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Histological evaluation of the lesion induced by inoculation of *Leishmania mexicana* in the cheek pouch of the hamster

Avaliação histológica da lesão induzida pela inoculação de *Leishmania mexicana* na bolsa jugal do hamster

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Abstract *We have studied the role of the immune response in the morphology of the leishmaniotic granuloma induced in the cheek pouch of hamsters, an immunologically privileged site, after inoculation of 3 x 10[5]* Leishmania mexicana*. Animals were histologically and immunologically evaluated until 120 days after inoculation. Independent of the time of sacrifice, the animals were always non-reactors to the footpad test (FPT). At histology, the introduction of* L. mexicana *in the cheek pouch leads to an abscess that evolves to a granulomatous reaction rich in amastigote forms, and later it leads to resolution, even in the absence of immune response detectable by FPT. Our results demonstrate that the development of immune response is not preponderant for the control of infection induced by* L. mexicana *inoculated subcutaneously in the cheek pouch of the hamster. It also suggests that the macrophages present in the leishmaniotic granuloma are capable of eliminating this parasite, even in the absence of immune response evaluated by FPT.*

Key-words: *Cheek pouch. Experimental leishmaniasis.* Leishmania mexicana*.*

Resumo *No presente estudo, investigamos o papel da resposta imune na morfologia do granuloma leishmaniótico induzido na bolsa jugal do hamster, um local imunologicamente privilegiado, após inoculação de 3x10[5]* Leishmania mexicana*. Os animais foram avaliados histológica e imunologicamente até os 120 dias da inoculação. Independente da época do sacrifício, os animais foram sempre não reatores ao teste do coxim plantar. Histologicamente, a inoculação de* Leishmania mexicana *na bolsa jugal resultou na formação de abcesso que evoluiu para reação granulomatosa rica em formas amastigotas e, posteriormente, para resolução. Esses resultados sugerem que o desenvolvimento da resposta imune não é preponderante no controle da infecção induzida pela* Leishmania mexicana *inoculada subcutaneamente na bolsa jugal do hamster. Sugerem ainda que os macrófagos que compõe os granulomas leishmanióticos são capazes de eliminar esse parasita, independente da presença de resposta imune avaliável pelo teste do coxim plantar.*

Palavras-chaves*: Bolsa jugal do hamster. Leishmaniose experimental.* Leishmania mexicana*.*

Similar to that which happens with other diseases in which the etiological agent is an intracellular parasite, the morphologic substrate of leishmaniasis is the granuloma. According to Adams[1], the granulomatous lesion initiates as an infiltrate of immature mononuclear cells, evolve to a mature granuloma after aggregation and maturation of these cells, and later results in an epithelioid granuloma.

The exact mechanism involved in the transformation of mature granulomas into epithelioid granulomas is not clear. Although some authors have suggested that all the epithelioid formations are delayed hypersensitivity reactions, it is known that non-immunogenic substances may elicit this type of reaction. Besides, epithelioid granulomas can be formed in immunosuppressed animals[9, 18] or in immunologically privileged sites[3, 4].

According to Isaza *et al*[10], the cells that mediate immune response would actively participate in the formation of the leishmaniotic granuloma, limiting the expansion of the infectious agent and acting in the control of the disease. In the present study, we investigated this hypothesis through inoculation of *L. mexicana* in the cheek pouch of the hamster.

The cheek pouch is an invagination of the oral mucous membrane with no lymphatic drainage[6] since the specific activities of lymphocytes, such as antigen

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recognition and interaction with other cells, occur in the secondary lymphoid organs, the absence of lymphatic drainage in the cheek pouch results in a blockage of the afferent branch of the immune response and generates a status of local immunologic tolerance.

In addition to this, the cheek pouch has a reduced number of Langerhan's cells that play a key role in the host immune system interactions, participating in events involving both initiation and maintenance of the T cell specific immune response. In the face of these peculiarities, the cheek pouch of the hamster is an alternative site for the development of studies regarding the host-parasite interactions in the absence of immune response[5].

