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Cell migration induced by *Leishmania (Leishmania) amazonensis*, *Leishmania (Leishmania) major* and *Leishmania (Viannia) braziliensis* into the peritoneal cavity of BALB/c mice

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ABSTRACT: In American cutaneous leishmaniasis, the initial infection phase is characterized by recruitment of neutrophils and monocytes. The migration of these cells in response to the presence of *Leishmania* in the peritoneum of affected animals remains unclear. The objective of this study was to investigate cell migration to the peritoneum of BALB/c mice after infection with *Leishmania (Leishmania) amazonensis, Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) major*. Initially, *Leishmania* spp. was intraperitoneally inoculated in five groups of six animals each and the cell migration was analyzed 0, 3, 6, 12, 24 and 48 hours after infection. Different cell counts were performed with a staining kit and showed a higher percentage of polymorphonuclear than mononuclear cells in all three species studied. The total cell count revealed peak migration in *L. (L.) amazonensis* and *L. (L.) major* at six hours, and in *L. (V.) braziliensis* at 12 hours. These results suggest that factors released from different cell types probably act by attracting polymorphonuclear cells, with the peak migration most likely depending on the species of *Leishmania* inoculated into the host.

KEY WORDS: BALB/c mice, *Leishmania* spp., American cutaneous leishmaniasis.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

In the initial phase of infection, American cutaneous leishmaniasis (ACL) lesions are characterized by the recruitment of blood neutrophils and monocytes (1). Leucoytes play an important role in host defense, and their recruitment to infected tissue may constitute a crucial event in the control of infections such as leishmaniasis. Peters *et al*. (2) reported that neutrophilis are rapidly mobilized to sandfly bite sites, where they phagocytose *L. major* parasites. While the saliva of the transmitter insect increases mouse macrophage chemotaxis, lipophosphoglycan (LPG) – the main component of the surface of *Leishmania* – reduces it, highlighting the capacity of this molecule to damage phagocyte responses (3-5). Chen *et al*. (6) reported that neutrophils may play an important role in the development of resistance to *T. cruzi* infection in BALB/c mice, despite the fact that neutrophil depletion reduces the degree of infection by *T. cruzi* in C57BL/6 mice, possibly through modulating the Th1/Th2 dichotomy in different directions. Other studies have shown that neutrophils may contribute to the immunopathology in experimental cerebral malaria (7, 8). These examples show the paradox and complexity of the roles of neutrophils in infections.

It is well known that the polymorphonuclear cells (PMN) are the first line of defense against infections and have a prolonged lifespan of approximately 6 to 10 hours in the bloodstream, after which they undergo spontaneous apoptosis (9). According to Aga *et al*. (10), these cells have their survival time increased by the inhibition of apoptosis induced by *Leishmania* parasite, predisposing them to serve as hosts to the parasites in the initial phase of infection. Lima *et al*. (11) have already reported that neutrophils present a leishmanicidal activity by holding back the spread of the parasite at the beginning of the infection. However, the precise immunoregulatory role of PMN infected with *Leishmania* is not completely understood. Previous findings document *in vitro* migration; however, in *in vivo* migration, in an air pouch model, the injection of *L. major* and *L. donovani* led to a rapid and transient accumulation of leucocytes (12). Although it seems ideal to use polymorphornuclear and mononuclear infiltrates in the injured skin to examine the function of these cells, it is almost impossible to obtain enough cells from local tissues for this purpose (13). Thus, we proposed the use of *in vivo* migration of these cells in response to *Leishmania* spp. in the mouse peritoneum.

The aim of this study was, therefore, to study the *in vivo* migration of polymorphonuclear and monocyte cells to the peritoneum of BALB/c mice in response to infection by *L. (L.) amazonensis*, *L. (V.) braziliensis* and *L. (L.) major.*

The procedures followed the regulations established by the Brazilian College of Animal Experimentation (COBEA) and the study was approved by the Ethics Committee for Animal Experimentation of the State University of Maringá. The results were analyzed by a non-parametric ANOVA using the Bonferroni test for multiple comparisons and t-tests, with Welch correction, for dual comparisons. A “p” value below 0.05 was considered significant.

