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LEISHMANIA MEXICANA AMAZONENSIS: HETEROGENEITY IN 5'-NUCLEOTIDASE AND PEROXIDASE ACTIVITIES OF MONONUCLEAR PHAGOCYTES DURING *IN VIVO* AND *IN VITRO* INFECTION

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The degree of maturation of cells of the Mononuclear Phagocyte System (MPS), during in vivo *and* in vitro *infection by* Leishmania mexicana amazonensis, *was evaluated in this study. The macrophages' differentiation was assayed by cytochemical characterization at the ultrastructural level, using two well-established markers: 5'-nucleotidase enzyme activity, for revealing the mature cells; and the peroxidase activity present in the cell granules to demonstrate immature mononuclear phagocytes. Only a few macrophages, demonstrating 5'-nucleotidase positive reaction in both the plasma membrane and within their cytoplasmic vesicles, were found scattered in the chronic inflammation at the* L. m. amazonensis *lesions in albino mice. However, by the peroxidase activity analysis, we were also able to demonstrate the presence of immature MPS cells, which predominate, together with parasitized vacuolated macrophages, in chronic lesions induced in this system by* L. m. amazonensis. *The implications of these results on the pathogenesis of murine cutaneous leishmaniasis are discussed.*

Key words: *Leishmania mexicana amazonensis (L. m. amazonensis)* – immature mononuclear phagocyte – mature macrophages – peritoneal macrophages – cutaneous lesions – 5'-Nucleotidase (E. C. 3.1.3.5) – Peroxidase (E. C. 1.11.1.7)

The MPS, which is widely distributed throughout the host, comprised a group of phagocytic cells with similar properties (Adams, 1976). They arise in the marrow from immature promonocytes, circulate briefly in the blood as monocytes, and migrate to the tissues and inflammatory foci where they mature into macrophages (Van Furth, 1970). In these foci these cells play a significant role in the host defense mechanism against invaders (Cohn, 1968). Their efficacy in protecting the host against these foreign intruders, specially for those chronic intracellular parasites such as protozoa of the genus *Leishmania*, is related directly to their state of maturation or differentiation (Adams, 1979; Mauel, 1979). Cells of this system showing different stages of maturation can be recognized at the inflammatory site of the host based on their ultrastructural morphological characteristics (Adams, 1974; Van der Rhee et al., 1979). Moreover a clear-cut distinction between immature and mature mononuclear phagocytes can be carried out by cytochemical characterization at the ultrastructural level (Edelson & Cohn, 1976; Edelson & Erbs, 1978; Beelen et al., 1979; Fahimi, 1970; Robinson & Karnovsky, 1983; Meirelles & De Souza, 1986).

In a morphological kinectic study, we have previously demonstrated, based only on ultrastructural morphology, that in typical chronic *L. mexicana* lesions in susceptible hosts, the main histopathological finding is an inflammation. This consisted mainly of a progressive infiltration of immature cells of the MPS which coalesced and evolved into well-developed macrophages forming loosely organized granulomas (Grimaldi et al., 1984). To corroborate these previous results, the present study was designed to define better the degree of maturation of cells of the MPS during infection of susceptible murine models *in vivo* and *in vitro* with *L. m. amazonensis*. The macrophage differentiation was assayed by cytochemical analysis at the ultrastructural level using two markers: an ectoenzyme, the 5'-nucleotidase for revealing the mature cells and analysis of the

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peroxidase activity in the cell granules to demonstrate immature mononuclear phagocytes.

MATERIALS AND METHODS

Parasite and host – The MHOM/BR/77/LTB0016 strain of *Leishmania mexicana amazonensis* used in all experiments was supplied by Dr. C. A. Cuba Cuba, Universidade de Brasília, Distrito Federal, Brazil. The identification as *L. m. amazonensis* has been confirmed by analysis of isoenzyme patterns (Momen & Grimaldi, unpublished data) and based on the characteristic reactivities with specific monoclonal antibodies (Grimaldi et al., 1987). The promastigote forms of the parasite used for experimental infection were obtained by culturing fragments of cutaneous lesions from albino mice in NNN blood agar medium with an overlay of modified LIT liquid medium (Jaffe et al., 1984). In the preparation of samples, the virulent parasites (promastigotes in late log phase of growth in culture, obtained by transformation of amastigotes and maintained *in vitro* for one week) were harvested by centrifugation (1,500 g for 10 min at 4°C), washed in tissue culture medium 199, and adjusted to the desired concentration.

Young adult female outbred mice albino Swiss-Webster, received from the Fundação Oswaldo Cruz animal house in a single lot, were inoculated subcutaneously in the perinasal region with 5×10^6 promastigotes in 0.05 ml. For *in vivo* study the animals were sacrificed one month after infection and the material from the lesions was processed for electron microscopy.

Macrophage culture – Resident peritoneal macrophages were collected from outbred albino mice. The animals were killed with ether, their peritoneal cavities were washed with 4 ml of the medium 199 and the macrophages thus recovered were plated in glass flasks. The cells were allowed to adhere to the glass surface for 1 h at 37°C, the saline solution and the non-adherent cells were then removed and culture medium (199 medium plus 10% foetal calf serum) was added. After incubation at 37°C for 24 h the macrophage cultures were rinsed with Ringer's solution (0,015 M-NaCl, 0,07 M-KCl and 0,05 M-$CaCl_2$) and used for the experiments.

Macrophage-parasite interaction – Promastigotes maintained *in vitro* for one week were suspended in Ringer's solution and maintained for 2 h in contact with the macrophages. After two hours the cultures were rinsed with Ringer's solution to remove extracellular parasites, and processed for cytochemical experiments and for electron microscopy.