MATERIAL AND METHODS

Inoculum. A suspension of *Leishmania mexicana* ssp (Parasitology Department - UNESP - Botucatu) was cultured in LIT medium at 25°C for 30 days[20].

Animals. Twenty-four hamsters (*Mesocricetus auratus*), male, eight weeks old, obtained from the laboratory animal facility, UNESP, Botucatu, were used. They were intraperitoneally anesthetized with Sodium Nembutal (Abbot Laboratory of Brazil, 40mg/kg body weight) and inoculated with $5x10^6$ viable *Leishmania mexicana*/ml in the distal part of right cheek pouch. Groups of three animals were sacrificed by ether inhalation at 20 hours, and 3, 7, 14, 30, 60 and 120 days post inoculation (pi).

Immunological evaluation. The development of cell mediated immune response was evaluated by the FPT[11]. Thus, 24 hours before the sacrifice, 0.1ml of Montenegro antigen, lot 19 – (Instituto Adolfo Lutz –SP, Brazil) was inoculated in the right footpad of the hamsters, the protein concentration used had 0.02181mg%. The same volume of sterile saline solution (0.85% NaCl) was inoculated in the left footpad of the animals being studied. At the time of sacrifice, samples from the footpads were fixed in formalin, embedded in paraffin and then stained with hematoxylin and eosin. Positivity was determined by the presence of mononuclear inflammatory infiltrate in the tissue.

Histology. After sacrifice, samples of the inoculated cheek pouches, footpads used in the immunological evaluation, lungs, hearts, livers, spleens and kidneys were collected. All samples were submitted to routine protocol for inclusion in paraffin, and hematoxylin and eosin and/or Giemsa staining.

RESULTS

Lesions from inoculation. Inoculation of *L. mexicana* in the cheek pouch of the hamster resulted in extensive abscess, with entire and degenerating neutrophiles and mononuclear cells in the periphery at 20 hours (Figure 1); at 3 days, the same histological picture was maintained, however, lesions were less extensive and plasmocytes and mononuclear cells could be observed on the borders of the abscess. At 7 and 14 days lesions were constituted by macrophagic granulomas with evident necrotic centers; many macrophages were vacuolated, others seemed to be modified, showing elongated nuclei and dense cytoplasm, nonetheless, without a definition of epithelioid cells. (Figure 2a) lymphocytes and plasma cell were also observed. Granulation tissue, represented by neovascularization and fibroblast proliferation, were also observed in the periphery of the lesion. From this time on, tissue changes were no longer observed. Amastigote forms of the parasite were observed in the cytoplasm of macrophages in lesions with 14 days of evolution (Figure 2b).

Lesions of dissemination. From the organs examined, only the liver exhibited histological changes. They were present after the 7th day of inoculation and were constituted by small infiltrates of mononuclear cells dispersed through the hepatic tissue. From the 14th day on some giant cells were also observed (Figure 3). Amastigote forms were not observed in these lesions.

Immunological evaluation. None of the footpads exhibited mononuclear cell infiltration, and therefore, the animals were considered FPT negative.

DISCUSSION

Leishmania major in the Old World and *Leishmania mexicana* in the New World can cause cutaneous leishmaniasis. The course of the disease is determined by a complex host-parasite interaction. In spite of the participation of the immune system in the infection caused by *L. major* being well established, the immunological mechanisms that modulate the infection by *L. mexicana* need to be better characterized.

In the present study we evaluated the participation of the immune response in the leishmaniotic lesion induced experimentally through inoculation of the parasite in the cheek pouch of hamsters. The animals used in this study were always FPT negative, which confirms that lesions developed in the absence of immune response.

