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Original Article

Leukocyte Behavior in Mesenteric Microcirculation upon Experimental By *Leishmania* Spp. in BALB/c Mice

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Abstract

Background: We aimed to determine the cellular recruitment (leukocyte rolling and adhesion) by which the *Leishmania (Viannia) braziliensis*, *L.* (*Leishmania*) *amazonensis*, and *L. (Leishmania*) *major* species in the mesenteric microcirculation of BALB/c mice.

***Methods*:** Five experimental groups were considered: group 1 (*L. braziliensis*); group 2 (*L. amazonensis*); group 3 (*L. major*); group 4 (control group with PBS); group 5 (negative control group), analyzed 3, 6, 12, and 24 h after parasite inoculation.

***Results*:** Infections by the different *Leishmania* species caused an increase in the number of rolling leukocytes: *L. braziliensis* a peak at 6 h; *L. amazonensis* and *L. major* a peak at 3 h. The *Leishmania* infections induced leukocyte adhesion: *L. major* and *L. amazonensis* showed an increase after 3 and 6 h, respectively.

***Conclusion*:** The kinetics of cellular recruitment in *Leishmania* infections, leading to infection susceptibility or resistance, indicates that distinct mechanisms regulate the initial response to *Leishmania* infection and determine its course.

Introduction

Leishmaniasis, caused by protozoa of the genus *Leishmania*, is distributed worldwide, in approximately 90 countries in the tropics, subtropics, and southern Europe (1). Cutaneous leishmaniasis (CL) has case reports in every Brazilian states (2). The localized form is generally identified in the ulcer cutaneous; the mucocutaneous form, which is secondary to the cutaneous form, can develop either silently or via the destruction of cartilage; and the diffuse and disseminated forms are rare and severe, with therapeutic difficulty, and multiple lesions (2). The diversity of the disease's clinical expression stems from the parasite's biological complexity and the host's immune response (3).

The development of an immune response is influenced by the primary protective response to infection (inflammation) via the recruitment of cells (leukocytes) to the site of infection in order to destroy, dilute, or isolate the damaging agent (4). The migration of these cells to the site of infection is essential for the control of intracellular pathogens (5). The number and the different populations of cells recruited in the initial phase following a stimulus will influence the susceptibility or resistance to infection (6,7).

Given the global importance to CL, which involves several *Leishmania* species, the aim was to determine the cellular recruitment by which the *L. (Viannia) braziliensis*, *L. (Leishmania) amazonensis*, and *L. (Leishmania) major* species (each responsible for different clinical manifestations) influence the control of infection (or susceptibility to it). The steps of leukocyte rolling and adhesion in the mesenteric microcirculation of BALB/c mice were evaluated to understand better, how the inflammatory response operates in the early stages of infection.

Materials and Methods

Animals

A total of 70 five-week-old female BALB/c mice weighing less than 21 g were used. The animals were housed under controlled temperature and humidity conditions (22 ± 2 ºC and 60%, respectively) and subjected to a 12 h/12 h light/dark cycle. The animals were provided with water and feed *ad libitum* but were fasted in the 2–4 h period preceding the surgical procedure.

This work followed the guidelines established by the Brazilian College for Animal Experimentation, and the protocol of the study was approved by the Committee of Ethical Conduct on the Use of Animals in Experimentation of the Universidade Estadual de Maringá (UEM) (protocol number 039/2013). All methods were performed in accordance with the relevant guidelines and regulations.

Experimental groups

The mice were randomly assigned to different groups. Five experimental groups were considered: group 1 - infection by *L. (V.) braziliensis* (20 animals); group 2 - infection by *L. (L.) amazonensis* (20 animals); group 3 - infection by *L. (L.) major* (20 animals); group 4 - control group with PBS (10 mM sodium phosphate-buffered saline; 0.15 M NaCl; pH 7.2) (5 animals); group 5 – negative control group (neither parasites nor PBS) (5 animals).

Groups 1, 2, and 3 were analyzed 3, 6, 12, and 24 h after parasite inoculation; group 4 was analyzed 3 h after PBS inoculation. Five animals were evaluated at a time.

Experimental infection

L. (V.) braziliensis (MHOM/BR/1989/M11272), *L. (L.) amazonensis* (MHOM/BR/1989/166MJO), and *L. (L.) major* (LV39) promastigotes were cultivated in 199 Medium (Gibco Laboratories, Grand Island, USA) supplemented with 1% L-glutamine, 1% human urine, and 10% fetal bovine serum, in a biological oxygen demand

(BOD) incubator at 25 °C. Culture maintenance was assured by weekly seeding without losing the infectivity of the parasites. Stationary phase cultures were used for the infection protocol: washing the parasites in PBS and inoculating 0.1 mL of suspension containing 2 × 10^8 parasites/mL of each species intraperitoneally into the mice in a single dose, according to the groups.