Cytochemistry – Cells and fragments of cutaneous lesions were incubated, for the detection of 5'-nucleotidase activity, in the medium described by Uusitalo & Karnovsky (1977) at 37°C for 60 min. The medium had the following composition: 100 mM Tris-Maleate buffer (pH 7.2), 5 mM adenosine 5'-monophosphoric acid (AMP) as substrate (Type III, Sigma Chem. Co.), 10 mM $Mg(NO_3)_2$ and 1 mM $CeCl_3$ (Sigma Chem. Co.). The following controls were used: 1) incubation in substrate-free medium; 2) incubation in medium containing β-glycerophosphate (B-GP) (Sigma Chemical Co.) instead of AMP; 3) incubation in the complete AMP or B-GP medium to which 0.5 or 2.0 mM levamisole (Sigma Chem. Co.), an inhibitor of nonspecific alkaline phosphatase, was added (Borgers, 1973). Cells and fragments of cutaneous lesion were incubated, for the detection of endogenous peroxidase activity, in a medium containing 10 mg of 3.3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chem. Co., St. Louis, Mo.), 10 ml of 0.1 M Tris-HCl buffer, pH 7.6 and 0.02% H_2O_2 (medium described by Graham & Karnovsky, 1966). The incubation was carried out at room temperature for 60 min. The following controls were used: 1) cells and tissues were incubated in the absence of DAB or H_2O_2 from the incubation medium; 2) incubation in the complete medium containing 10 mM of 3-amino 1:2:4-triazole, inhibitor of catalase.

Electron microscopy – Cells and tissues were fixed in 2.5% glutaraldehyde and 0.1 M Na cacodylate buffer pH 7.2 (to reveal peroxidase activity) or in 1% paraformaldehyde + 1% glutaraldehyde in 0.1 M Tris-maleate buffer pH 6.0 (to reveal 5'-nucleotidase activity) both for 30 min at 4°C. They were then washed with buffer and used for the cytochemical experiments. After incubation of the cells and tissues in one of the media described above, followed by glutaraldehyde fixation, the cells (peritoneal macrophages) were rinsed in cacodylate buffer and left in this buffer overnight at 4°C. The cells were then gently scrapped off the wall of

the culture vessel with a rubber policeman, post-fixed with 1% OsO_4, dehydrated in acetone, and embedded in Epon. Thin sections were obtained in a Reichert Ultramicrotome OmU3, stained with aqueous uranyl acetate and lead citrate and examined in a EM 10B Zeiss electron microscope.

RESULTS

The 5'-nucleotidase activity of MPS cells – Only a few macrophages scattered in the chronic inflammatory reaction at the site of the *L. m. amazonensis* lesions in albino mice showed a positive enzymatic reaction at both the plasma membrane projection (Fig. 1) and within the cytoplasmic vesicles (Fig. 2). Splenic cells which are very reactive for this ectoenzyme are shown in Fig. 3 and were used as a positive control for the specificity of 5'-nucleotidase. On the other hand, cell incubation in medium containing β-glycerophosphate or levamisole inhibited completely the reaction in these cells (data not shown). The 5'-nucleotidase-positive reaction was not present in any parasitized monovacuolated macrophage.

Peritoneal macrophages infected for 2 h with *L. m. amazonensis*, parasitized or not, showed an electrondense reaction at their plasma membrane and in some cytoplasmic vesicles (Figs. 4 and 5). In infected macrophages, although the endocytic vacuole containing intact or even degenerated organism had no positive reaction in its membrane, the degenerated parasites displayed a positive reaction for this enzyme in their plasma membrane (see Fig. 6).

The peroxidase activity in MPS cells – Immature MPS cells, like the exsudate monocyte shown partially in Fig. 7, were detected in the mouse lesion with a strong positive peroxidase activity in the granules near the Golgi apparatus. Immature macrophages, as shown partially in Fig. 8, also revealed a positive reaction in the granules located at the periphery of their cytoplasm, sometimes found together with a clear microfilamentar system. On the other hand, neither in non-infected mature nor in heavily vacuolated macrophages could peroxidase activity in the cytoplasmic granules be detected (data not shown). However, parasitized immature macrophages exhibit with strong peroxidase activity, mainly around the parasitophorous vacuole. Some of the granules apparently transfer the peroxidase content into the parasitophorous vacuole (Fig. 9).

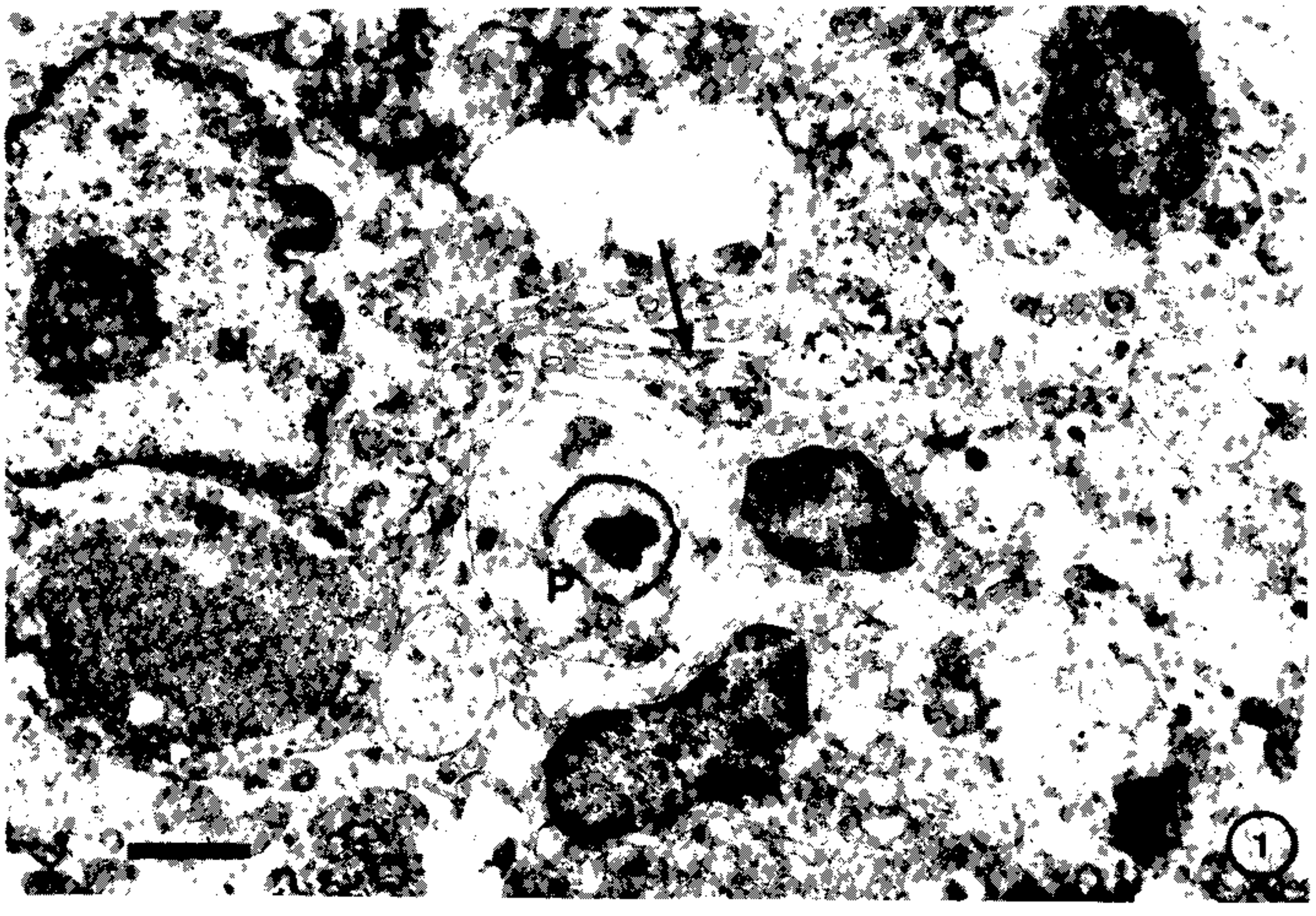
No reaction product was observed when DAB or H_2O_2 were omitted from the incubation medium. The addition of aminotriazole to the incubation medium did not affect the pattern of peroxidase distribution in the cells (data not shown).

