Immunopathology of Experimental Cutaneous Leishmaniasis

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Relatively susceptible BALB/c and relatively resistant A/J mice were infected subcutaneously in the right hind footpad with promastigotes of *Leishmania mexicana amazonensis*. A large localized lesion developed within 2 months after infection in the BALB/c mice, while A/J mice exhibited a small discrete fibrotic nodule. Sequential immunologic and histologic examination demonstrated that BALB/c mice developed a nodular foam-cell type of lesion and progressive depression of a delayed-type hypersensitivity (DTH) response to leishmania antigen, while the A/J mice had a mixed cellular fibrosing and encapsulating reaction and developed and maintained positive DTH responses to leishmania antigen. Anti-leishmania antibody responses were positive at similar levels in both strains. The lesions in BALB/c mice were found in bone marrow, tendon, skin appendages, and regional lymph nodes, with a tendency toward cutaneous metastases. Lesions in A/J mice remained localized. Fibrosis, focal fibrinoid necrosis, and lymphocytic and macrophagic infiltration were the outstanding features. Light and transmission electron microscopic studies indicated that no outstanding destruction of leishmanias seemed to occur within macrophages of either mouse strain. In the more resistant A/J mice, however, parasitized macrophages were frequently necrotic, and degenerating leishmanias were often seen free in the interstitial tissue. These observations help the interpretation of the histologic features, as well as the pathogenesis, of cutaneous and mucocutaneous leishmaniasis in man. (Am J Pathol 1984, 114: 137–148)

MICE infected with *Leishmania* organisms are proving to be valuable models for the human diseases caused by these parasites. Depending upon the parasite species and mouse strain chosen, a considerable spectrum of disease patterns may be produced. Inbred mouse strains have been repeatedly shown to display relative resistance or susceptibility to both visceral[1] and cutaneous[2,3] leishmaniasis. They could thus offer an interesting experimental approach to the investigation of histologic and immunologic correlations and histologic indicators of resistance or prognosis. Such a possibility has been little explored, and it could offer additional information to studies of human leishmaniasis in which attempts are made to correlate histologic features with clinical presentation, prognosis, and response to therapy.[4–6]

Agents of American cutaneous leishmaniasis have been divided into the *L mexicana* and *L braziliensis* complexes. The former is rarely associated with mucocutaneous disease and is generally represented by single or few lesions. However, *L mexicana amazonensis* has been associated with severe and debilitating diffuse cutaneous leishmaniasis, and visceralization has been known to occur in some infections. Diffuse disease is often accompanied by unresponsiveness to the leishmania skin test and is represented in an animal model by infection in the BALB/c mice. In the present study relatively resistant and relatively susceptible inbred strains of mice were infected with a human isolate of *L mexicana amazonensis*. Immunologic, histopathologic, and ultrastructural events were monitored during the course of the disease.

Materials and Methods

Mice

Inbred BALB/c and A/J mice were reared in our animal facility and used at 6–8 weeks of age. The animals were maintained on wood shavings in a tempera-

Supported by Grant 16282 from the National Institutes of Health.

Electron-microscopic studies were performed at the Institute of Pathology, Case Western Reserve University (Dr. M. Aikawa), Cleveland, Ohio, during the tenure of a Rockefeller Foundation Fellowship awarded to Dr. Andrade.

Accepted for publication July 28, 1983.

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ture-controlled room and fed a balanced mouse ration and water *ad libitum*.

Parasite

A human isolate of *L mexicana amazonensis*, typed by isoenzyme and DNA buoyant density analysis, was provided by Dr. Philip Marsden (University of Brasilia). The parasite is maintained in our laboratory by subcutaneous passage in BALB/c mice. Promastigotes used for infection were obtained by culturing amastigotes from a foot lesion in Yaeger's liver infusion tryptose medium supplemented with 5% fetal bovine serum (LIT-FCS) for 10–14 days at 24–26 C. The animals were infected by subcutaneous inoculation of 1.5×10^7 promastigotes in 0.025 ml in the right hind footpad.

Antibody Tests

Indirect fluorescent antibody (IFA) titers were determined with the use of formalin-fixed *L mexicana* amastigotes, separated from the footpads of infected mice by the method of Chang,[7] and rabbit anti-mouse IgG (Cappel, Worthington, Pa). There were 5 mice in each group.

Skin Testing

Parasite antigen used to detect delayed-type hypersensitivity (DTH) was prepared according to Bryceson et al[8] from promastigotes maintained in LIT-FCS. Briefly, parasites were washed in phosphate-buffered saline (PBS), pH 7.2 5–6 times resuspended in water, then broken by 12–15 rapid freeze-thaw cycles (liquid N_2, 37 C). The parasites were suspended in urea to a final concentration of 8 M and incubated at 37 C for 2 hours, then dialyzed at 4 C for 48 hours against 0.1 M ammonium acetate, 0.1 M sodium carbonate buffer, pH 7.4, then 24 hours against water. The antigen was centrifuged at 4000*g* for 30 minutes, and the supernatant was analyzed for protein content (Lowry) and filter-sterilized before use. One hundred micrograms protein in 0.025 ml was injected subcutaneously into hind footpads. Change in thickness was noted 24 hours later with a dial gauge micrometer ("Schnelltaster") and expressed as the difference in millimeters before and after antigen injection.

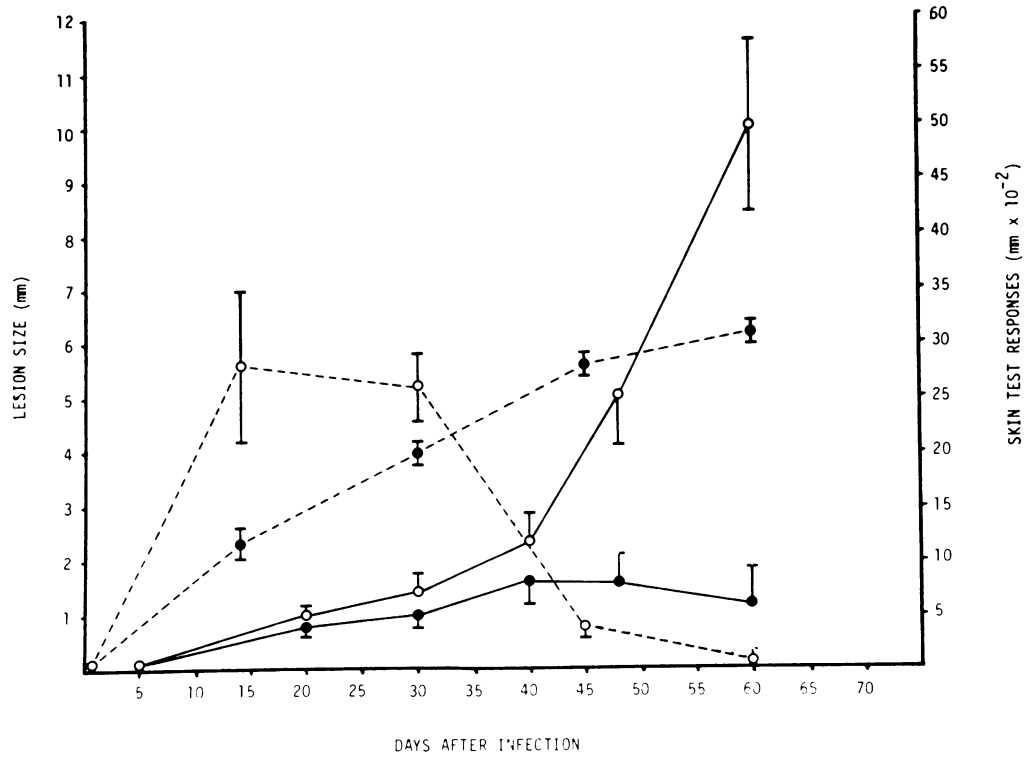


Figure 1 – Growth of primary lesion and skin test responses to leishmania antigen during the course of infection in BALB/c and A/J mice. Lesion size (mean ± SD) is the size of the right hind footpad following subcutaneous inoculation of 1.5×10^7 promastigotes, expressed as the difference between infected and uninfected footpads. The skin test response (mean ± SD) is expressed as the difference in footpad thickness before and 24 hours after subcutaneous injection of 100 μg leishmania antigen into the left hind footpad. Seven mice per group. o, BALB/c; •, A/J; ----, skin test response; ——, lesion size.