In vivo in situ determination of leukocyte rolling and adhesion in mesenteric microcirculation

For the surgical procedure, the animals were anesthetized via intramuscular administration of 120 mg/kg ketamine and 20 mg/kg xylazine. Laparotomy was performed via a longitudinal incision (1.5 cm) in the right lateral abdominal wall to expose the intestine and observe the mesenteric microcirculation *in situ*.

The procedures to evaluate rolling and adhesion were performed according to those described by Baez (8) and Fortes et al. (9), with some modifications. The animals were kept on a heating plate (37 °C) with a transparent cover, allowing for transillumination of the mesentery, which was kept moist by irrigating with Ringer–Locke solution (pH 7.2–7.4). The heating plate was mounted on a tetra-ocular optical microscope (Motic), coupled to a digital camera with a magnifying lens system and a computer for image projection and recording (3400× magnification). The images were acquired through image capturing and editing software and were used to measure vessel diameters. The vessels selected for the study were post-capillary venules, with diameters between 23–30 µm and extension of 75 µm (Fig. 1).

The interaction between circulating leukocytes and the luminal surface of the endothelium was studied at 5-min intervals.

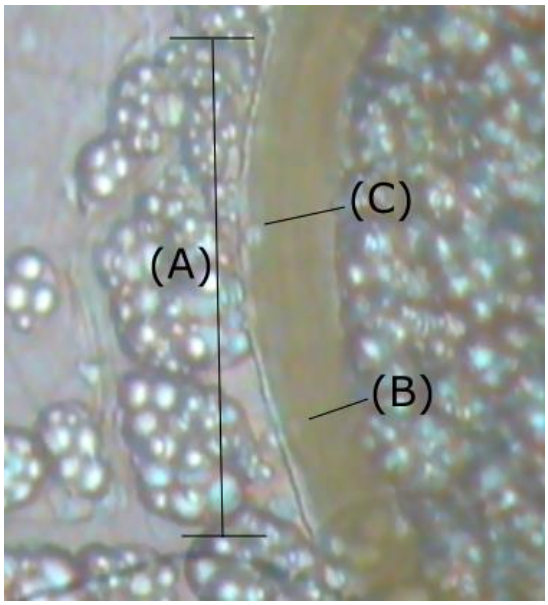

Fig. 1: In vivo *in situ* determination of leukocyte rolling and adhesion in mesenteric microcirculation, in post-capillary venule of the mesentery of BALB/c mice, superfused with Ringer-Locke solution which the *L.* (*L.*) *amazonensis*. (A) leukocyte counting site along 100 µm venule length; (B) leukocyte rolling; (C) leukocyte adhesion

The cells at the periphery of the blood stream that rolled over the endothelium (rolling) or remained fixed to the endothelium for a minimum of 30 s (adhesion) were taken into account.Each examined vascular segment was used only once, and only one numerical determination was carried out per animal. After each experiment, the animals were euthanized by increasing the dose of anesthetic.

Total and differential leukogram

Blood samples obtained from the tail end were used to determine the total and differential number of leukocytes. The total leukocyte count was determined by adding 10 µL of blood to 190 µL of Turk's liquid. The cells were counted directly using a Neubauer chamber. The differential leukocyte count was performed using a blood swab on a glass slide, fixed with methanol, stained with Giemsa, and

examined under a common optical microscope (1000× magnification).

Statistical analyses

Results were expressed as the mean ± standard deviation (μ ± σ). Statistical analyses were performed using GraphPad Prism 5.0 software (Graph Prism Inc., San Diego, CA), applying the Kruskal-Wallis test to compare three or more populations, or analysis of variance (ANOVA) to compare variation within and between groups, and the Dunn's test *post hoc* to compare each group of samples with each other where $P \leq 0.05$ was considered statistically significant.

Results

Leukocyte rolling

In our study, relative to the results of the control groups (4 and 5), infections by the different *Leishmania* species caused an increase in the number of rolling leukocytes (Fig. 2A). Mice infected with *L. (V.) braziliensis* showed an increased number of rolling after 3 h, with a peak at 6 h; there was a gradual decrease after 12 h and a return to the basal level after 24 h. Animals infected with *L. (L.) amazonensis* and *L. (L.) major* also showed an increase in the number of rolling at all timepoints (3, 6, 12, and 24 h), with a peak after 3 h, followed by a gradual decline until 24 h. This increase was significant after 3 h in the *L. (L.) major*-infected group compared to the control groups (4 and 5). There was no difference in the number of rolling leukocytes between the control groups (4 and 5) (Fig. 2A).