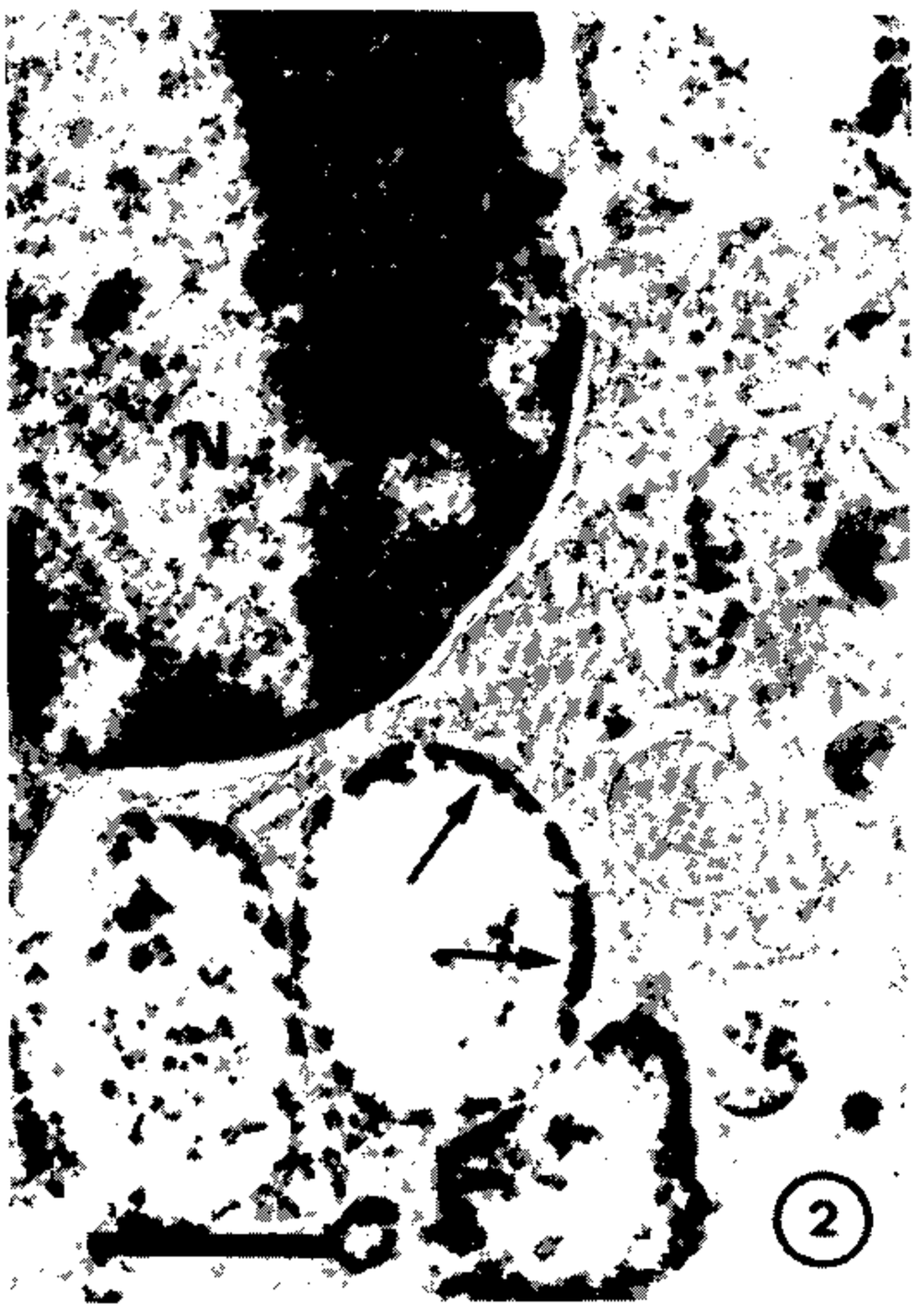
Histological and ultrastructural features of *L. mexicana* infection in susceptible and resistant inbred mice are described in detail elsewhere (Grimaldi et al., 1980; 1981; 1984). Here the histopathological finding in chronic *L. m. amazonensis* lesions in outbred albino mouse confirmed our previous results. Namely, that the inflammation consists mainly of an infiltration of cells of the MPS, manifestating itself in a range from immature cells to well-developed macrophages. In this loosely organized granulomatous reaction there was also an infiltration of granulocytes together with an accumulation of parasitized, vacuolated macrophages. These latter cells together with immature cells of the MPS predominated in the inflammatory reaction of this susceptible host.

DISCUSSION

Maturation or differentiation of mononuclear phagocytes has been extensively investigated *in vitro* (Cohn, 1968; Gordon & Cohn, 1973) and has also been directly demonstrated *in vivo* (Adams, 1974; 1975). In both systems macrophages can be stimulated to develop altered functional and metabolic characteristics, a state often termed activation (Adams, 1976). Macrophage activation in this broad sense can be induced by many stimuli, including parasites of the genus *Leishmania*. Cells of this system showing different maturational stages can be recognized at the inflammatory site of the host by the size, shape and the characteristic distribution of the specific granules in their cytoplasm (Adams, 1974; 1979; Van der Rhee et al., 1979), as well as by their different functional capacities (Cohn, 1968).