In the current study, stationary-phase promastigotes (2 x 10^6 parasites in 0.1 mL of PBS) of *L. (L.) amazonensis* (MHOM/BR/1989/166MJO), *L. (V.) braziliensis* (MHOM/BR/1987/M11272) and *L. (L.) major* (LV39) were intraperitoneally inoculated in six BALB/c mice (7 to 12 weeks old) per experimental group. At different moments after the inoculation [0 (control), 3, 6, 12, 24 and 48 hours], the animals were lethally anesthetized [80 mg/kg of ketamine plus 16 mg/kg of xylazine (14)], and the peritoneal contents were washed with a total of 5 mL of Hank’s solution to collect leukocytes from the exudates. The only two experiments were performed on different days.

The mean viability of the cells obtained from the peritoneal exudates at the different times, verified by trypan blue exclusion (0.1%), was found to be 96%. Cellular differentiation between polymorphonuclear and mononuclear cells was performed microscopically in the smears on a plate stained with a Panotico® kit (Laborclin, Brazil). The percentage of polymorphonuclear cells that migrated to the peritoneum was greater than that of the mononuclears for the different *Leishmania* species. *L. (L.) major* was not statistically different from the control group after 3, 6 and 24 hours of infection. Statistical difference was observed in *L. (L.) amazonensis* at the times of 6, 12 and 24 hours, whereas *L. (V.) braziliensis* differed statistically after 12 hours of infection in relation to controls. However, there was no significant difference among the three species of *Leishmania* evaluated (Table 1).

Table 1. Percentage of polymorphonuclears that migrated to the peritoneum of BALB/c mice intraperitoneally inoculated with *L. (L.) amazonensis*, *L. (V.) braziliensis* and *L. (L.) major* at different times after intraperitoneal inoculation

Parasite	Polymorphonuclears (%; means)					
	0 hour	3 hours	6 hours	12 hours	24 hours	48 hours
L. (L.) amazonensis	82.5 ± 6.72	91.5 ± 1.06*	86.5 ± 3.9**	86.5 ± 3.18*	81.5 ± 3.89**	64.0 ± 7.78
L. (V.) braziliensis	78.0 ± 9.19	89.5 ± 1.06	88.5 ± 3.2	86.0 ± 8.49*	78.0 ± 11.3	84.5 ± 4.46
L. (L.) major	73.0 ± 9.9	79.5 ± 9.55	88.0 ± 5.0*	85.0 ± 0.0	82.5 ± 3.89*	82.5 ± 6.01

The values are means ± SEM and are representative of two separate experiments with six mice per group (each time period). *p < 0.05 and **p < 0.01 compared to control group (0 hour); (ANOVA followed by Bonferroni's t test).

Recruited cells were counted directly with Turk's solution in a Neubauer chamber. The peaks in cell migration into the peritoneal cavity were observed at six hours in mice inoculed with *L. (L.) amazonensis* and *L. (L.) major*, and at 12 hours in those inoculed with *L. (V.) braziliensis*. When the time periods of 3, 6, 12, 24 and 48 hours were compared among the three *Leishmania* species, no statistically significant differences were found. When the controls (0 hour) were compared with the different time periods for *L. (L.) amazonensis*, statistical differences were observed at six hours (39 x 10^6 cells/mL), 12 hours (11 x 10^6 cells/mL) and 24 hours (14 x 10^6 cells/mL); *L. (V.) braziliensis* differed statistically at 12 hours (18 x 10^6 cells/mL), whereas *L. (L.) major* presented differences at three hours (12 x 10^6 cells/mL), six hours (12 x 10^6 cells/mL) and 24 hours (5.4 x 10^6 cells/mL) (Table 2).