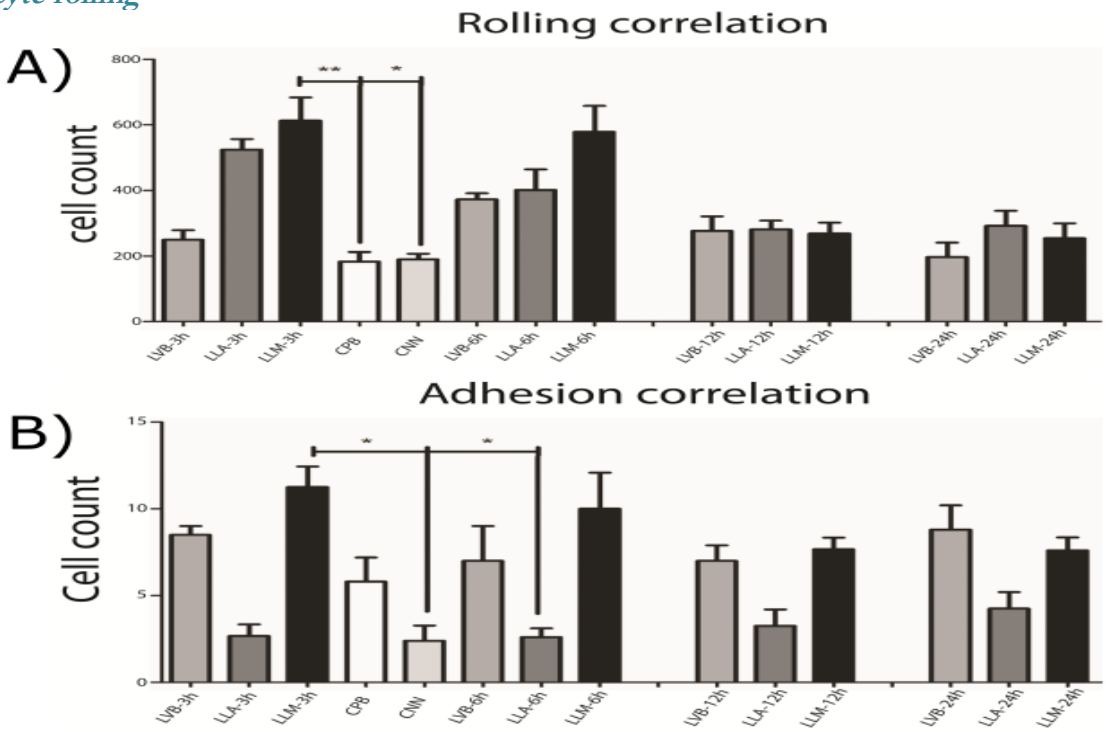

Fig. 2: Correlation of rolling (A) and adhesion (B) between the species and the times. In rolling, there was a significant increase in the *L. (L.) major*-infected group compared to the control groups after 3 h. In adhesion, there was a significant increase in the *L. (L.) major*-infected group and controls after 3 h and in the *L. (L.) amazonensis*-infected group after 6 h. LVB: *L. (V.) braziliensis*. LLA: *L. (L.) amazonensis*. LLM: *L. (L.) major*. CPB: Control with PBS. CNN: Negative Control. *$P < 0.05$. **$P < 0.01$ by Dunn's test

Leukocyte adhesion

The *Leishmania* infections induced leukocyte adhesion on the endothelial surface. Groups infected with *L. (L.) major* and *L. (L.) amazonensis* showed a significant increase in the adhesion after 3 and 6 h, respectively. There was no difference in the adhesion between control groups (4 and 5) (Fig. 2B).

Total and differential leukogram

The total number of leukocytes in the peripheral blood was increased in the animals infected with *L. (V.) braziliensis* after 3 h, and in the animals infected with *L. (L.) amazonensis* after 3 and 12 h. No increase in the total number of leukocytes was observed at any other timepoint, either in the control or in the *L. (L.) major*-infected animals.

On the differential count in the peripheral blood, both experimental and control groups were predominant mononuclear leukocytes (MNs) (75%) compared to polymorphonuclear (PMNs) ones (25%) at all times. It was observed that lymphocytes were predominant (70%), followed by neutrophils (24%), monocytes (5%), and eosinophils and basophils (1%).

