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Saliva of laboratory-reared *Lutzomyia longipalpis* exacerbates *Leishmania (Leishmania) amazonensis* infection more potently than saliva of wild-caught *Lutzomyia longipalpis*

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ABSTRACT

In order to compare the saliva effect from wild-caught and lab-reared *L. longipalpis* on the development of experimental cutaneous leishmaniasis, C57BL/6 mice were inoculated subcutaneously into the hind footpads with promastigotes of *L. (L.) amazonensis* plus salivary gland lysate from wild-caught (SGL-W) and lab-colonized (SGL-C) vectors. Lesion sizes were significantly larger in the mice infected with both saliva compared to mice infected with parasites alone; moreover, the lesions caused by parasite + SGL-C were significantly larger than the lesions caused by parasite + SGL-W. Histopathological morphometric studies regarding the acute phase of infections showed lower numbers of polymorphonuclear cells, greater numbers of mononuclear cells and parasites in SGL-C infected mice compared to SGL-W infected mice. In the chronic phase of infection, the number of mononuclear cells was lower and the number of parasites was greater in SGL-C infected mice than SGL-W infected mice. *In vitro* studies showed increased infection index of macrophages infected with parasites plus saliva compared to infection with parasites alone, with no difference between the saliva infection indices. SDS-PAGE gel for SGL-C and SGL-W showed differences in the composition and quantity of protein bands, determined by densitometry. These results call attention to the experimental saliva model, which shows exacerbation of infection caused by sandfly saliva.

1. Introduction

Leishmaniasis is an important tropical disease transmitted by sandflies. The parasites are transmitted by the female vector bite during blood feeding on the vertebrate host, which includes humans. Sandfly vector saliva plays an important role in *Leishmania* transmission [1,2]. The capacity of vector saliva to increase *Leishmania* infectivity is restricted to sandfly saliva since the saliva obtained from other bloodsucking arthropods cannot mediate the same phenomenon under experimental conditions [3,4]. Saliva of *L. longipalpis* and *P. papatasi*, the two most widely colonized sandflies and vectors of *Leishmania* in the New and Old World, respectively, is known to possess immunomodulatory activities [5,6].

The increase in *Leishmania* infectivity coinoculated with saliva has been demonstrated using inbred mouse models infected with *L. major* [3], *L. braziliensis* [7] and *L. amazonesis* [8]. Both parasite- and sandfly-derived factors contribute to the increase in infectivity [9]. Lesion sizes and parasite numbers dramatically increase when salivary gland lysate of *L. longipalpis* is added to the inoculum [3]. Increased infectivity has also been shown in susceptible and resistant mice inoculated with *L. major* in the presence *P. papatasi* saliva [10,11]. Sandfly saliva also has the capacity to inhibit macrophage activities during parasite antigen presentation to T lymphocytes [12]. Moreover, *P. papatasi* saliva is chemotactic to mouse peritoneal macrophages [13,14]. During natural infections, this activity could be one of the mechanisms that assist parasite phagocytosis by host macrophages, reducing extracellular parasite exposure to natural immunity components in the inoculation site.

In addition to the exacerbation effect of sandfly saliva in naive mice, protection against *L. major* infection has been shown in mouse models when they are preexposed to noninfected sandfly bites, or preimmunized with saliva [10,15]. Host antibody responses to sandfly saliva are potential markers of the risk of *Leishmania* transmission in endemic areas [16–18]. Furthermore, salivary gland proteins or their cDNAs are also being considered as viable vaccine targets against leishmaniasis [19–21].

All the reports concerning the effects of saliva exacerbation or protection against *Leishmania* infection have been developed using saliva from lab-colonized vectors, likely due to the difficulty of working with wild-caught sandflies. To this date, no consideration has been given

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to the fact that individuals at risk of exposure to *Leishmania*-harboring sandflies are actually bitten by wild sandfly vectors originating from their endemic areas. Thus, the main objective of this study was to compare the effect of saliva from wild-caught and lab-colonized vectors on the development of cutaneous leishmaniasis.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice 6 to 8 weeks-old from the General Colony of São Paulo University Medical School were kept in our laboratory during the experiments, according to the methods approved by the Animal Care and Use Committee of São Paulo Medical School.

2.2. Parasites

Leishmania (Leishmania) amazonensis, MHOM/BR/73/M2269 strain, recently recovered from chronically infected BALB/c mice were cultivated as promastigotes in supplemented RPMI 1640 medium (10% fetal calf serum, 5 mM HEPES, 50 μg/ml gentamicin and 100 U/ml penicillin). Stationary-phase promastigotes were harvested and washed three times in sterile PBS for use.