Our main purpose here was to characterize better the degree or state of maturation of MPS cells, parasitized or not, during infection of susceptible murine models with *L. m. amazonensis*. As the maturation process of MPS cells at any inflammatory site is a gradual event, a clear-cut distinction between these





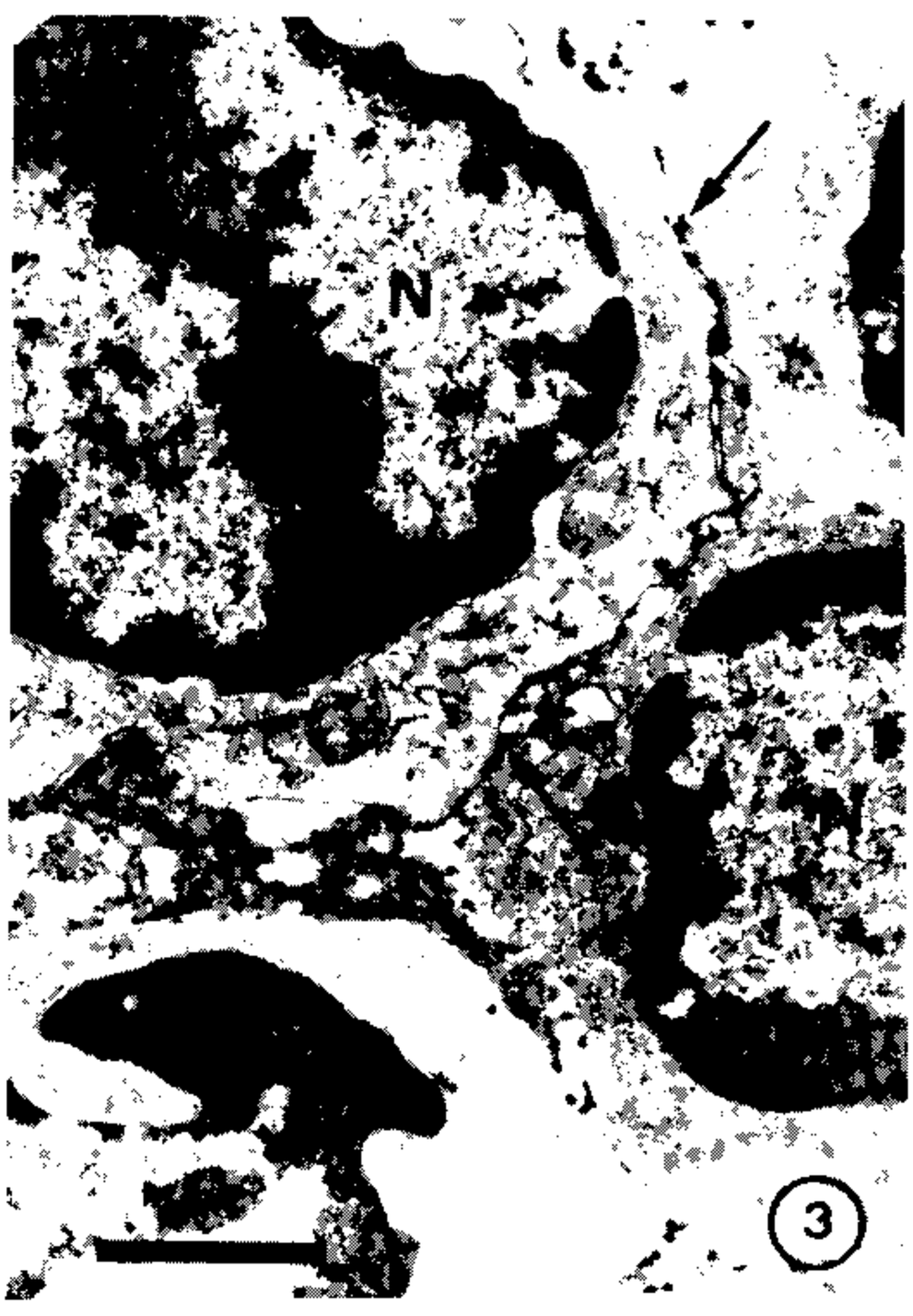
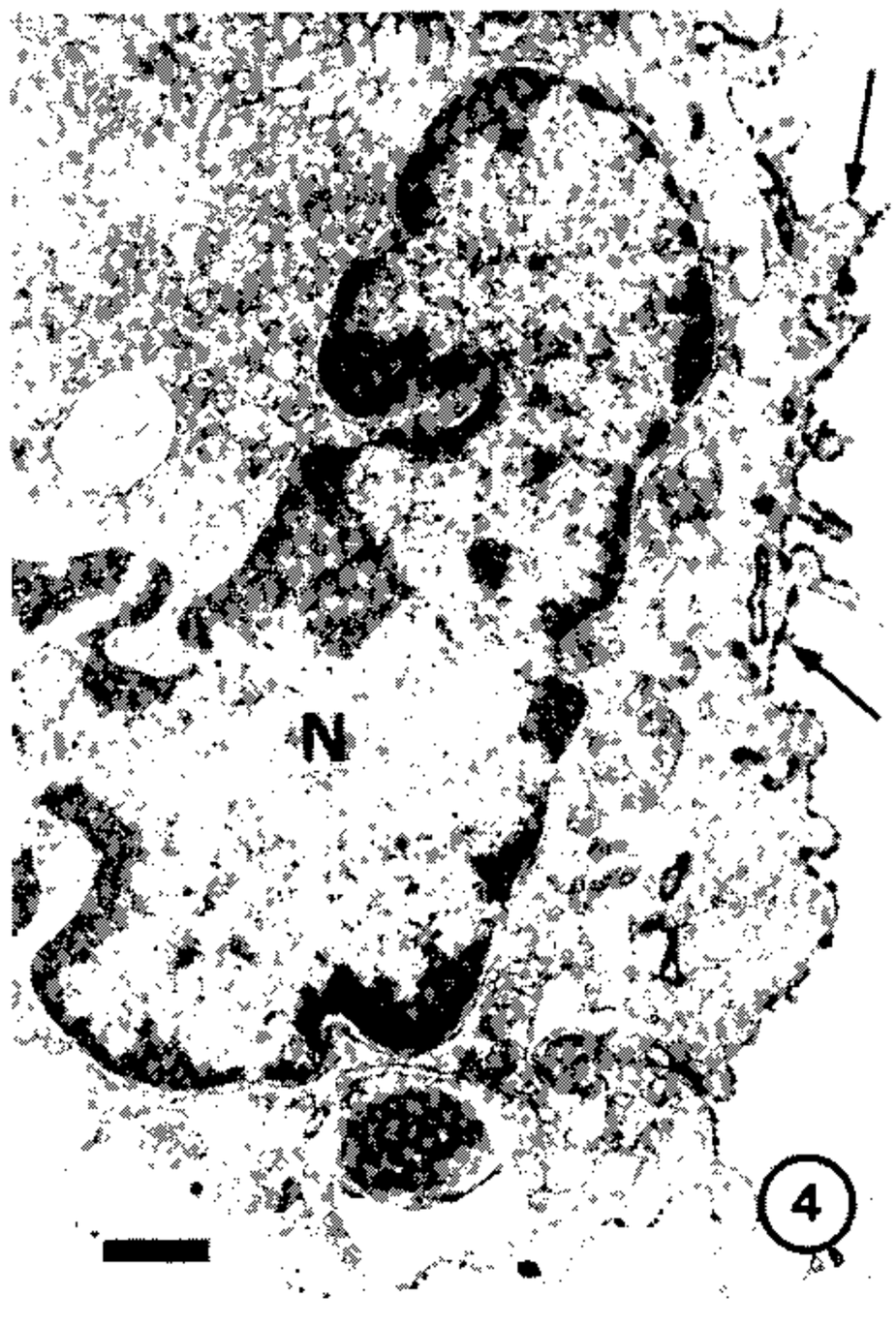
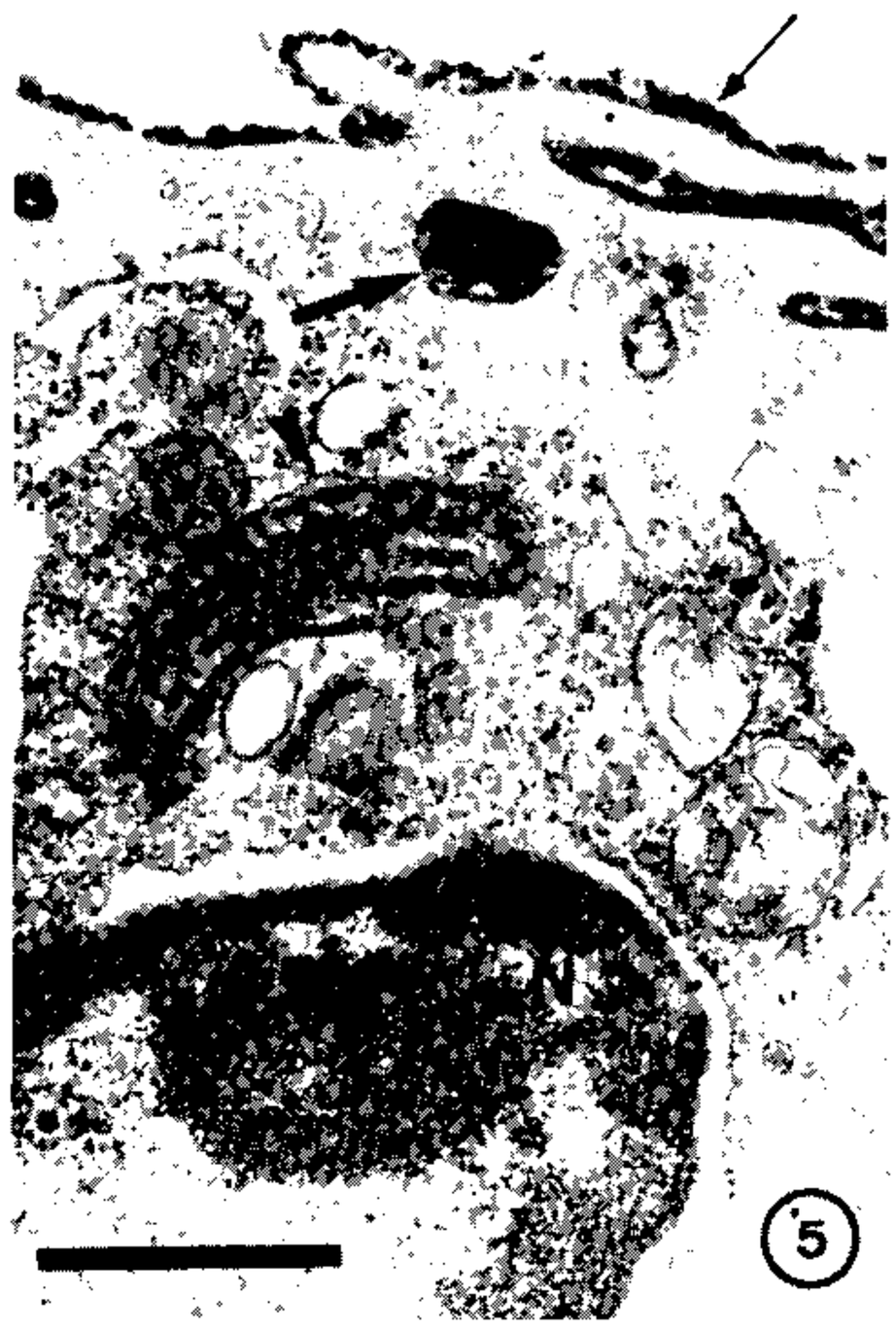
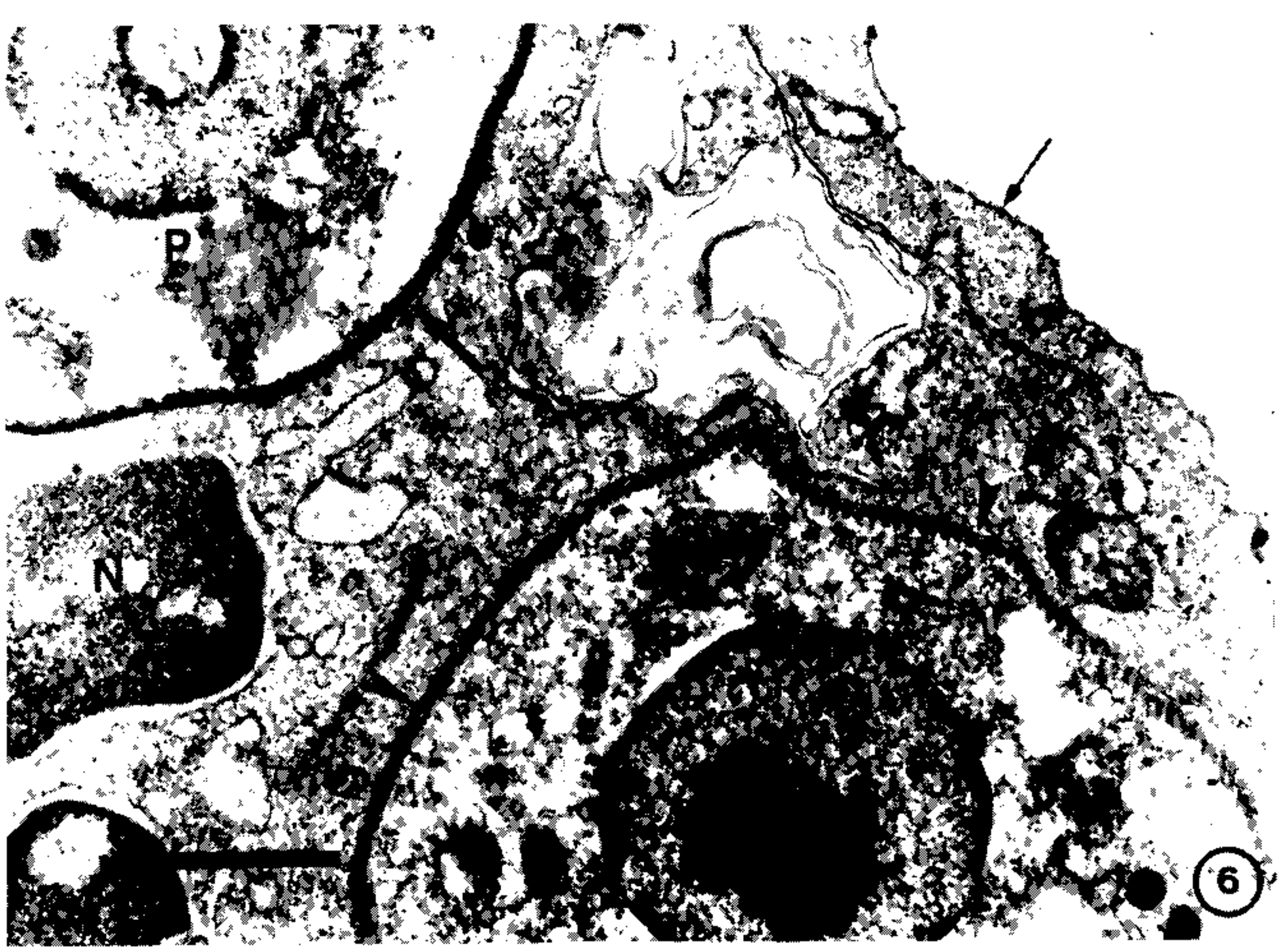