Pathologic Examinations

Growth of primary lesions was determined with a dial gauge micrometer. At various times (daily during the first 7 days, weekly during the first month, and at 2 months) during infection, feet, lymph nodes, spleen, and liver were removed and placed in Bouin's fixative. Control animals received inoculation of culture medium alone or of an equivalent number of heat-killed (60 C, 30 minutes) promastigotes in the opposite footpad. The groups consisted of 4–5 mice each.

After fixation, the foot lesion was sectioned longitudinally. The two halves were decalcified in EDTA and embedded in paraffin, and the sections were stained with hematoxylin and eosin (H&E) and, in special cases, Masson's trichrome stain, Gomori's reticulum impregnation, and periodic acid–Schiff (PAS).

One- and 2-month-old footpad lesions from both mouse strains were subjected to ultrastructural analysis. Tissue fragments were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, washed in the same buffer, and postfixed in 1.0% osmium tetroxide for 1 hour. The samples were embedded in Spurr's medium, and the blocks were cut with a Porter-Blum MT-2 ultramicrotome.

Multiple sections stained with 1.0% uranyl acetate and lead citrate were examined with a JEOL 100 Cx electron microscope at 60 mv.

Results

Growth of Primary Lesion

The course of the growth of the primary lesion differed dramatically between BALB/c and A/J mice (Figure 1). The lesion increased progressively in BALB/c mice through approximately 90 days, after which time necrosis generally occurred. In A/J mice, the lesion reached maximum size on about Day 40, following which healing occurred, which sometimes resulted in a scarred, deformed foot (Figure 2).

Antibody Production

Anti-leishmania antibody was produced in both mouse strains without significant difference. Titers of 40–80 were observed at 14–30 days after infection, and increased to 80–160 by 60 days after infection.

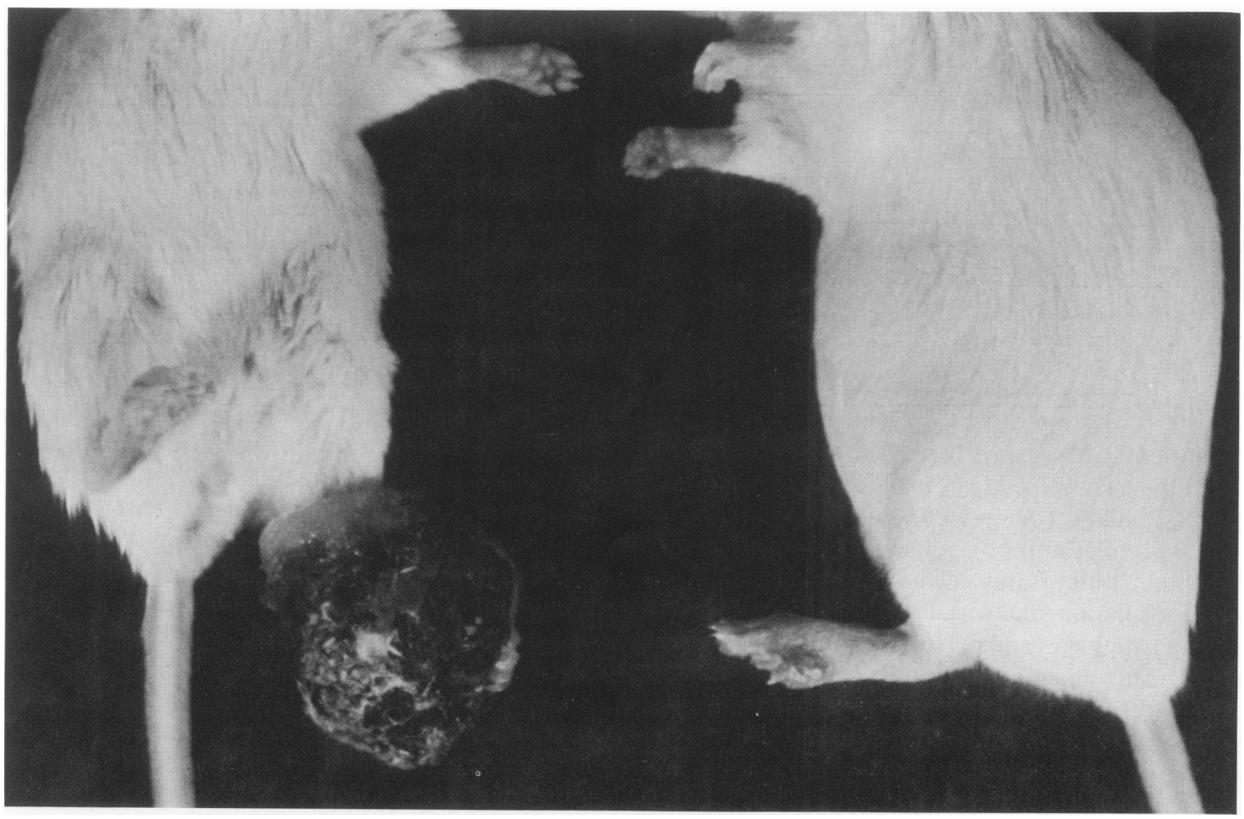


Figure 2— BALB/c and A/J mice 2 months after inoculation of *L mexicana amazonensis* into the footpad. While a huge dark mass formed in BALB/c mouse, only a small nodule developed in the A/J mouse.

