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Kinetics of an experimental inflammatory reaction induced by *Leishmania major* during the implantation of paraffin tablets in mice

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Abstract In leishmaniasis, macrophages play important but potentially divergent roles. They act as the host cell in which the parasite may reside and replicate, and, at the same time, they act as an effector cell with the potential to eliminate the parasite. In this work, we experimentally induced an inflammatory model that provokes a continued recruitment of the monocytes to the site of inflammation. This model was carried out by means of implanting paraffin tablets under the skin of Balb/c or C57BL/6 mice. Mice were then infected with *Leishmania major* to determine how the monocyte inflammatory response to paraffin could influence the course of infection with *L. major.* Mice were sacrificed 15, 21, 30, and 45 days after infection, and skin and inflammatory capsule were collected for histopathology. At 15 days and 21 days, the lesions induced by *L. major* in combination with paraffin contained markedly increased numbers of parasites relative to lesions in parallel control animals infected with *L. major* (without paraffin). Both Balb/c and C57BL/6 mice exhibited high parasite numbers in their lesions. The intense parasite burden observed following paraffin implantation would suggest that the monocytes–macrophages that are recruited to the lesion are acting more as a host cell permitting parasite growth than as an effector cell capable of eliminating *L. major*. At later times, the two strains of mice stratified according to their genetic susceptibility/resistance profiles. Susceptible Balb/c mice continue to have large parasite burdens, whereas the resistant C56BL/6 mice begin to control parasite numbers. This later observation indicates that the genetic difference between susceptible and resistant strains is not due to differences in monocyte recruitment and cannot be reversed through the altering of monocyte inflammation.

Keywords Balb/c and C57BL/6 mice · *Leishmania major* · Paraffin tablets · Inflammation · Monocytes–macrophages

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Introduction

Leishmania is an intracellular protozoan parasite that is delivered to the vertebrate host by the bite of an infected sandfly. Following injection into the skin, the extracellular promastigote form of the parasite rapidly enters its host cell, the macrophage. It is within macrophages that *Leishmania* survive and replicate as amastigotes, the intracellular form of the parasite. Amastigotes are responsible for the clinical manifestations of infection. Both the species of *Leishmania* and the host–immune response to the parasite can determine the clinical outcome of the disease. In the New World, cutaneous leishmaniasis, in which parasites are localized to the skin, is caused mainly by *L. braziliensis* and *L. mexicana.* Visceral disease, in which parasites disseminate to the bone marrow, liver, spleen, and lymph nodes, is generally caused by *L. chagasi* [5, 25].

In the initial stages of infection, *Leishmania* are phagocytosed by tissue macrophages. It is generally accepted that these kinetoplastid parasites are able to enter and successfully replicate within unstimulated resident tissue macrophages. Both in vitro and in vivo studies have confirmed that resident macrophages can support the growth of *Leishmania* and are the primary cell type infected within *Leishmania* lesions [13, 18, 26]. It is also generally accepted that immunologically activated macrophages can restrict the intracellular growth of *Leishmania*. Macrophage activation to exert antileishmanial

activity is regulated by interferon (IFN)-γ, which is produced by T- and natural killer (NK) cells [17, 19, 20]. Animals that fail to make IFN-γ, or those rendered deficient in IFN-γ, are unable to control parasite growth. Conversely, animals given exogenous IFN-γ or IFN-γ-inducing cytokines are invariably more resistant to infection [2, 6, 20, 21]. As is the case with other intracellular pathogens, the presence of parasites in tissue also provokes the appearance of bone-marrow-derived blood monocytes [12].

The role of inflammatory macrophages in leishmaniasis has been less well defined. Using a visceral model of *L. donovani* infection, Murray and colleagues have suggested that infiltrating monocytes–macrophages are the effector cells that eliminate *Leishmania* from tissue [14]. Conversely, Soong and colleagues, working in a cutaneous model of *L. amazonensis* infection, have suggested that infiltrating monocytes–macrophages are permissive to parasite growth and are actually necessary to permit optimal parasite growth in tissue [23].