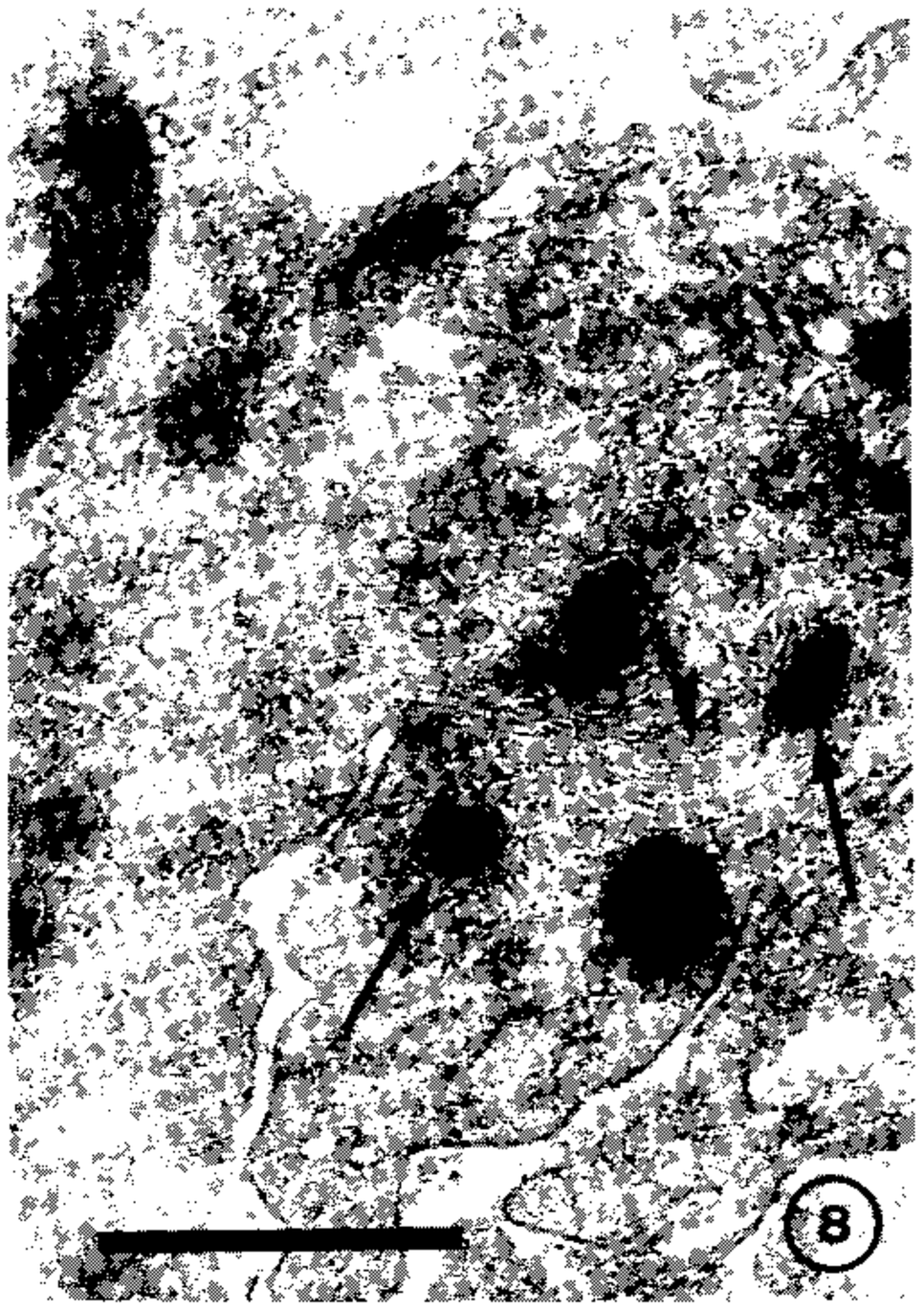
Fig. 1: Few macrophages present at the site of the lesion one month old induced by *Leishmania mexicana amazonensis* showed a positive reaction for 5'-nucleotidase in the projections of their plasma membrane (arrow). Fig. 2: A precipitate from the enzymatic reaction for 5'-nucleotidase in seen within cytoplasmatic vesicles of a few macrophages (arrows). Fig. 3: Splenic cells of the normal mice display an electron dense reaction indicative of the activity of the 5'-nucleotidase at their plasma membrane (arrows). Scale: bar = 1,0 μm.







