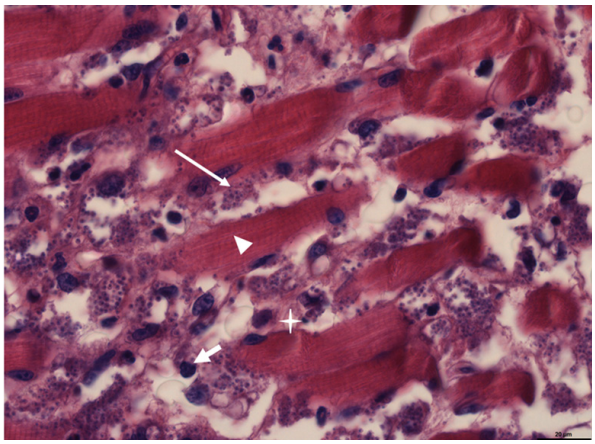

Figure 10: Migration of *Leishmania mexicana* from the plantar pad to the heart. Thin arrow: parasite, thick arrow: macrophage, arrowhead: cardiac muscle, and star: preadipocyte.

Figure 11a depicts the life cycle of *Leishmania spp*. as currently known. The vector infects the mammal while feeding. The parasite is phagocytosed by macrophages, loses its flagellum, transforms into an amastigote, and multiplies inside the macrophage. The parasite is released into the bloodstream and migrates to the organs. The vector ingests amastigotes. The amastigotes transform into promastigotes in the vector's gut. The mosquito bites and infects the mammal, completing the cycle.

Figure 11b illustrates the proposed life cycle of *Leishmania spp*. presented in this work, where the vector infects the mammal while feeding. The parasite is phagocytosed not only by macrophages but also by adipocytes, loses its flagellum, and transforms into an amastigote. The parasites, macrophages, and adipocytes multiply, and adipose tissue becomes essential. The parasite is released into the bloodstream, leaving behind remnants of empty macrophages and adipocytes. The parasite migrates to the organs. The following is what has already been reported for the life cycle: the vector is ingested by amastigotes. The amastigotes transform into promastigotes in the vector's gut. The mosquito bites the mammal, infects it, and the cycle is completed.

Discussion

The H&E stains alone demonstrate the histopathological nature of both murine models. We know that adipose tissue is an energy reservoir (white adipose tissue), and in this study, the presence of brown adipose tissue is evident. This tissue is responsible for producing energy and fulfills its function very well, as it allows the parasite to multiply and subsequently migrate to different organs.

It will be interesting to know what mechanisms cause this to happen. The preadipocytes present in this tumor lead us to consider their possible role in the production of macrophages and adipocytes,

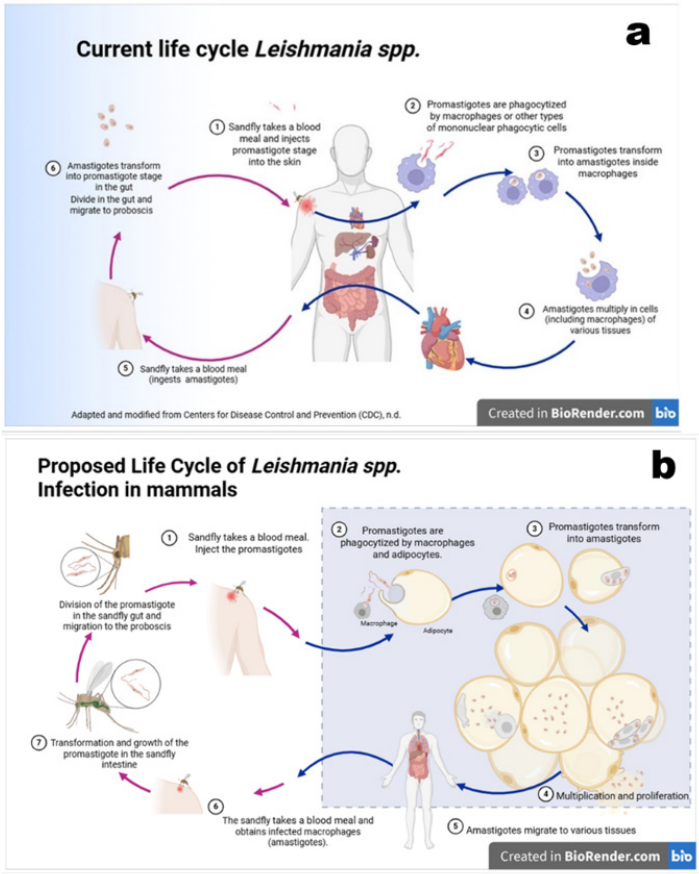

Figure 11: Life cycle of *Leishmania spp*. a) The life cycle known so far (Centers for Disease Control and Prevention. Leishmaniasis. DPDx. (https://www.cdc.gov/dpdx/leishmaniasis/index.html); b) The cycle we propose, in step 2 we note the presence of adipocytes phagocytizing parasites alongside macrophages; in step 3 the flagellum is lost and the parasites transform into amastigotes; in step 4 there is proliferation of parasites, macrophages and adipose tissue (See Figure 9). (https://www.biorender.com).

since, as shown in the results, both macrophages and adipocytes phagocytize the parasites. The various figures in this text show parasites outside and inside macrophages, as well as inside adipocytes; many of these originate from the hatching of parasites that have multiplied within the macrophages. Preadipocytes were difficult to identify due to their close resemblance to macrophages.

The histology of the adipocytes and their nuclei correspond to the brown fat type, which is responsible for utilizing stored energy to support the survival, multiplication, and migration of the parasites.

Conclusion

The results obtained in the murine model of the plantar pad inoculated with *Leishmania mexicana*, show a tumor formed by brown adipose tissue, where the parasite is housed inside and outside the macrophages, the parasites in their amastigote form. The histopathological nature of this tissue led us to conclude that it is a tumor whose characteristics correspond to a lipoma, which gives the parasite every chance of survival and propagation.

With this finding, we suggest that the life cycle of *Leishmania spp*. includes not only macrophages phagocytizing the parasite, but also adipocytes phagocytizing them, and where these cellular elements multiply forming adipose tissue that becomes necessary as an energy source for the invasion, proliferation, and migration of the parasite.

The abundance of adipose tissue suggests an active dynamic. It's difficult to imagine adipose tissue migrating to other tissues with all its cellular components; however, it is known that the parasite itself does move to different organs such as the liver, kidneys, and heart, among others. We suggest that where the parasite migrates, there will be proliferation of adipose tissue. Our histopathological results obtained in the murine model inoculated at the base of the tail, although they do not develop lipomas, support the idea of abundant adipose tissue harboring parasites in macrophages and adipocytes, thus satisfying the energy source to form the ulcers or lesions seen in this model, which are very similar to those that occur in humans, especially in cutaneous leishmaniasis.

Acknowledgments

Thanks to Irma Elena López Martínez (Unidad de Microscopia Facultad de Medicina/ Universidad Nacional Autónoma de México); to Edith Álvarez León (CORELAB del Instituto Nacional

de Cardiología/ Universidad Nacional Autónoma de México) for their service in the confocal microscope LSM 700 Zeiss.

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