In this work, we examine the role of inflammatory monocytes–macrophages during the initial stages of infection with *L. major*. We have developed a model of monocyte inflammation using an immunologically inert particle, the paraffin tablet, to induce monocyte inflammation. This inert irritant induces the sustained recruitment of monocytes to the site of inflammation and a local immobilization of monocytes–macrophages in tissue. We examine infection in Balb/c and C57BL/6 mice, which are susceptible and resistant to *L. major*, respectively. Our objective was to determine how a monocyte–macrophage inflammatory reaction would affect lesion progression following infection with *L. major* and to determine whether inflammatory macrophages infiltrating into lesions were permissive or restrictive to *Leishmania* growth.

Materials and methods

Parasites

The Friendlin strain of *L. major* clone IV (MHOM/IL/80/Friedlin) was used in all experiments. Promastigotes were grown at 250 C in Grace's insect cell culture medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20% heat-inactivated fetal bovine serum, glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 µg/ml). Stationary-phase promastigotes, obtained from 7-day to 9-day cultures, were used for all experimental infection.

Balb/c and C57BL/6 mice

Mice (4–6 weeks old) were obtained from our animal facility at ICB/UFMG. Mice were separated into three groups of eight. The first group had paraffin tablets implanted, the second group was experimentally infected with *L. major* promastigotes, and the third group received paraffin tablet implantation and was infected with promastigotes of *L. major* immediately thereafter.

Subcutaneous implantation of paraffin tablets

Paraffin tablets (220.0 mg; Queel – Indústrias Químicas Ltda, São Paulo, Brazil) with the following dimensions – 1.0×0.5 cm (H×L) – were prepared as described [16]. These tablets have a smooth surface and are maintained under aseptic conditions. To implant the paraffin tablets, mice were anesthetized, shaved, and then a skin incision (1.5 cm) was made on the dorsum of the animals using blunt-point scissors. Tablets were inserted into a small subcutaneous cavity, and the wound was stitched.

Experimental infection

Animals were injected with 1×10^7 promastigotes in 0.1 ml in the dorsum nearest the paraffin tablet immediately after implantation. Control mice (without paraffin) were infected at the same time.

Disease progression and collection of tissue samples for histopathology

Two animals from each group were sacrificed at 15, 21, 30 and 45 days after the experimental infection. At each of the designated days, fragments of skin tissue and the tissue surrounding the paraffin tablets (inflammatory capsule) were collected for histopathology analyses. Tissues were fixed in 10% buffered formalin solution, dehydrated, cleared, embedded in paraffin, cut (3- to 4-µm thick) and stained with hematoxylin and eosin (H&E).

Results

The mononuclear response to paraffin implantation (group 1)

Balb/c and C57BL/6 mice were implanted with paraffin and their inflammatory lesions were observed over time. In both strains of mice, there was an inflammatory capsule of tissue formed around the paraffin tablets as a result of a granulation tissue deposition. At 15 days, the inflammatory capsule was observed as a delicate granulation tissue constituted by numerous newly formed blood vessels, edema, and mononuclear cells (macrophages and lymphocytes). Fibroblast proliferation with a loose collagen deposition was also observed (Fig. 1a). Giant cells around or near paraffin fragments were only rarely observed. At 21 days, the inflammatory capsule was observed as a mature granulation tissue constituting a proliferation of blood vessels and fibroblasts with a deposition of collagen (Fig. 1b). The mononuclear cell exudation was moderate, and the size and number of giant cells was increased relative to 15 days. Giant cells were mainly of foreign body type, and they were always present around paraffin fragments. At 30 days and 45 days, the inflammatory capsule was characterized by an intense fibroblast accumulation with a high degree of collagen deposition. Blood vessel neoformation and the exudation of mononuclear cells and giant cells were observed.