Figs. 4-5: Normal peritoneal macrophages or infected with *Leishmania mexicana amazonensis* showed positive reaction for 5'-nucleotidase in the plasma membrane (thin arrow) and in some cytoplasmic vesicles (large arrow). No reaction product indicative of 5'-nucleotidase was seen in the membrane of the endocytic vacuole (arrow head). Fig. 6: The membrane of the endocytic vacuole of macrophages containing intact or degenerated parasites was negative for 5'-nucleotidase (arrow head). A weak reaction is observed at the plasma membrane of the macrophages (thin arrow). The plasma membrane of the degenerated parasites displays a positive reaction for this enzyme (large arrow). Scale: bar = 0,5 μm.



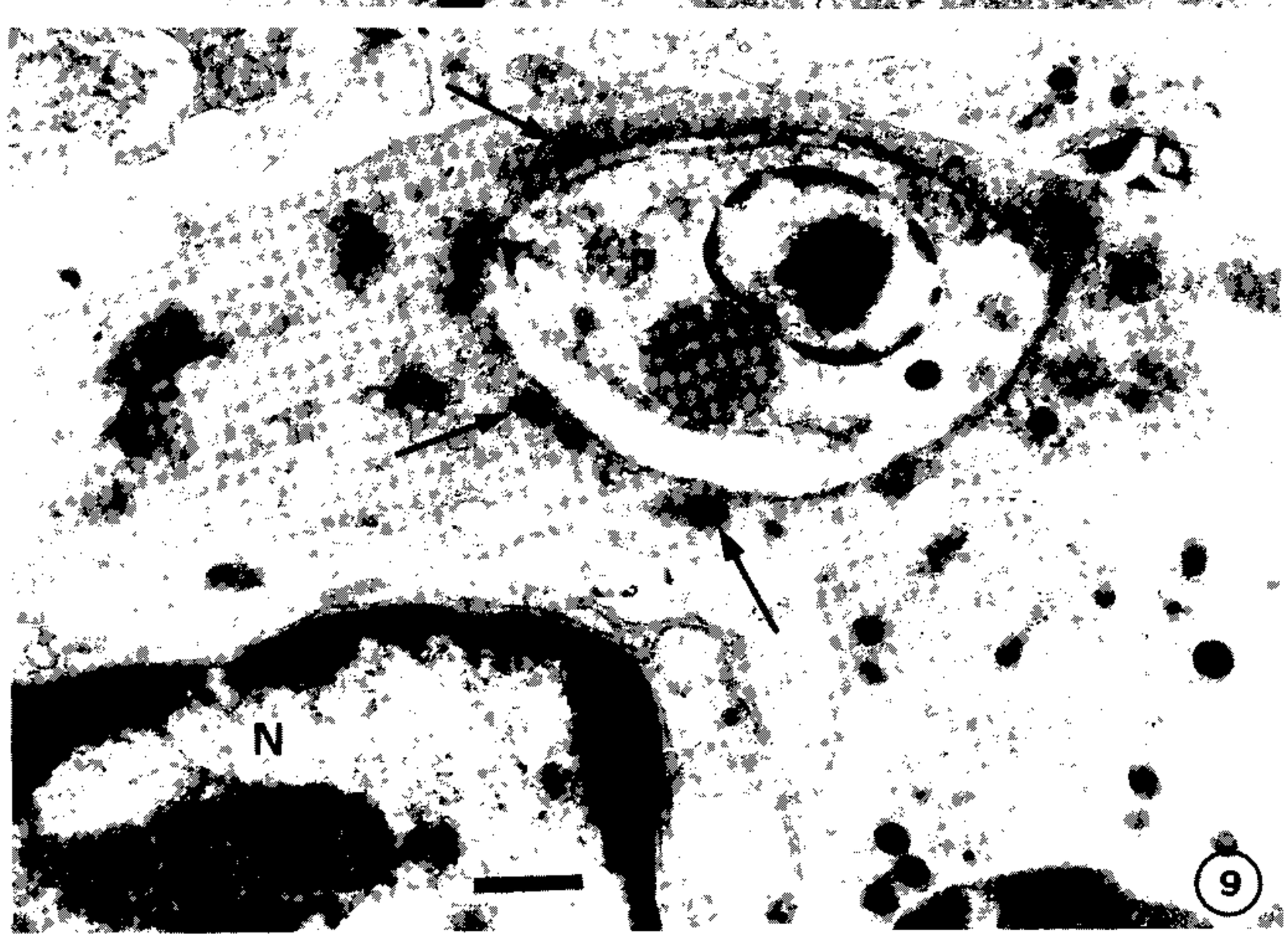


Fig. 7: In mouse cutaneous lesions one month old induced by *Leishmania mexicana amazonensis*, the exudate monocyte showed a strong positive peroxidase activity in the granules near the Golgi apparatus (arrows). Fig. 8: A positive reaction for peroxidase is localized in granules at the periphery of immature macrophage's cytoplasm (arrows). Also a clear microfilamentar system is found close to these granules (arrow-head). Fig. 9: An immature macrophage infected with *Leishmania mexicana amazonensis* exhibits granules in this cytoplasm with strong enzymatic activity, and apparently is degranulating the peroxidase content into the parasitophorus vacuole (arrows). Scale: bar = 0,5 μm.