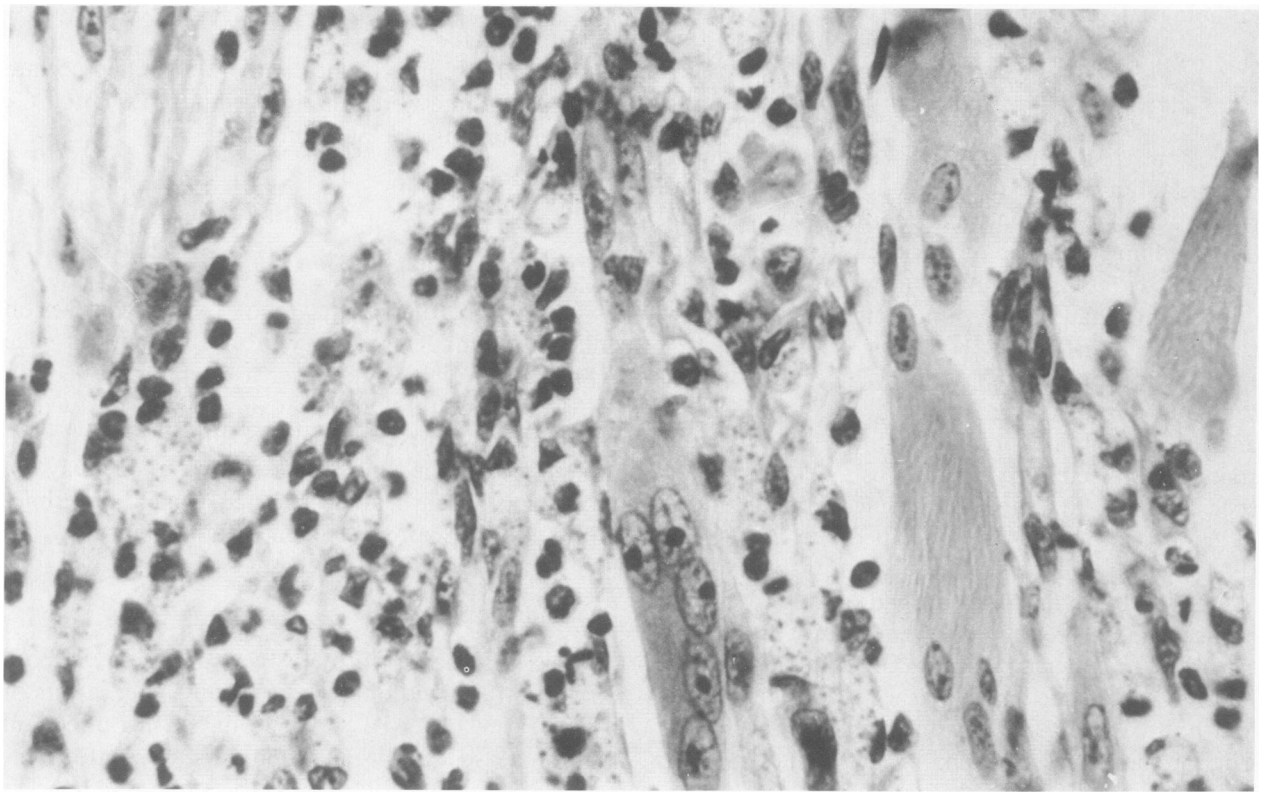


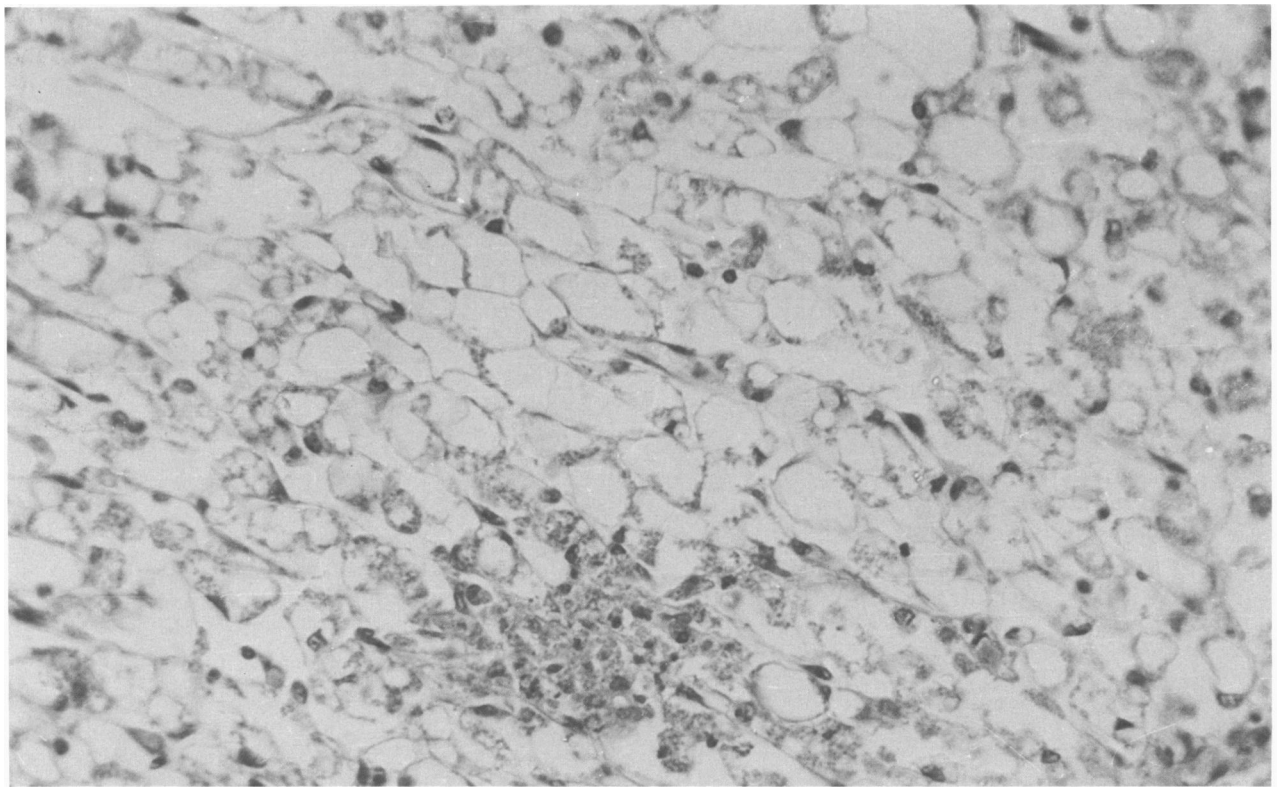
Figure 3—Early reaction to leishmania in the mouse. Macrophages laden with parasites are seen among other mononuclear and polymorphonuclear leukocytes dissociating striated muscle fibers, which show degenerative and regenerative features. BALB/c, 2 days after inoculation. (H&E, ×400)

Delayed-Type Hypersensitivity Responses

Measurable DTH responses appeared in both BALB/c and A/J mice between 2 and 3 weeks of infection. The response remained positive in A/J mice during the course of observation (90 days) but became negative in BALB/c mice by 45–60 days after infection (Figure 1).

Histopathology

During the first 4 days there were no differences between the changes seen in the inoculated footpads of BALB/c and A/J mice. Both showed an acute reaction, with infiltration of polymorphonuclear neutrophils, congestion, and edema present by 24 hours after inoculation, and with leishmanias present inside both macrophages and polymorphonuclear cells (Figure 3). This acute reaction also appeared 24 hours after injection of either culture supernatant or dead parasites, although it was then transient and less intense. By the fourth day many macrophages containing phagocytized parasites appeared at the inoculation site, and the microscopic appearance of the lesions started to show differences between the two mouse strains. In the BALB/c mice there was accumulation of macrophages around an area of microabscess that soon disappeared to give rise to the characteristic monotonous, one-cell-type Virchowian infiltration with numerous parasites (Figure 4). In the A/J mice the reaction revealed a mixed-cellular type of infiltration with scattered parasitized macrophages, as well as lymphocytes, plasma cells, and fibroblasts (Figure 5). From the seventh day on, the BALB/c lesion increased progressively in size, always disclosing a monomorphic type of reaction with numerous vacuolated parasitized macrophages forming an extensive collection of cells packed together, simulating the appearance of fatty tissue (Figure 4). This picture was only changed when relatively extensive areas of coagulative necrosis ensued. Necrotic areas were seen free of cellular infiltration, but soon a dense collection of polymorphonuclear neutrophils occurred and formed focal areas of purulent necrosis (Figure 6). These changes were prominent after the fourteenth day of infection and continued thereafter, causing the gross lesion to present areas of collapse and bosselation that sometimes assumed a variegated appearance due to concomitant hemorrhages. Collection of parasitized macrophages were seen infiltrating between