Leishmania infection of Balb/c mice and C57BL/6 inoculated in the dorsum skin (group 2)

Control mice (without paraffin) were infected in the skin, and their lesions were examined over time. By 15 days, the skin of Balb/c mice showed a chronic inflammatory

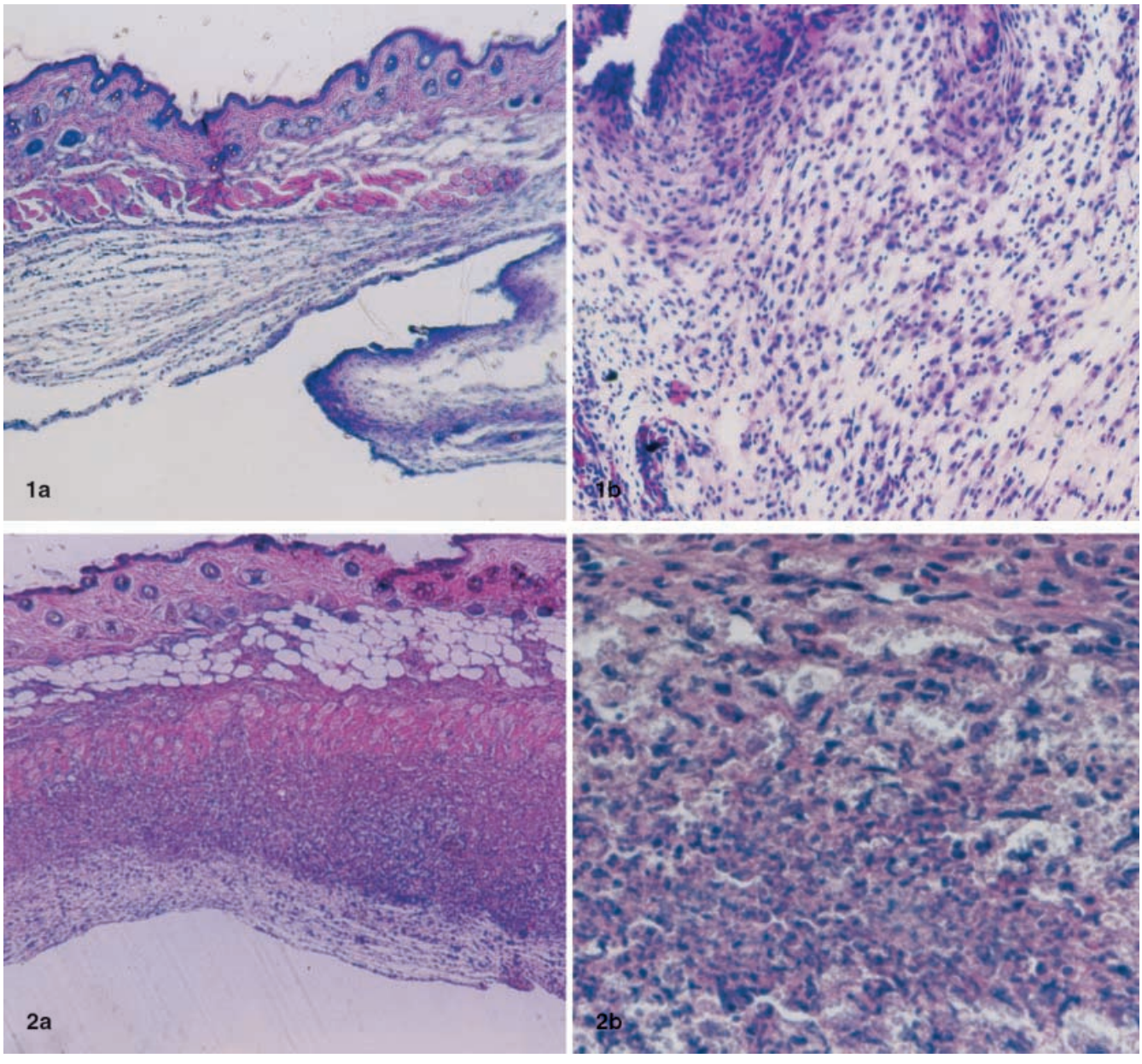


Fig. 1 Balb/c mice implanted with paraffin. **a** 15 days: note the presence of an inflammatory capsule in the deep dorsum skin dermis. Hematoxylin and eosin (H&E) ×40. **b** 21 days: an intense inflammatory reaction in deep dermis with an exudate of mononuclear cells. H&E ×100