Table 2. Number of cells that migrated to the peritoneum of BALB/c mice intraperitoneally inoculated with *L. (L.) amazonensis*, *L. (V.) braziliensis* and *L. (L.) major* at different times after intraperitoneal inoculation

Parasite	Total cells (x 10^6; means)					
	0 hour	3 hours	6 hours	12 hours	24 hours	48 hours
L. (L.) amazonensis	9.1 ± 0.07	3.8 ± 0.78	39 ± 21.9*	11 ± 4.1*	14 ± 6.01*	12 ± 0.71
L. (V.) braziliensis	9.8 ± 0.03	1.8 ± 0.05	6.8 ± 0.08	18 ± 0.46*	1.1 ± 0.04	8.4 ± 0.29
L. (L.) major	3.2 ± 0.14	12 ± 5.44*	12 ± 7.78*	4.1 ± 1.13	5.4 ± 2.8*	15 ± 1.34

The values are means ± SEM and are representative of two separate experiments with six mice per group (each time period). *p < 0.05 compared to control group (0 h); (ANOVA followed by Bonferroni's t test).

In the current study, the presence of a greater number of PMN, compared to mononuclears, at the beginning of the infection agrees with the results obtained *in vitro* using a chemotactic assay of human leucocytes (15). Furthermore, in agreement with the findings from the *in vivo* air pouch model obtained by Matte and Olivier (12), from the paw inoculation of animals and draining lymph nodes, it was reported herein that the PMN had been found in large numbers in infiltrates at the lesion sites after inoculation with *L. (L.) major* in BALB/c mice (11, 16).

The migration peaks may be related to the release of inflammatory factors at the infection site, and direct the type of immune response from the host, while the cellular diversity and accumulation may be associated with the expression pattern of the chemokine genes (12). Similarly, Romani *et al*. (17) reported that the production of IL-10 or IL-12 by neutrophils in mice with candidiasis is associated with development of a Th1 or Th2 response, respectively. The immune response to leishmaniasis can result in a polarization of a subpopulation of T lymphocytes, which leads to a different cell phenotype and results in immune protection or exacerbation of the disease (18). Furthermore, Tacchini-Cottier *et al*. (19) reported that the influx of neutrophils in the first 24 hours modifies the response of T cells, via production of IL-4, and the susceptibility to infection by *L. major*, by developing a Th2 response. In addition, van Zandbergen *et al*. (15) demonstrated that *Leishmania* promastigotes induce the migration of PMN by releasing *Leishmania* chemotactic factor (LCF), and that the co-incubation of *Leishmania* with PMN inhibits the CXC chemokine interferon gamma-inducible protein 10 (IP-10), suggesting that *Leishmania* inhibits the activity of Th1 or

NK cells and, consequently, interferes with the development of the protective immune response.

Matte and Olivier (12) reported that the inflammatory events of leishmaniasis that occur at the inoculation site could direct the type of species-specific pathogenesis that will be developed subsequently. Tacchini-Cottier *et al*. (19) suggested that the initial accumulation and persistence of PMN could be linked to the possible immunoregulatory role of these cells, or even better, to the development of the Th2 response that is characteristic of mice susceptible to *L. major*. Furthermore, in resistant mice (C57BL/6), the percentage of neutrophils infected with *L. major* fell from 60% to less than 10%, 72 hours after infection, by predisposing the mice to the development of the Th1 response. In the present study the peak at six hours coincided with that obtained by Matte and Olivier (12) in the air pouch model, after inoculation with *L. (L.) major*. It was deduced that the longer PMN peak (at six hours) in BALB/c mice susceptible to *L. (L.) major* and *L. (L.) amazonensis* corresponds to the shorter *Leishmania* retention time presented by the polymorphonuclear cells. Conversely, in this mouse lineage, which is resistant to *L. (V.) braziliensis*, the 12-hour peak corresponds to the longer *Leishmania* retention time displayed by the PMN.

In conclusion, compared with other studies, these results suggest that factors released by different cell types act by attracting cells, mainly polymorphonuclears, to the peritoneal exudate of BALB/c mice in the initial phase of infection, predisposing the animals to either susceptibility or resistence to the infection. The migration of a large number of cells to the infection site – which occurs at six hours for *L. (L.) amazonensis* and *L. (L.) major* and at 12 hours for *L. (V.) braziliensis* – suggests that the release of these factors is greater at these times and depends on the species of the parasite involved and on the immune response of the host.

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