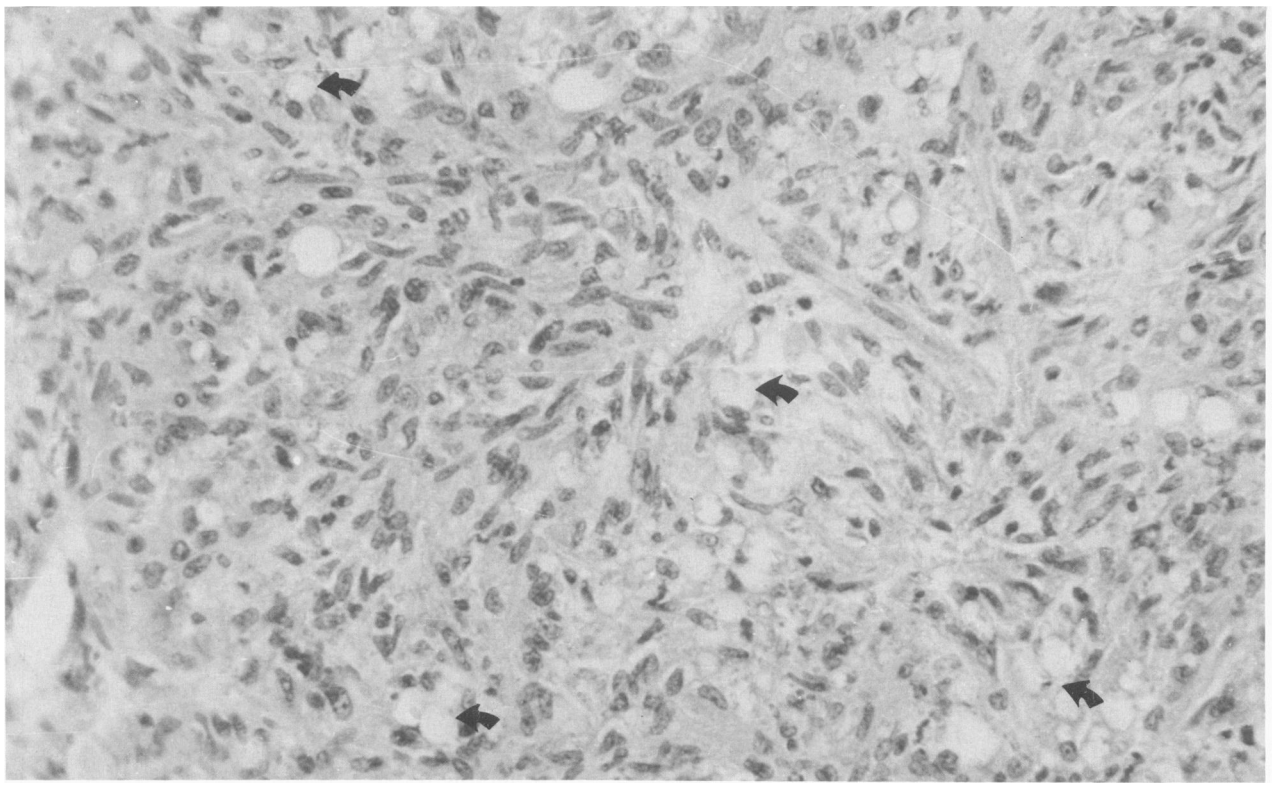


Figure 4 — One-month-old lesion in the BALB/c mouse. Collection of vacuolated parasitized macrophages assumes a fatty-tissue-like appearance. (H&E, ×400) **Figure 5** — One-month-old lesion in the A/J mouse. There are some vacuolated parasitized macrophages present (*arrows*), but the bulk of the lesion is made up by nonparasitized macrophages, some lymphocytes, and fibroblasts. (H&E, ×400)

striated muscle bands, and by the thirtieth day even the dense connective tissue of the tendon sheath and the local bone marrow appeared infiltrated (Figures 6 and 7). Also, hair follicles and sweat and sebaceous glands were infiltrated and dissociated, although not destroyed. However, although collections of vacuolated macrophages were seen in the superficial dermis, no invasion of the epidermis was ever detected.

The draining popliteal lymph node showed nonspecific changes from the beginning of infection, but by 2 weeks there was dilatation of cortical sinuses where several isolated, distended macrophages containing poorly stained leishmania organisms could be observed (Figure 8). However, no evidence of *in situ* parasite multiplication could be seen. In the medullary areas there sometimes were foci of epithelioid cells, and plasmocytosis and hypercellularity of the medullary cords. The histologic character of the spleen and liver was within normal limits, although these organs yielded parasites when cultured in LIT-FCS.

In the A/J mice the footpad lesion assumed a distinctive histologic pattern from the fourth day of infection. Parasitized macrophages were numerous, but these cells were isolated among a variety of other cell types, including lymphocytes, plasma cells, eosinophils, polymorphonuclear neutrophils, and fibroblasts. The periphery of the lesion was soon surrounded by granulation tissue and a thin layer of fibroblastic proliferation and collagen deposition, so that no invasion of tendon, bone marrow, or adnexal skin structures was ever seen. Up to the seventh day many microabscesses were formed, but they were not observed at later times. By the twenty-first day the reaction was dominated by fibroblastic proliferation, the presence of a few small granulomas, and an absence of polymorphonuclear cells. By the second month the lesion had become distinctively fibrotic, and the number of parasites and parasitized cells had decreased considerably. The parasites did not, however, disappear in any case. Focal areas of fibrinoid necrosis occurred 30–60 days after infection, affecting areas with dense parasitism (Figure 9). These necrotic areas apparently underwent liquefaction and resorption and fibrous replacement rather than neutrophilic infiltration, as was the case with BALB/c mice.

Draining popliteal lymph nodes showed variable degrees of nonspecific changes, but parasites were not detected. At later stages, plasmocytosis of the medullary cords and simple dilatation of medullary sinuses appeared as constant and prominent findings. Changes in spleen and liver were not remarkable.