Fig. 2 Balb/c mice with *Leishmania major* infection (15 days). **a** Diffuse and chronic inflammatory reaction containing macrophages and lymphocytes in the deep dermis. Hematoxylin and eosin (H&E) ×40. **b** Note the presence of macrophages with intracellular amastigotes of *L. major*. H&E ×400

reaction characterized by a cellular exudate with lymphocytes, macrophages, and rare neutrophils (Fig. 2a). Macrophages parasitized with intracellular amastigotes of *Leishmania* were observed in the lesions (Fig. 2b). Skin tissue of C57BL/6 mice showed a discrete inflammatory reaction, constituting a cellular exudate with lymphocytes and macrophages in the deep dermis (Fig. 3a). Some macrophages with intracellular amastigotes of *Leishmania* were observed (Fig. 3b). By 21 days in Balb/c mice, the inflammatory reaction was more intense and the fibroblast proliferation with collagen fiber deposition was observed mainly in the deep dermis. Large numbers of parasitized macrophages were present throughout the lesion. In C57BL/6 mice, a moderate chronic inflammatory process localized in the deep dermis was observed. Only few parasitized macrophages with *L. major* were observed. In all Balb/c mice, an intense inflammatory lesion persisted and, by 30 days and 45 days, innumerous amastigotes were observed inside of macrophages. In contrast, there was a discrete chronic inflammatory reaction in the skin of all C57BL/6 mice with rare macrophages parasitized with amastigotes of *L. major* (Table 1).