Discussion

The mechanisms that control cellular recruitment at the initial stages of *Leishmania* infection are yet to be defined (10,11). The few existing studies mainly deal with *L. (L.) major* infections (5,6,10-15). Moreover, literature on *L. (L.) amazonensis* and *L. (V.) braziliensis,* the two main species detected in Brazil and other parts of Latin America, is scarce. Since the parasite influences disease pathogenesis and tissue damage, studies on the different cell recruitment patterns are important to understand the mechanisms of the immune response with respect to the various *Leishmania* species.

The initial events of *Leishmania* infections have been investigated using experiments involving mice (16,17). Although the skin is *Leishmania*'s site of infection, the in vivo *in situ* study of cell recruitment into the cutaneous blood vessels of experimental animals presents certain limitations (18,19). In contrast, cellular recruitment in the peritoneal cavity of mice may reflect the initial stages of cellular migration upon *Leishmania* infection (19).

In the present study, there was an increase in the number of rolling leucocytes upon infection differed among the different *Leishmania* species. Studies have shown that in experimental *Leishmania* infections, cellular migration occurs in the skin after intradermal inoculation (12,20), in the peritoneum after intraperitoneal inoculation (19), and in the lymphoid organs (11,12). Matte and Olivier (11) reported that *L. (L.) donovani* and *L. (L.) major* induce the recruitment of different quantities of cells to the infection site.

After inoculation of *Leishmania* promastigotes into the skin by infected sand flies, cells migrate to the site of infection (7,20). The initial cellular recruitment and its maintenance depend on immunoregulatory mediators (11,13,21,22). However, some *Leishmania* species can modulate these mediators using virulence factors, thus interfering with cellular recruitment (23) and influencing the severity of the disease (21). Charmoy et al. (6) showed that different *L. (L.) major* strains may induce different PMN responses, suggesting the involvement of different virulence factors. This indicates that different species antigens characterize the type of cellular response.

The highest rolling numbers were observed in *L. (L.) amazonensis* and *L. (L.) major* infections, which were both higher than that in *L. (V.) braziliensis*. In addition to the BALB/c mice being resistant to *L. (V.) braziliensis*, this finding could be explained by the fact that some leukocytes can act as a reservoir, causing the late release of *Leishmania* parasites, inhibiting the activation of microbicidal mechanisms, and, thus, maintaining the infection and allowing the survival of the parasite in susceptible

animals (24). Moreover, *Leishmania* parasites have escape mechanisms that enable them to survive transiently within leukocytes (16). Experiments involving *L. (L.) major* in resistant animals resulted in the complete disappearance of the disease, but it was possible to detect viable parasites in several organs throughout the animal's life (25).

In this study, leukocyte adhesion was observed to be higher in *L. (L.) major*-infected animals, and an association between rolling and adhesion numbers was found. However, this association most likely arises because of the type of methodology used, as only one part of a single blood vessel was analyzed. In principle, a higher rate of adhesion improves diapedesis and, thus, enhances the efficiency of leukocyte migration to the site of infection. This represents a superior innate immune response and, consequently, superior infection control. We observed adhesion followed by diapedesis in some analyses. This represents the subsequent steps of the innate immune response process. Further studies are needed to determine the role of adhesion molecules in the processes preceding tissue migration with respect to *Leishmania* virulence.

In this study, a peak of rolling was observed after 6 h for *L. (V.) braziliensis* and after 3 h for *L. (L.) amazonensis* and *L. (L.) major*. The peak of the cellular migration process may be related to the release of inflammatory factors at the infection site, which direct both cell diversity and type of immune response (11). The events that define the course of the *Leishmania* infection occur in the first hours or days after parasite inoculation. Wakimoto et al. (19) reported cellular migration studies in peritoneal exudates after intraperitoneal infection, in which peaks occurred after 12 h for *L. (V.) braziliensis* and after 6 h for *L. (L.) amazonensis* and *L. (L.) major*. Under similar conditions, Matte and Olivier (11) also observed a peak after 6 h for *L. (L.) major*. It is important to re-emphasize that cellular recruitment begins with rolling, followed by adhesion and subsequent tissue migration, which explains the observed timeframes.

In BALB/c mice, which are resistant to *L. (V.) braziliensis* infection, the occurrence of the peak of cellular migration after 6 h may indicate that the longer retention time of the parasites in PMNs is responsible for the activation of the microbicidal mechanisms in these cells, contributing to the efficient elimination of *Leishmania*. In contrast, BALB/c mice, which are susceptible to *L. (L.) major* and *L. (L.) amazonensis* strains, the peak of cell migration occurred after 3 h, corresponding to a shorter retention time of *Leishmania* within the PMNs. This indicates that the infection by these *Leishmania* species stimulates mechanisms that contribute to early cellular migration, but that are not efficient at parasite elimination, leading to their persistence and, subsequently, resulting in disease.