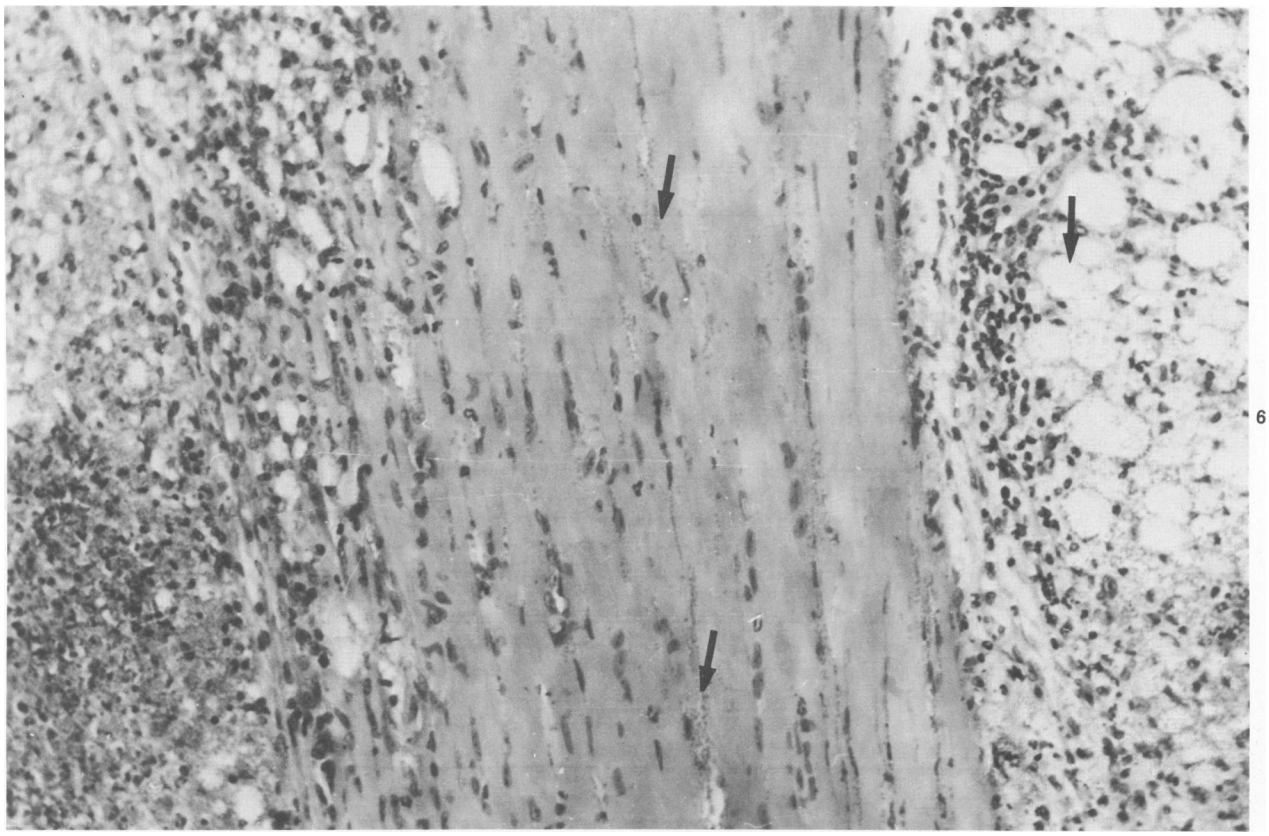
On electron-microscopic examination the leishmanias were found, similarly, within the parasitophorous vacuoles of the macrophages in both BALB/c and A/J mice. Some parasites were attached to the vacuolar membrane, while others were free within the vacuoles. The leishmanias were well preserved, and only a few exhibited degenerative changes, appearing shrunken, dark, and vacuolated.

Well-preserved, vacuolated, parasitized macrophages formed extensive collections in BALB/c lesions (Figure 10), while in A/J lesions there were frequent foci of necrosis associated with parasitized cells. In the presence of necrosis, leishmanias were found free in the interstitial tissue and sometimes phagocytosed by polymorphonuclear neutrophils or eosinophils. The parasites in interstitial tissue exhibited variable degrees of vacuolization and disintegration (Figure 11).

Groups of 6–8 BALB/c and A/J mice, infected in the footpad like the other experimental animals, were left under observation to allow the normal course of infection to continue. In all of the BALB/c mice disseminating fatal infections developed with multiple metastatic lesions on the nose, ears, tail, and feet. Splenomegaly was present in these animals, and parasites were recovered from the spleen and liver. By approximately 100–120 days after injection wasting and death occurred in all animals. The A/J mice, as indicated above, had nearly resolved primary lesions by approximately 60 days after infection. By 120 days 2 of 6 animals had developed ulcerating, nonhealing metastatic lesions on their ears and noses.

Discussion

Although the polar forms of cutaneous leishmaniasis present quite distinct histologic pictures comparable to those found in leprosy,[9,10] frequently both cutaneous leishmaniasis and mucocutaneous leishmaniasis in man show a rather nonspecific chronic inflammation with some histologic features that could have prognostic significance. Ridley et al[4,11] observed that in such cases the histologic aspects varied from an almost normal skin up to dense cellular infiltration, granulomas, and necrosis, parasites being scanty or absent. These authors classified their cases into five groups that could be designated as nonreactive, reactive, infiltrative, tuberculoid, and hyperergic, but no clear-cut clinical or immunologic correlations were evident. In keeping with most cases of human leishmaniasis, our A/J mouse strain exhibited a considerable degree of resistance to leishmania while producing a nonspecific mixed-cellular inflammatory reaction. Although some giant cells and a few focal accumulations of epitheliod cells did appear, a



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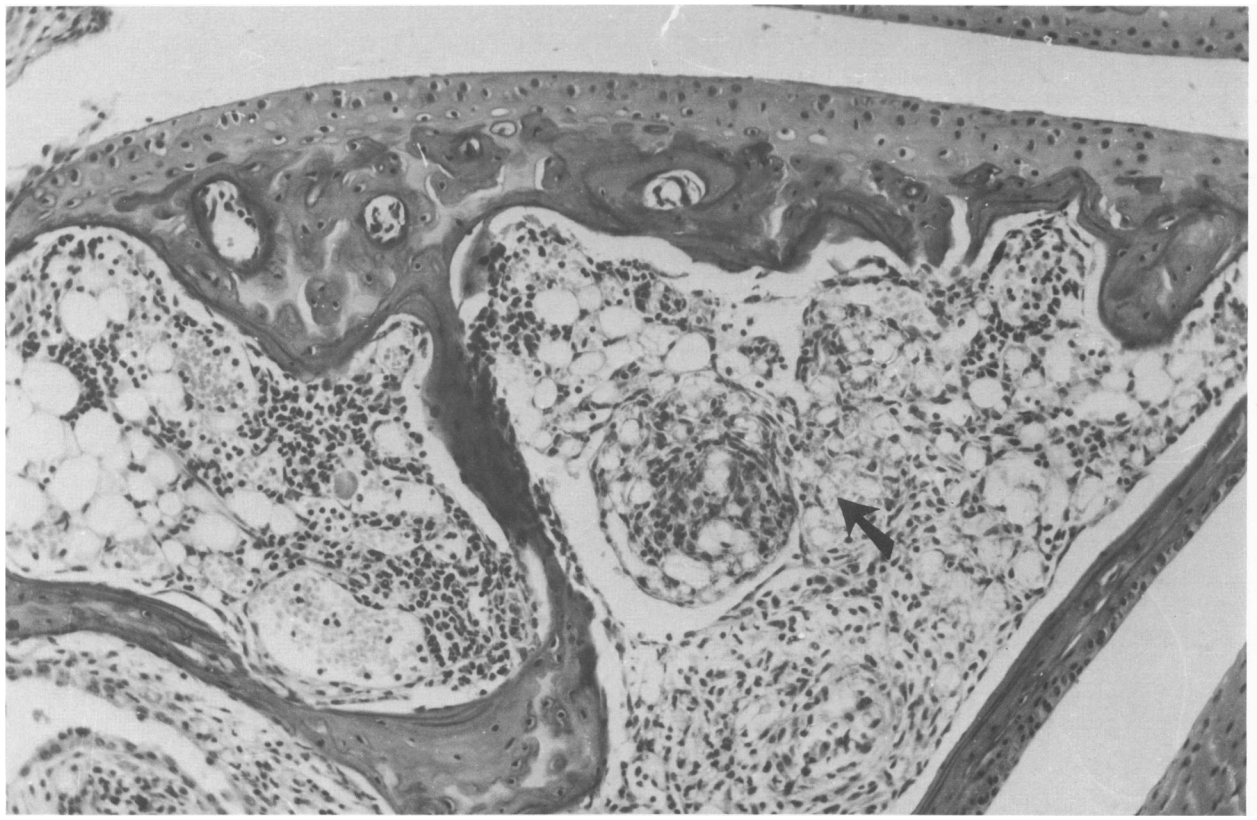