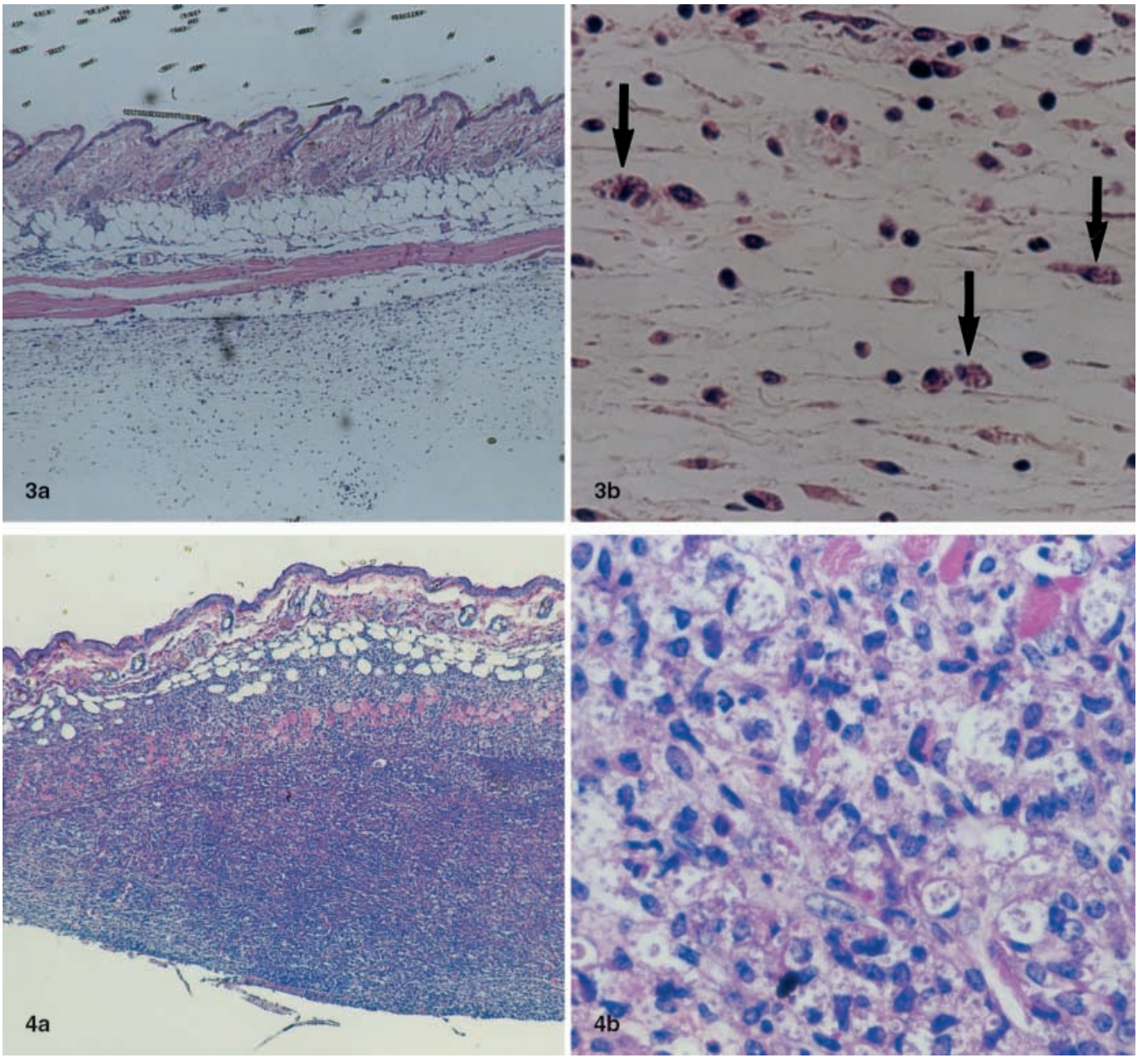


Fig. 3 C57BL/6 mice with *Leishmania major* infection (15 days). **a** Note the presence of a discrete inflammatory reaction constituted by a cellular exudate with lymphocytes and macrophages in the deep dermis. Hematoxylin and eosin ×40. **b** Some macrophages with intracellular amastigotes of *L. major* ×400 (*arrows*)

Fig. 4 Balb/c mice implanted with paraffin tablets and infected with *Leishmania major* (15 days). **a** An intense chronic inflammatory reaction with a diffuse cellular exudate of mononuclear cells. Hematoxylin and eosin (H&E) ×40. **b** Many macrophages with intact *L. major* amastigotes within them. H&E ×400

Leishmania infection following paraffin implantation in Balb/c and C57BL/6 mice (group 3)

Mice were implanted with paraffin tablets and then inoculated with parasites adjacent to the implantation site. By 15 days, both groups of animals showed an intense chronic inflammatory reaction mainly localized to the deep dermis with a diffuse cellular exudate of macrophages, lymphocytes, and some neutrophils and eosinophils (Fig. 4a). In both species of mice, numerous macrophages were loaded with amastigotes of *Leishmania* (Fig. 4b). New blood vessel formation, edema, and a loose collagen tissue with a discrete fibroblast proliferation were formed as granulation tissue. By 21 days, an intense and diffuse chronic inflammatory reaction was observed throughout all of the skin dermis with areas of necrosis in all animals of both Balb/c and C57BL/6 groups examined (Fig. 5a, b). Macrophages were vacuolated and loaded with innumerous *Leishmania* amastigotes (Fig. 6a, b). Blood vessel neoformation and fibroblast proliferation with a mature collagen deposition were observed. Some neutrophils and eosinophils were present in tissue. In Balb/c mice, some giant cell forma-

Table 1 Inflammatory reaction in mice infected with *Leishmania major* with or without paraffin tablet implantation. + to ++++ represents increasing degrees of intensity of the chronic inflammatory reaction and the presence of amastigotes, such that + is the lower and ++++ is the highest observed

	Chronic inflammatory reaction		Presence of amastigotes	
	Balb/c	C57BL/6	Balb/c	C57BL/6
Paraffin implanting				
15 days	+	+	–	–
21 days	+–++	+–++	–	–
30–45 days	++–+++	++–+++	–	–
L. major infection				
15 days	+	+	++	+
21 days	++–+++	++	++–+++	++
30–45 days	+++	+	+++	+
L. major infection and paraffin implanting				
15 days	+++	++++	+++	+++
21 days	+++–++++	+++–++++	++++	++++
30–45 days	++++	++++	++++	+

tion was observed, and some of these cells had parasites within their cytoplasm (Fig. 5c).