Clinically, the lesions of inoculation showed a rapid initial development manifested as a nodule that reached its maximum size at 14 days pi, and after that coalesced. This model of clinical evolution is similar to the one described in hamsters inoculated with *L. braziliensis* and *L. mexicana* through subcutaneous and/or intratesticular and/or intraperitoneal via. All together, these results suggest that the absence of immune response does not interfere with the clinical

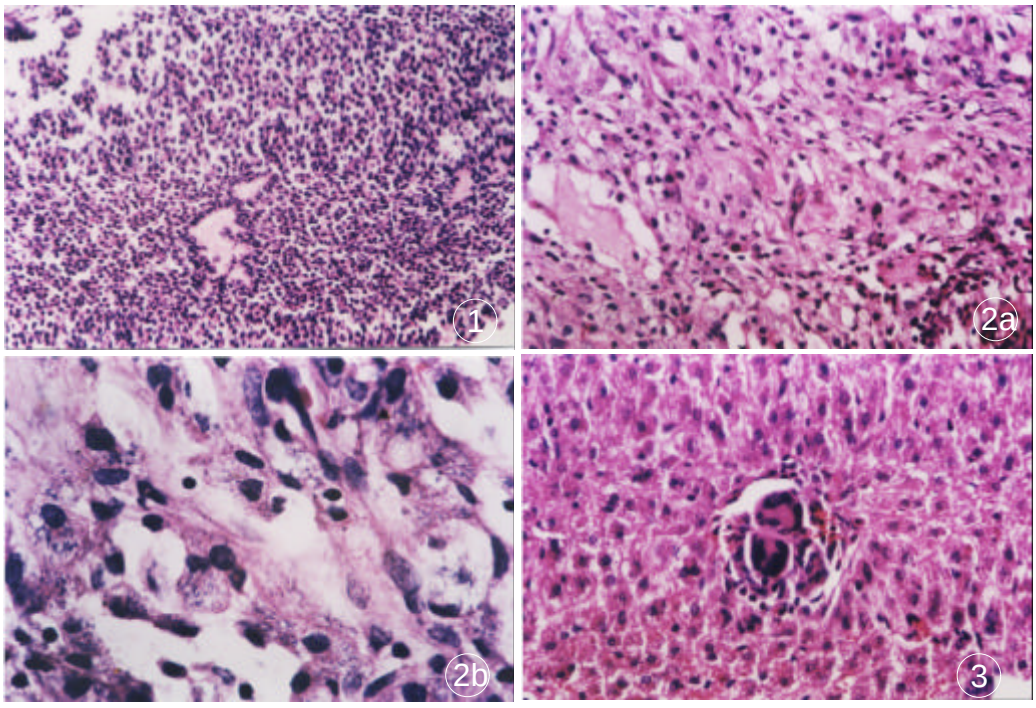

Figure 1 - Hamster inoculated with L. mexicana*. Cheek pouch. 20 hours pi. Abscess formed by accumulated neutrophiles. Whole and degenerated polymorphonuclear cells. Figure 2: Hamster inoculated with* L. mexicana*. Cheek pouch. 14 days pi. A: Macrophagic granuloma with foci of necrosis. B: Detailed view of the granuloma showing modified macrophages with elongated nuclei and dense cytoplasm. Amastigote forms observed in the cytoplasm of macrophages. Figure 3: Hamster inoculated with* L. mexicana*. Liver. 14 days pi. Note small infiltrate of mononuclear and giant cells.*

evolution of infection induced through experimental subcutaneous inoculation of *L. mexicana* in hamsters.

Histologically, lesions were present until the 14th day of infection, exhibited a compact granulomatous aspect, with multivacuolated macrophages harboring many amastigote forms in their cytoplasm. In some cases, there were foci of necrosis. Focal necrotizing inflammation was also observed in the cheek pouch of hamsters inoculated with *L. brasiliensis*[19] Coutinho & Coelho[8] obtained similar results in hamsters subcutaneously inoculated with *L. mexicana* in the dorsum, nose and base of the tail. These authors stated that foci of abscessed necrosis and parasitized macrophages are common findings in hamsters inoculated with *L. mexicana*. Observing a great number of eosinophils associated with this picture Tafuri *et al*[19] suggested that the necrotizing changes observed in the cheek pouch could be due to products liberated from eosinophilic degranulation.