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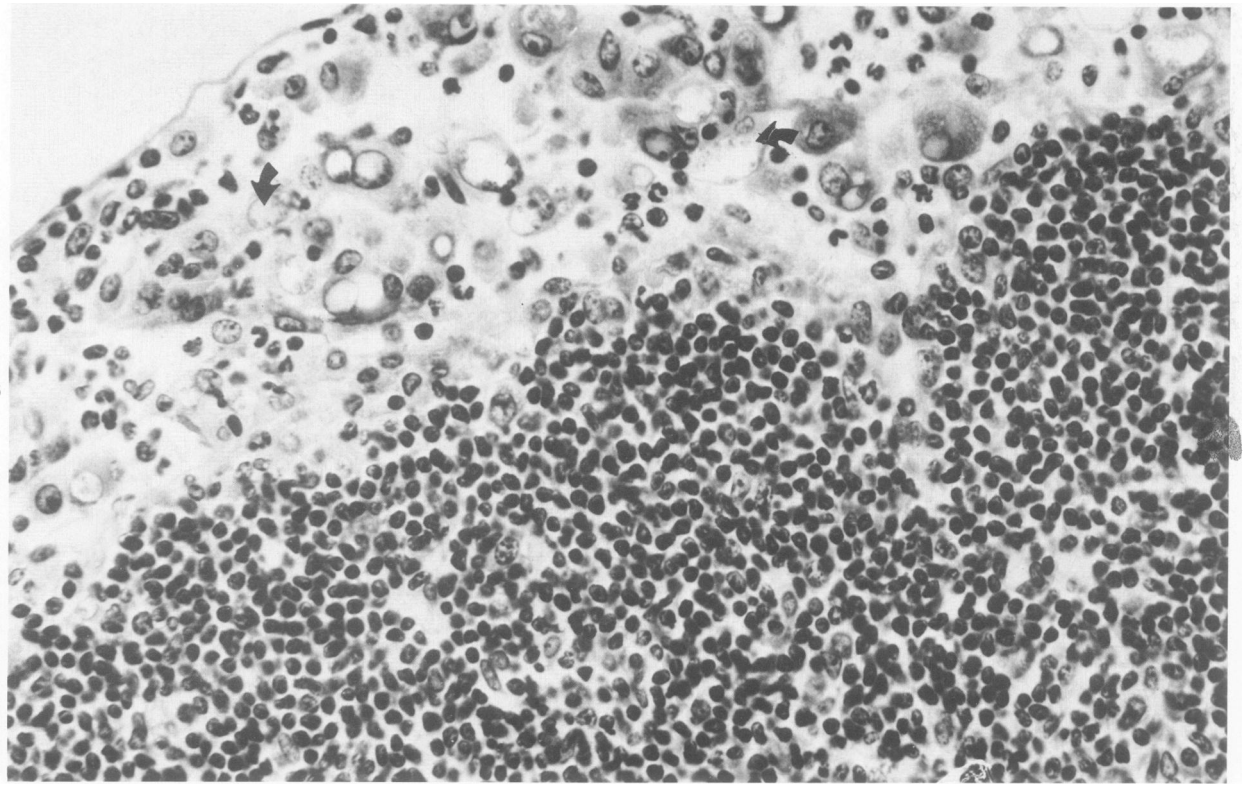
Figure 6 – Extensive collections of vacuolated and parasitized macrophages appear on both sides of a tendon. At the lower right of the picture there is a focus of purulent necrosis. Parasitized macrophages can be seen in between the dense collagen fibers of the tendon. BALB/c, 2-month-old lesion. (H&E, × 150) **Figure 7** – Bone marrow invasion by leishmanias in a BALB/c mouse. There are many parasitized macrophages and focal accumulations of inflammatory cells. (H&E, × 120)



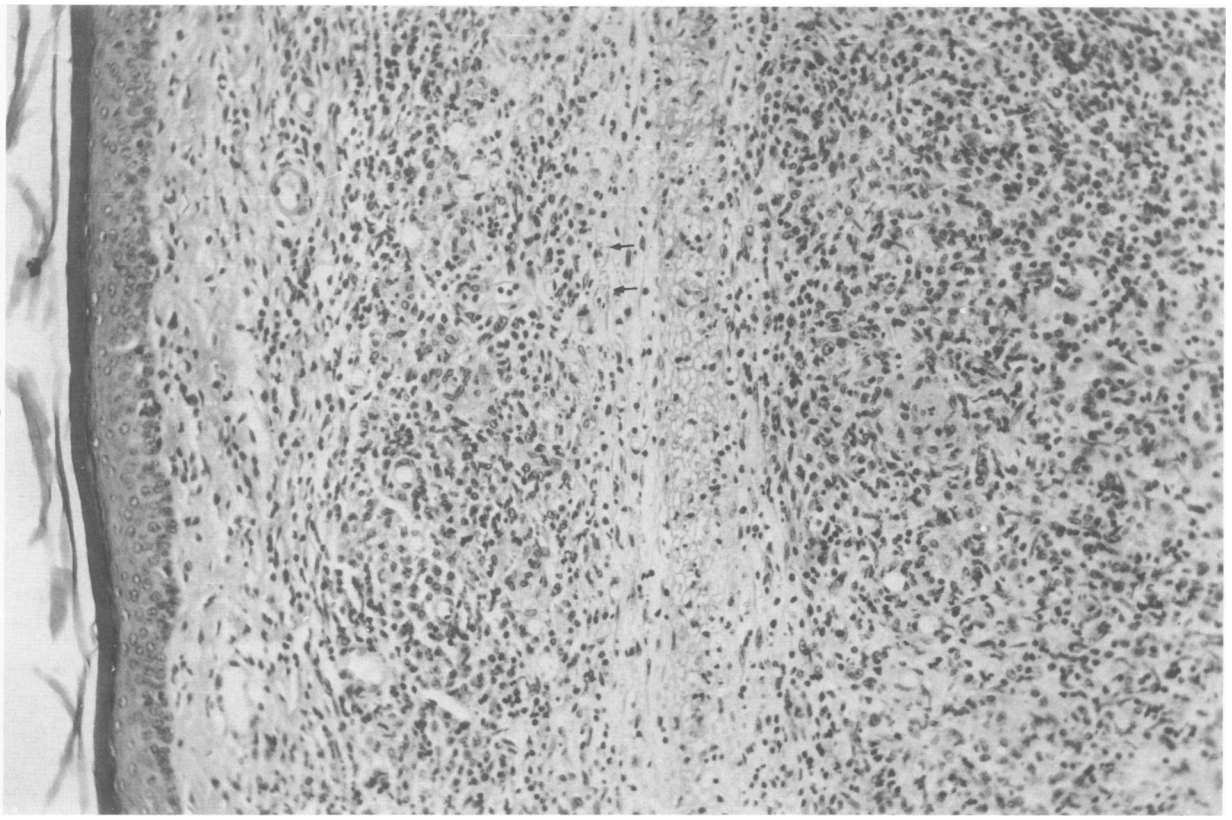


Figure 8 – Popliteal lymph node draining a 2-month old footpad lesion in a BALB/c mouse caused by *L mexicana amazonensis*. Cortical sinus is dilated and contains poorly stained parasites (*arrows*) within the cytoplasm of macrophages. (H&E, × 150) **Figure 9** – A representative late leishmania lesion in the A/J mouse. Intracellular leishmanias can be seen (*arrows*). The dermis shows a mixed-cellular reaction with lymphocytes and macrophages predominating. In the middle of the field there is a band of fibrinoid necrosis. (H&E, × 100)