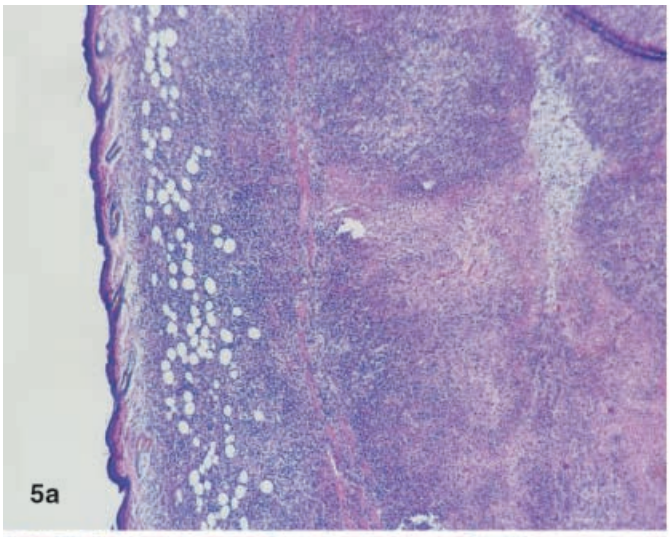
By 30 days and 45 days, the lesions in the two strains of mice began to show marked differences. In Balb/c mice, they continued to progress, while in C57BL/6 mice, the parasite numbers began to resolve. The skin of Balb/c mice showed areas of necrosis with degenerated extracellular parasites associated with an intense mononuclear cell exudation and some neutrophils. Many macrophages were vacuolated with multiple intracellular amastigotes. Lesions contained more giant cells, some of which were parasitized. Angiogenesis, fibroblast proliferation, and collagenesis were also observed. In the skin of C57BL/6 mice, a chronic inflammation with an exudate of mononuclear cells and fibroblast proliferation persisted. However, the parasitism was markedly lower at these later times. The prominent parasitism and necrosis observed in Balb/c mice at this time was not observed in C57BL/6 mice (Table 1). Thus, despite the initially high parasite infectivity and the intense mononuclear cell infiltration, the resistant C57BL/6 strain of mice were eventually able to control their lesions and eradicate the parasites.