Andrade *et al*[2], comparing lesions observed in sensitive and resistant strains of mice challenged with *L. mexicana*, described that necrosis was a late event in the course of the disease, and it was associated with delayed hypersensitivity. Susceptible animals exhibited coagulation type necrosis that appeared in focal areas and had no cellular infiltrate. A similar picture was observed in our animals and therefore, in lesions that developed in the absence of delayed hypersensitivity.

As has been observed in resistant mice[2], the lesions in our animals also exhibited fibroblast proliferation among histiocytes and in the periphery of the lesion, producing a pseudo-capsule. Thus, the lesions observed in the cheek pouch of the hamster demonstrated some characteristics that were already observed in strains of resistant mice and others observed in susceptible mice. Lesion that developed in hamsters in the absence of immune response can therefore be characterized as borderline.

The bases of susceptibility or resistance to cutaneous leishmaniasis are not well understood. The macrophages indeed phagocytose the *Leishmania*, and in spite of the lysossome and phagossome fusion, the parasite survives and multiplies intracellularly[7,13] Electron microscopy studies could not detect the differences between parasitized macrophages from susceptible and resistant animals, but suggest that tissue destruction of parasitized cells was much more frequent in resistant than in susceptible animals.

It has been suggested that macrophage activation and consequent restraining and/or elimination of the parasite could be related to production of cytokines, mainly release of IFNγ (gamma interferon) in the first week of infection. According to Laskay *et al*[12], the main

source of IFNγ in this period is the NK cell (natural killer). Considering that our animals apparently recovered from infection in the absence of immune response, it is possible that release of IFNγ by NK cells may have been sufficient to eliminate parasites under the experimental conditions performed.

Similarly to what was observed in the cheek pouch, the inoculation of *L. mexicana amazonensis* and *L. brasiliensis* through subcutaneous[2] and intradermic via[15] in BALB/c mice and hamster, results in lesions that evolve to resolution. Thus, development of immune response does not seem to be preponderant for the control of infection by *L. mexicana* subcutaneously inoculated. It is also suggested that macrophages that constitute leishmaniotic granulomas are capable of eliminating the parasite, regardless of the presence of immune response.

We investigated the dissemination of *L. mexicana* inoculated in the cheek pouch, Michalick *et al*[14] in a similar study suggested that the parasites of the mexicana complex are not able to infect golden hamster by cheek pouch route. In the present study, from all the organs examined, only the liver showed changes, which were nodular accumulations of mononuclear cells. However, hepatic lesions have also been observed in BALB/c mice inoculated with *L. mexicana amazonensis* subcutaneously. Solis *et al*[17] inoculating this parasite in the nose of hamsters reported that none of the animals used presented visceral lesions, with the parasites remaining circumscribed to the site of inoculation.

The possible visceral dissemination of this parasite from the initial lesion has raised some discussion. Some researchers have mentioned that we may find the parasite in several organs in the hamster model, however, others question these findings since *Leishmania* is very susceptible to temperatures over 36°C. It should be considered that in the present study we did not observe amastigote forms in any hepatic lesion, therefore these lesions may not be specific.

Our data inidcates that the introduction of *L. mexicana* in the cheek pouch leads initially to abscess formation; and then courses to a granulomatous reaction, rich in amastigote forms, and posteriori to resolution, even in the absence of immune response detectable by the FPT. Our results agree with the description of other authors who used a different strain of *Leishmania* and other experimental models[16], which demonstrates that the cheek pouch of the hamster is an alternative site for studies involving the role of the lymphatic drainage and the immune response in the modulation of the leishmaniotic lesion.

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