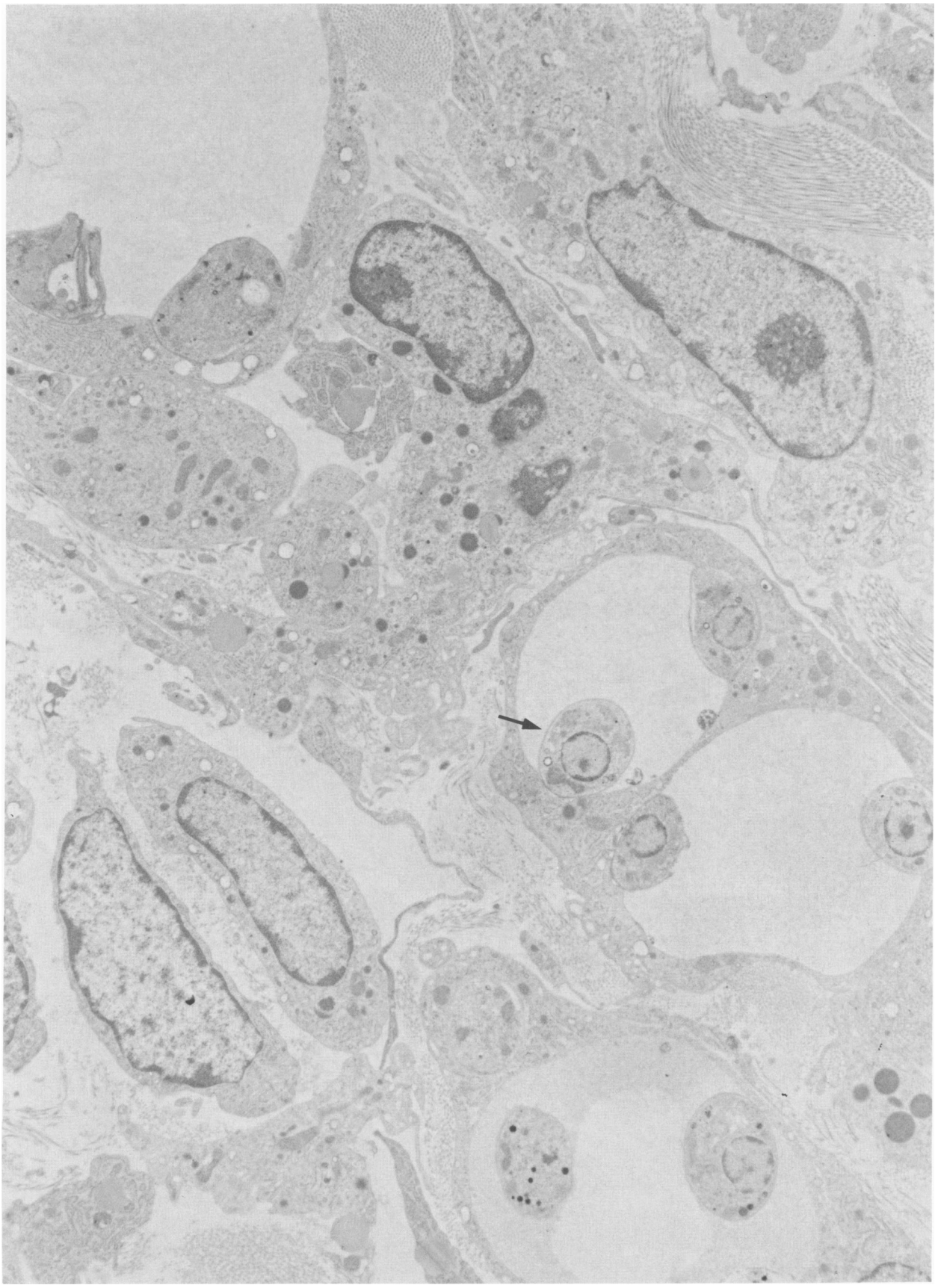


Figure 10 – Electron micrograph showing well-preserved leishmanias within the parasitophorus vacuoles of macrophages (*arrows*), a representative aspect seen in 1- and 2-month-old footpad leishmania lesions, in the BALB/c mouse. (×2000)