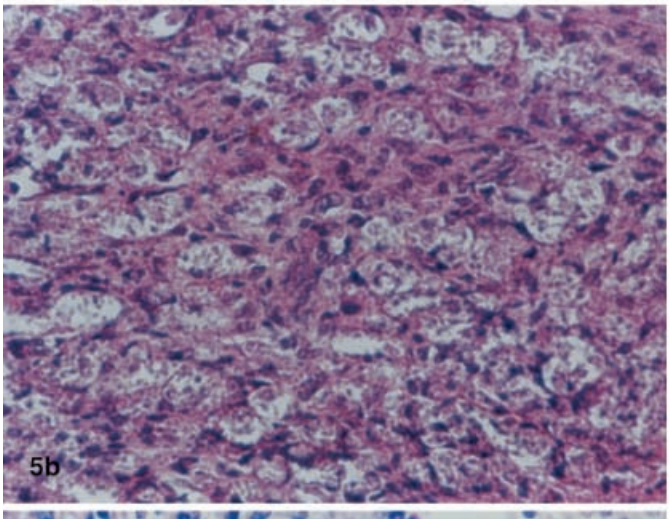
Discussion

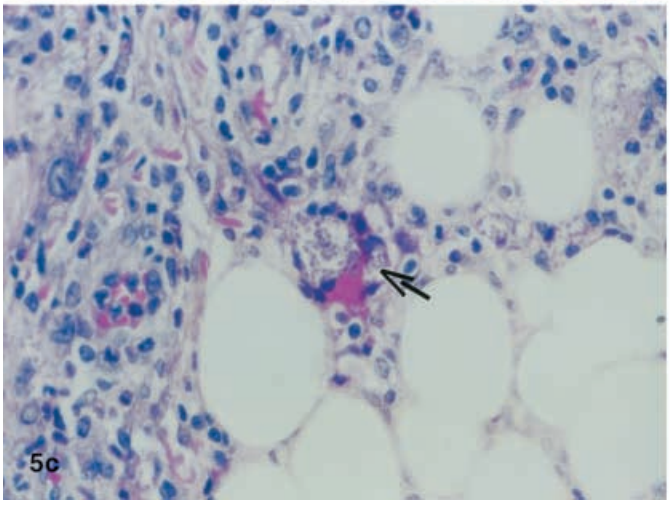
Resident tissue macrophages have been incriminated as a principal host cell that allows the intracellular replication of *Leishmania*. These parasites have evolved strategies that allow a significant percentage of them to survive in the hostile environment of the macrophage phagolysosome [11, 13, 18]. Intracellular amastigotes are able to proliferate and spread the infection to neighboring cells, causing the clinical spectrum of disease. Activated macrophages, however, are able to kill *Leishmania*. Treatment of macrophages with lymphokines, such as IFN-γ, leads to both the in vitro [4] and in vivo [1, 20, 24, 27] eradication of parasites and clinical cure. In cutaneous leishmaniasis, a murine model has been developed that illustrates the importance of cell-mediated immunity and macrophage activation to *Leishmania* eradication. C57BL/6 mice, which produce IFN-γ in response to *Leishmania* infection, resolve their infection and develop long-lasting immunity. Balb/c mice, in contrast, produce interleukin (IL)-4 rather than IFN-γ, and fail to resolve their lesions. These mice ultimately succumb to fulminant infection [21].

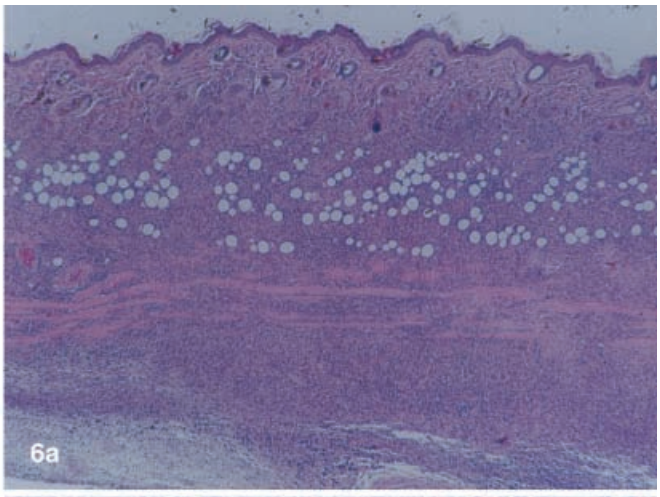
In this study, we examined how one inert inflammatory agent, paraffin, can modulate the host response to *L. major*. In this inflammatory model [16], the paraffin induced a sustained recruitment of the monocytes to the site of inflammation and a local immobilization of monocytes–macrophages. Our results showed that *L. major* lesions were more intense when induced along with this inert inflammatory agent. In both the Balb/c and C57BL/6 strains of mice, an early increase in the numbers of parasites in lesions accompanied this inflammation. This increase in parasite numbers in the lesion was observed over the first 3 weeks of infection (Table 1). In Balb/c mice, the lesions continued to progress and, at later times, were characterized by large numbers of parasites and extensive tissue necrosis. These observations are consistent with previous reports, which demonstrate that this strain is unable to control infection by *L. major* [7].