2.3. Sandflies

Wild-caught and lab-colonized *L. longipalpis* sandflies were used. The wild-caught sandflies were collected in Lapinha Cave located in Minas Gerais State, Brazil (longitude 43°57′W and latitude 19°03′S) over a period of 3 days, although those caught in the first collection were not used in order to work with newly released sandflies of similar age. These sandflies were maintained in the insectary for two days until the time of the experiments. The wild-caught sandflies were checked for the absence of blood meal in their guts and egg development in their ovaries in order to confirm that they had no previous blood intake. The lab-colonized sandflies were from a colony initiated with insects collected in the same place and they were reared at least for ten generations before being used in the experiments. Three to five day-old sandfly females from the colony were used in the experiments and were considered of similar age to the wild-caught sandflies. All the sandflies received 5% sugar solution *ad libitum* until the time of the experiments. They were maintained in the insectary of the Laboratory of Medical Entomology at Institute René Rachou (FIOCRUZ-MG) according to the conditions described by Killick-Kendrick et al. [22].

2.4. Salivary gland lysates

Salivary gland lysates were obtained from wild-caught (SGL-W) and lab-colonized (SGL-C) sandfly females. At the moment of the dissection, all the sandfly midguts were verified regarding the absence of blood meal. The dissected salivary glands were collected in phosphate buffered saline (PBS), pH 7.2, and stored at −70 °C. At the time of the experiments, the salivary glands were disrupted by freeze-thawing, vortex and rapid centrifugation to discharge possible tissues. Half of a pair of salivary glands was used for each inoculation point for both SGL-C and SGL-W.

2.5. SDS-PAGE of salivary gland lysates

SDS-PAGE gel electrophoresis was used to separate and characterize the molecular weights of the SGL-C and SGL-W proteins. After determining the protein concentrations by the Lowry method, 10 μg of the total protein was applied in each line in 12% acrylamide gel under denaturing conditions. Molecular weight standards from 205 kDa to 29 kDa (Sigma Chemical Co. USA) were used. After electrophoresis, the gel was stained with silver nitrate. Densitometric analysis of band intensity for all visible bands was measured by the Eagle Eye gel documentation system (Stratagene, USA).

2.6. Effect of SGL on ex vivo Leishmania-infected macrophages

Mouse resident peritoneal macrophages (1×10^5) were allowed to attach to sterile round coverslips (13 mm) and were then placed in 24-well plates in complete RPMI medium. Stationary-phase *L. (L.) amazonensis* promastigotes were added to the macrophage monolayers (5:1) in the presence and absence of half of a pair of salivary glands, for both SGL-C and SGL-W. The cultures were maintained in a 5% CO_2 incubator at 35 °C and the infection index was evaluated 24 h after the interaction. The infection index (percentage of infected macrophages × average number of amastigotes per macrophage) was determined in the coverslips stained by Giemsa under bright-field illumination using immersion objective lens.

2.7. Effect of SGL on in vivo Leishmania-infected mice

2.7.1. Mouse infection

Promastigotes (1×10^6 in 50 μl) with or without SGLs were inoculated subcutaneously into one of the mouse hind footpads, using a total of three experimental groups of mice infected as follows: parasites (P group), SGL-W plus parasites (SGL-W + P group) and SGL-C plus parasites (SGL-C + P group). The control groups were mice inoculated with PBS, SGL-W and SGL-C.

2.7.2. Lesion measurement

The hind-footpad swelling in each infected mouse was monitored weekly by measuring the thickness of infected footpad with a metric caliper and subtracting the thickness of the noninfected, contra-lateral footpad.

2.7.3. Histopathological study

Five mice from each experimental group were euthanized at 3, 24, 72 h, 7 and 60 days postinfection. Hind-footpad fragments were collected and fixed in 10% formaldehyde solution in PBS and routinely processed for paraffin embedding. The sections were stained with hematoxylin-eosin for analysis by light microscopy.

2.7.4. Quantitative morphometric analysis

Morphometric analysis was performed on three different levels of skin section per mouse using an eyepiece graticule in an area of 0.01 mm^2 adapted to an Olympus planapochromatic immersion objective lens (100×). Three types of analysis were performed: quantification of cell types, polymorphonuclear (PMN) and mononuclear (MONO), by counting 900 cells, where each cell type constituted of at least 10% of the total, in order to keep the relative standard error below

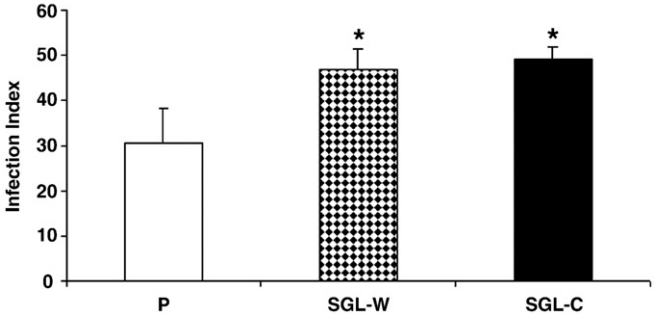

Fig. 1. Infection index of macrophages infected with *Leishmania* parasites (P group), SGL-W plus parasites (SGL-W + P group) and SGL-C plus parasites (SGL-C + P group). (*) $p<0.05$ between P and W-SGH + P and C-SGH + P. Data are from one representative experiment performed in triplicate.