elements is extremely difficult to estabish based only on morphological parameters (Van der Rhee et al., 1979). However the demonstration of cytochemical markers, such as 5'-nucleotidase and peroxidase enzymes activities, is a relatively simple and efficient procedure which distinghishes between mature and immature mononuclear phagocytes respectively (Edelson & Cohn, 1976; Werb & Cohn, 1976; Edelson & Erbs, 1978; Beelen et al., 1979; Meirelles & De Souza, 1986).

We demonstrated that, while MPS cells present in the inflammatory reaction showed low levels of 5'-nucleotidase activity, peritoneal macrophages infected *in vitro* with the same parasite had positive activity in their plasma membrane. Previous reports have also pointed out to these differences between peritoneal macrophages and MPS cells during an inflammatory reaction (Unkeless et al., 1974; Bianco et al., 1975; Edelson & Cohn, 1976). The absence of this enzyme in the phagosomal membrane of parasitized peritoneal macrophages could be due to its membranal redistribution during the phagocytosis process, as occurs in granulocytes (Cramer et al., 1980), or inactivation when the phagosomes fuse with lysosomes (Werb & Cohn, 1976). It has been shown that 5'-nucleotidase can be interiorized with latex particles (Werb & Cohn, 1976) but not with the parasite *Trypanosoma cruzi* (Meirelles & De Souza, 1986). We can not exclude the possibility that the components of the cell which are being interiorized vary according to the particles which are going ingested.

Peroxidase, in granules, is a cytochemical marker for monocytes and immature macrophages. As confirmed in this study, granules positive for peroxidase are numerous at the Golgi area of monocytes or close to the plasma membrane in immature mononuclear phagocytes. The microfilamentar system found close to these granules in some immature cells is probably participating in their translocation in the cytoplasm or degranulation process as suggested by some authors (Porter, 1973; Kern, 1975).

In a sequential morphological study, using light and electron microscopy, we have previously demonstrated that in C57B1/10J inbred mice resistant to infection with *L. mexicana* the tissue reaction was basically a granulomatous process. This chronic inflammation first increased and then diminished in intensity as the number of parasites decreased. Initially there was an accumulation of granulocytes and immature cells of the MPS. Some parasites penetrated into macrophages with the consequent formation and accumulation of parasitized vacuolated macrophages. Around these cells developed a progressive infiltration of monocytes which evolved into mature macrophages and swirling nests of epithelioid cells. In association with this granulomatous reaction, there was a progressive infiltration of lymphocytes, plasma cells and granulocytes. In later lesions, there was a progressive destruction of parasitized macrophages with marked reduction of parasite number or resolution of the lesion (Grimaldi et al., 1981). In contrast, confirming previous results (Grimaldi et al., 1984) the present study demonstrated that in chronic *L. mexicana* lesions, in a susceptible host, the main histopathological finding was a progressive accumulation and predominance of immature mononuclear phagocytes including heavily parasitized vacuolated macrophages. These results suggest that the development of a well-organized granulomatous reaction, with participation of immunocompetent cells, probably represents the factor which is responsible for destruction of parasites and control of the infection. Also, the degree of vacuolization of the host cell, typical of *L. mexicana* infection, may reflect processes involved in inactivation of host destructive capacities (Grimaldi, 1982). As demonstrated in this study, such mechanisms could be due to the precoceous parasite invasion of immature mononuclear phagocytes present at the inflammatory site.

Thus it is clear that, using these two cytochemical techniques, we have confirmed our previous results on chronic *L. mexicana* lesions in susceptible hosts. The main inflammatory cells participating in this process are immature macrophages. These cells have, as precursors, exsudate monocytes continuously infiltrating the inflammatory site evoked by presence of *Leishmania* amastigotes.

We could observe in this present study that, as these cells phagocytose the parasites, they degranulate peroxidase into the phagosome and then loose this cytochemical marker. This may explain why we also do not find this enzyme in mature cells (Nacy & Diggs, 1981). Also the parasites which show high levels of multiplica-

tion are continuosly interiorized by new immature mononuclear phagocytes, transforming them into parasitized vacuolated macrophages thus avoiding their natural maturation.

Studies with other markers such as adenylcyclase and sodium potassium ATPase of the mononuclear cell membrane would clarify in a better way the changes in stages of activation of these cells that may occur during the process of differentiation of an immature to a mature cell.

RESUMO

Leishmania mexicana amazonensis: **heterogeneidade na atividade da 5'-Nucleotidase e da peroxidase em fagócitos mononucleares durante infecção** *in vivo* e *in vitro* – Um estudo sobre o grau de maturação das células do Sistema Fagocítico Mononuclear foi realizado durante a infecção *in vivo* e *in vitro* com a *Leishmania mexicana amazonensis.* A caracterização da diferenciação das células fagocíticas foi obtida com a localização ultraestrutural de dois marcadores enzimáticos bem conhecidos: a enzima 5'-Nucleotidase marcadora de membrana plasmática de células maduras e a enzima peroxidase, presente em grânulos, marcadora de células imaturas. A atividade da enzima 5'-Nucleotidase foi encontrada apenas em alguns macrófagos, presentes no foco inflamatório, em projeções da membrana plasmática e em algumas vesículas citoplasmáticas. Macrófagos peritoneais de camundongo apresentaram a mesma reatividade para este marcador. Contudo a análise da atividade peroxidásica demonstrou a predominância da presença de fagócitos mononucleares imaturos nas lesões crônicas induzidas neste sistema por *Leishmania mexicana amazonensis.*

Palavras-chave: *Leishmania mexicana amazonensis (L. m. amazonensis)* – fagócito mononuclear imaturo – macrófago maturo – macrófago peritoneal – lesões cutâneas – 5'-Nucleotidase (E. C. 3.1.3.5) – Peroxidase (E. C. 1.11.1.7)

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