The increased parasite burden due to paraffin implantation was also true for C57BL/6 mouse over the first 21 days. These mice showed an intense inflammatory reaction with macrophages loaded with parasites. These results were unexpected because C57BL/6 mice have been characterized as being resistant to *Leishmania* infection. Relative to control infected C57BL/6 mice, littermates implanted with paraffin and then infected with *Leishmania* showed elevated parasite burdens during the first 15–21 days after infection. This increase in parasites was transient, however, and at later times these mice eventually began to resolve their infections. By 30 days and 45 days after infection, the number of parasites in their lesions was markedly reduced relative to Balb/c mice (Table 1). This control of parasite numbers at these later days occurred despite the persistence of the inflammato-









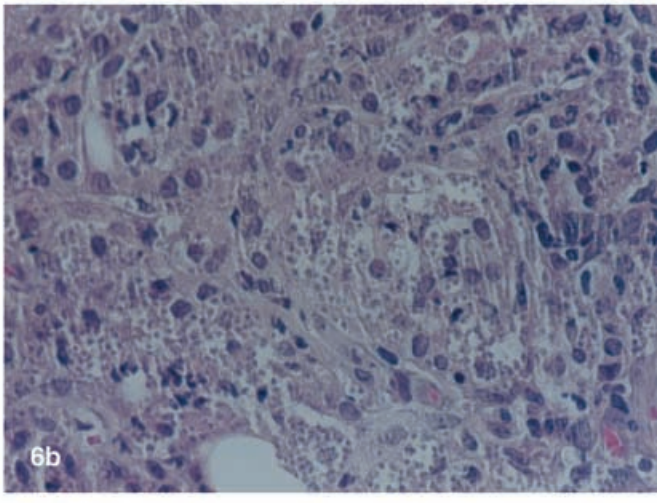


Fig. 5 Balb/c mice implanted with paraffin-tablets and infected with *Leishmania major* (21 days). **a** An intense and diffuse chronic inflammatory process throughout the dermis with mononuclear cells and some eosinophilic areas of necrosis. Hematoxylin and eosin (H&E) ×40. **b** Note the presence of innumerous parasitized macrophages with amastigotes of *L. major*. H&E ×400. **c** Formation of giant cells, with some of them vacuolated and showing intracellular *L. major* amastigotes (*arrow*) H&E ×400

Fig. 6 C57BL/6 mice implanted with paraffin tablets and infected with *Leishmania major* (21 days). **a** An intense and diffuse chronic inflammatory reaction with a diffuse cellular exudate of mononuclear cells. Hematoxylin and eosin (H&E) ×40. **b** Note the presence of numerous macrophages loaded with amastigotes of *L. major.* H&E ×400

ry reaction induced by the paraffin. Our results indicate that, over the first 3 weeks of infection, immigrating monocytes–macrophages act more like host cells that are permissive to *Leishmania* growth than effector cells that restrict parasite replication. This permissiveness is true even in the face of the intense inflammatory reaction that occurs in response to paraffin.

Mice are generally characterized as being either resistant or susceptible to infection with *L. major*, based on whether they heal their infection or develop progressive disease [7, 9, 15]. It is now well established that a T helper (Th) 1-like immune response is required for mice to heal infections, while mice that develop Th-2-like responses develop progressive disease [3, 6, 8, 10, 17, 21,

22]. The nature of the factors that govern the specific type of immune response that develops is incompletely understood. In the present study, we attempted to augment leukocyte infiltration in each group by the administration of paraffin tablets. Our goal was to determine whether an alteration in cell migration could influence the course and severity of infection with *L. major*. We report that following paraffin implantation, Balb/c and C57BL/6 mice were similar with regard to monocyte infiltration. In addition, the animals had increased numbers of parasites in their lesions during the first 3 weeks of infection. However, despite the early increase in parasite numbers, C57BL/6 mice retained their resistant phenotype and eventually began to resolve their infections. Thus, this resistant phenotype of C57BL/6 mice is not affected by differences in the initial inflammatory response to the parasite, nor is it influenced by differences in the initial number of parasites within the lesion.

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