The total leukocyte count in peripheral blood was increased in the animals infected with *L. (V.) braziliensis* after 3 h, and in the animals infected with *L. (L.) amazonensis* after 3 and 12 h. The total leukocyte count did not increase at any other timepoint for the aforementioned infections, either in the *L. (L.) major*-infected animals or in the control animals. The increase in the number of leukocytes in the blood is linked to parasitemia and the time of infection.

Because it is an analysis in vivo in vitro, the differential leukocytes in mesenteric vessels was not analyzed. In contrast, in peripheral blood, there was no difference with reference values, with predominance of mononuclear cells. However, Wakimoto et al (19) observed a predominance of polymorphonuclear cells in peritoneal exudate. The first cells migrating to the infection site can influence subsequent events such as cellular differentiation and targeting of the immune response towards one of two types: Th1, consisting of a strong cellular response that leads to cure, or Th2, with a low cellular response resulting in uncontrolled parasite proliferation and cutaneous lesions at the site of inoculation (17).

Neutrophils contribute to the initial defense against infectious agents (26-28), and cooperate with other cell types to determine the effector immune response (15,23,29). The degree of suppression of neutrophil chemotaxis by infectious agents is associated with disease severity. This suggests that a dysfunction in neutrophil migration is a marker of poor prognosis in bacterial diseases (26). Studies have suggested that the protective function of neutrophils occurs by directing the Th1 response (18,24,30). In resistant mice, neutrophils present at the early stages of *L. (L.) major* infection direct the Th1 response. However, this pattern is not observed in susceptible mice, where neutrophil depletion results in lesion exacerbation and parasite survival (15,18). In resistant mice, PMN depletion induced by the injection of neutrophil and eosinophil inhibitors at the time of infection or during the first week of infection led to a significant increase in the parasitic load. In contrast, in susceptible animals, PMNs constitute the primary cellular population recruited within the first ten days following parasite inoculation (15,18,19,30).

Macrophages are important in the late phase (24 h post-infection), contributing to the antipathogen defense (12) as well as releasing important innate immunity mediators and developing adaptive immunity (31). Macrophages, which is found in most vertebrate tissues, performs biological functions such as host defense and tissue homeostasis (32). Inflammatory macrophages selectively traffic to the sites of inflammation and produce inflammatory cytokines and chemokines, resulting in the orchestration of both local and systemic inflammation (33). In resistant animals, macrophages predominate after three days (15,19). Souza Carmo et al. (29) observed that in susceptible animals, macrophages predominated in the first few hours after infection with *L. (L.) amazonensis*; however, resistant animals exhibited an extensive infiltration of neutrophils in the initial phase. Teixeira et al. (7) showed that a higher number of macrophages at the site of infection during the initial phase resulted in severe cases of the disease.

Furthermore, *Leishmania* infection is also associated with the recruitment of eosinophils, lymphocytes, natural killer cells, plasma cells, mast cells, and dendritic cells to the inflammatory sites (16,19,21). These cells can activate different immune and inflammatory mediators, which attract and activate distinct cell populations, which, in turn, can modulate the *Leishmania* infection in diverse ways and, subsequently, determine its fate.

Parasitic infection stimulates leukocyte tropism towards the affected region, which is determinant at the first phase of the infection. The kinetics of cellular recruitment in *L. (V.) braziliensis*, *L. (L.) amazonensis*, and *L. (L.) major* infections, leading to infection susceptibility or resistance, indicates that distinct mechanisms regulate the initial response to *Leishmania* infection and determine its course. The different antigens among the species stimulate cellular migration differently, characterizing the type of cellular response.

This study is, to our knowledge, the first to employ intravital microscopy to evaluate leukocyte migration kinetics in the mesenteric microcirculation of mice infected by different *Leishmania* species. Thus, further studies showing the relationship between different cellular migrations to the site of infection and infection control (or lack thereof) are necessary, as well as analyses of the virulence factors that may interfere with cellular recruitment. Furthermore, the knowledge of cellular recruitment activators as well as inhibitors may provide novel strategies for therapeutic design, acquired protective immunity (vaccines), and the minimization of the immune response responsible for tissue damage.

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Conflict of interest

The authors declare that there is no conflict of interest.

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