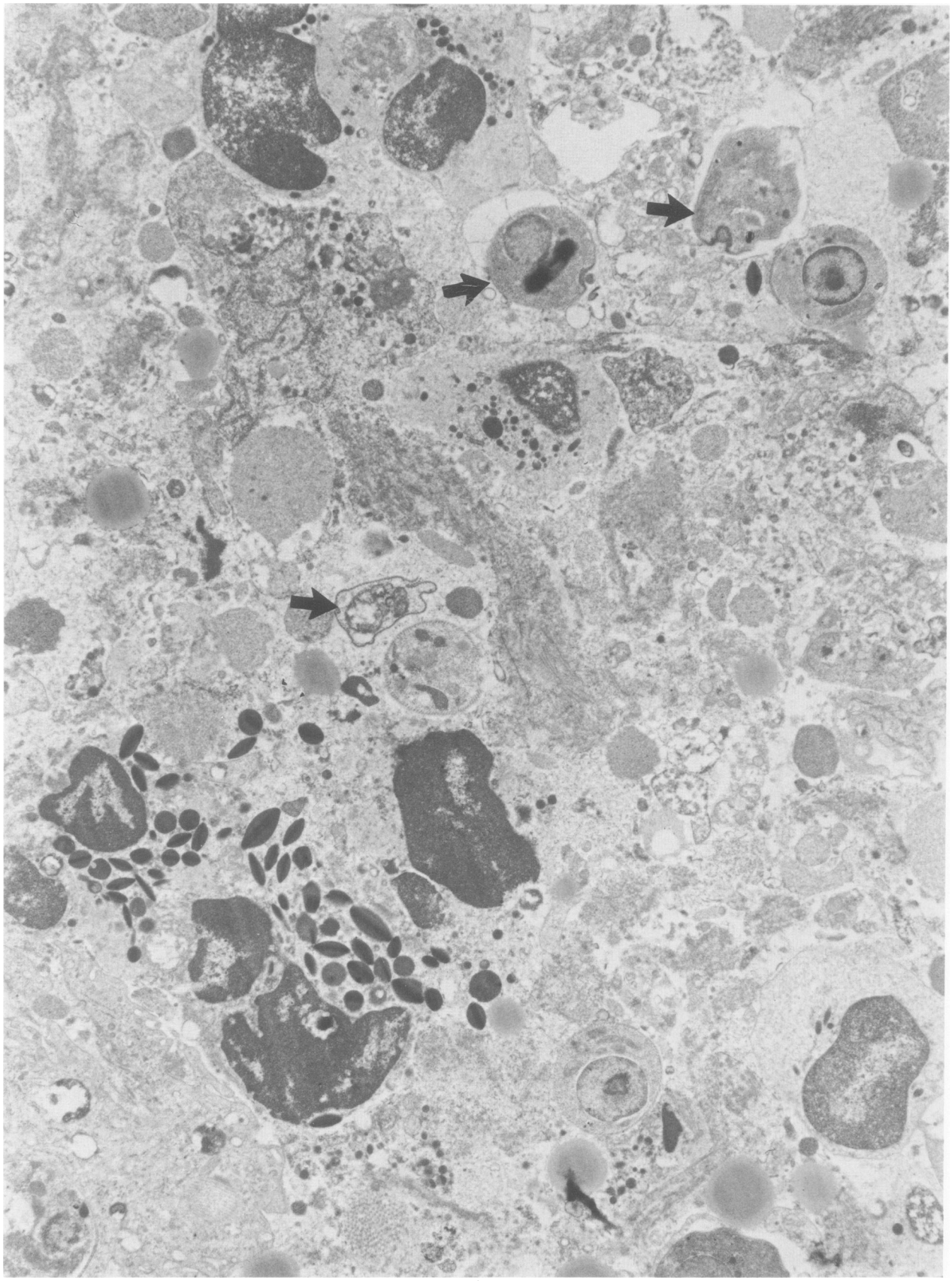


Figure 11 – Disintegration of parasitized macrophages and other cell types, including eosinophils, in a A/J leishmania lesion of 2 months' duration. Leishmanias free in the desintegrating tissue show evident degenerative changes (*arrows*), while others appear better preserved. (× 2600)

clear-cut tuberculoid type of reaction was not a consistent feature. Ridley[11] has suggested that necrosis of parasitized macrophages seems to be the most effective host defense mechanism in human leishmaniasis. It is interesting to note that in our resistant strain necrosis appeared relatively late in the course of infection, when a footpad delayed hypersensitivity response was becoming more evident. Similar to the necrosis seen in man,[4,12] a fibrinoid pattern was also observed in the mouse. In the susceptible BALB/c mice extensive areas of necrosis were frequently observed. However, these appeared earlier, were of coagulative type, and seemed to be ischemic, appearing in focal areas devoid of cellular infiltration. Necrotic areas were soon infiltrated by polymorphonuclear neutrophils and presented progressive collapse and resorption, while the fibrinoid necrosis appearing in resistant mice underwent fibrous replacement. Indeed, fibroblastic proliferation occurred throughout the lesion and at the periphery produced a capsulelike formation that turned the lesion into a discrete nodule. Thus, the resistant lesion was characterized by a localized nodule with a mixed-cellular infiltration and many lymphocytes and other mononuclear cells, focal fibrinoid necrosis, and interstitial and peripheral fibrosis. Fibrosis in parasitic infection can be interrelated with the immune host response, fibroblastic activation being dependent on lymphokine production[13] or liberation of secretory factors of activated macrophages.[14,15] On the other hand, the nodular foam-cell lesions in BALB/c mice were infiltrative and extended to tendon, bone marrow, and skin appendages, although sparing the epidermis, which did not ulcerate. Focal cellular infiltration of the epidermis did occur in the more resistant mice, and this could represent a potential basic mechanism for skin ulceration seen by others[16,17] in later stages of leishmaniasis in resistant mouse strains. Such invasive, destructive lesions have been observed in human mucocutaneous leishmaniasis[4,12] and have been considered one histologic correlate of DTH.

Levels of anti-leishmania IF antibody did not differ significantly between the two mouse strains. DTH responses to parasite antigen were present in both strains relatively early in the infection, but the response became negative in the BALB/c mice as the infection progressed. Similar findings were reported by Howard et al[18] in BALB/c mice infected with *L tropica*. Pérez et al[2] also reported decreased specific DTH responses in BALB/c mice infected with *L mexicana*. However, it was noted by these authors that agglutinating antibody titers were lower in BALB/c mice than in more resistant strains.[2,19] In the present study, like that of Nasseri and Modabber,[3] we used the IFA test to detect IgG antibodies. As a matter of fact, IFA test has been considered as the most sensitive method of detecting antibody in leishmania infections.[20]

The basis of the relative susceptibility to cutaneous leishmaniasis is not well understood, although Howard et al[21] used bone marrow chimeras of resistant and susceptible strains to demonstrate that resistance and suceptibility to *L tropica* was determined by the source of bone marrow cells and not by their environment. In general, the BALB/c mouse is the strain most susceptible to visceral and cutaneous leishmaniasis. However, to suggest that this is a result of a peculiar defect in the ability of the BALB/c mouse to recognize or respond to leishmania antigens in general may be quite misleading. For example, C57Bl/6 or C67Bl/10 mice are highly susceptible to *L donovani* or *L donovani chagasi* but are "resistant" to cutaneous species.[12]

Macrophages readily phagocytose leishmanias; and although lysosomal fusion with the parasitophorus vacuoles occurs,[22,23] the parasites manage to survive and multiply intracellularly. Our electron-microscopic findings did not detect any ultrastructural differences between parasitized macrophages from susceptible versus resistant mouse strains. However, they suggested that destruction of parasitized cells and tissues was more frequent in resistant than in susceptible strains. In addition, we observed that leishmanias exhibited more degenerative changes extracellularly than intracellularly. Although necrosis did occur in both mouse strains, it had different appearances and probably a different pathogenesis and protective effect, being of an ischemic nature in BALB/c and relatively more disseminated and probably immunologically mediated in A/J mice. It has been demonstrated that macrophages can express parasite antigens in their membranes when infected with leishmanias.[24] It remains to be demonstrated whether susceptibility and resistance to leishmanial infection in our mice are somewhat related to the ability of the immune system to recognize and react to parasite antigens in macrophages. Our data suggest that destruction of parasitized macrophages, thereby exposing the leishmanias to adverse conditions in interstitial tissue, may be one important mechanism leading to resistance in the A/J mice. However, it is possible that there are multiple genetically determined resistance factors not yet understood that affect disease outcome in different mouse strains.

With regard to the relative resistance and susceptibility of mouse strains to cutaneous leishmaniasis, it has become increasingly apparent that such patterns may overlap somewhat. We have observed, for example, that in A/J mice, after a period of 4–6

months, metastatic lesions may develop, even after resolution of the initial lesions is long completed. A similar observation has been made in the relatively resistant C57BL/6 mouse infected with *L mexicana*.[17] It should be noted, however, that under the same conditions 100% of the infected BALB/c mice develop metastases. Recently, Scott and Farrel[25] showed that in mice that are relatively resistant to cutaneous *L tropica* when infected subcutaneously, disseminated infection develops when they are infected intravenously. Perhaps in a similar context, humans with diffuse cutaneous leishmaniasis frequently exhibit evidence of local lymphatic damage.[10] Such observations seem to indicate a barrier role for the draining lymph node in localized leishmaniasis. In this regard the draining lymph nodes in both our mouse strains disclosed reactive changes. However, while no parasites were ever seen in A/J mice, in BALB/c mice they were present within macrophages in cortical and paracortical sinuses, which were markedly dilated. Initially the phagocytosed parasites seemed poorly stained and nonviable; but later on, with mounting cellular immune depression (see Table 1), it is probable that many of them could bypass the lymph node barrier, thus accounting for disseminated metastases.

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