THE ROLE OF PHLEBOTOMINE SALIVA ON THE ENHANCEMENT AND CONTROL OF *LEISHMANIA* INFECTION
O papel da saliva de flebotomíneos na exacerbação e controle da infecção por *Leishmania*

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Resumo

A leishmaniose é uma enfermidade infecciosa causada por parasitos do gênero *Leishmania* que são transmitidos por insetos vetores. A infecção no hospedeiro vertebrado se estabelece no momento em que a fêmea do flebotomíneo infectada, ao realizar o repasto sanguíneo, regurgita na pele do mamífero as formas promastigotas metacíclicas do parasito juntamente com parte do conteúdo da glândula salivar do vetor. Tem sido descrito que componentes do conteúdo da saliva do vetor tem propriedades imunomodulatórias facilitando o estabelecimento da infecção no hospedeiro. Por outro lado, outros estudos mostram que a pré-sensibilização do hospedeiro vertebrado com saliva de flebotomíneo leva a proteção da infecção por *Leishmania*. Esta revisão teve como principal objetivo, revisar o papel da saliva na evolução da infecção por *Leishmania*, quer seja na exacerbação ou na proteção.

Palavras-chaves: Leishmaniose, *Leishmania*, Phlebotomíneo, Saliva.

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Abstract

Leishmaniasis is an infectious infirmity caused by parasites of the genus *Leishmania*, which are transmitted by sand fly vectors. Infection of the vertebrate host is established when an infected female phlebotomine regurgitates the metacyclic promastigotes of the parasite into the mammalian skin during blood feed, together with part of the vector salivary gland contents. It has been reported that components of vector saliva content possess immunomodulatory properties that facilitate the establishment of infection in the host. On the other hand, other studies show that presensitization of the vertebrate host to phlebotomine saliva leads to protect against *Leishmania* infection. The main aim of this report was to review the role of saliva in the evolution of *Leishmania* infection, with regard to exacerbation and protection.

Keywords: Leishmaniasis, *Leishmania*, Phlebotomine, Saliva.

Introduction

Leishmaniases are a group of noncontagious infectious diseases, with chronic evolution, caused by different species of parasitic protozoans of the genus *Leishmania*. Visceral leishmaniasis principally affects cells of the mononuclear phagocyte system of the bone marrow, lymph nodes, spleen and liver[1,2]. Cutaneous leishmaniasis is mainly characterized by skin tissue compromise and secondarily, by mucosa tissue lesions, depending on the parasite species and host immunogenetic factors[3,4].

Leishmaniases are amply distributed worldwide, occurring in numerous countries in the Americas and Europe. The disease has also been reported in regions in Africa, in Middle Eastern countries and China. In the Americas, leishmaniases are widely distributed from southern United States, through Central America to northern Argentina. In the American continent, Brazil represents the largest territorial extension with endemic areas and one of the countries with the highest rates of notification for this infection[5,6].

In the Americas, leishmaniases are considered primary zoonoses of wild mammals, such as rodents, marsupials, edentates and primates, among which different species of *Leishmania* are transmitted by the bite of phlebotomine insect vectors. Humans acquire the infection when they enter in contact with forest areas where the wild enzootic cycle of different parasite species occurs. Thus, the disease assumes an occupational character, occurring in professional categories that expose humans to intimate contact with the forest. Moreover, in regions that undergo expressive environmental changes, domestic animals can exert an important role in the biological cycle of the parasites; as reservoirs that maintains the parasites in the natural environment. In these regions, environmental changes also exert a major influence on the biological behavior of species of phlebotomine vectors, as the most resistant species are capable of adapting to the environmental transformations, establishing new ecological niches for their survival in peridomestic settings[7].

Leishmaniasis agents are protozoan parasites of different species belonging to the genus *Leishmania*. Currently, approximately 30 *Leishmania* species are known worldwide, of which 22 are considered agents of human leishmaniases. Species of the genus *Leishmania* are classified into two subgenera according to the evolutionary behavior of the parasite in the digestive tract of the phlebotomine vector: *Viannia*, which develops from the posterior to the anterior midgut; and *Leishmania*, which develops from the middle to the anterior midgut[7]. It should be noted that all the species of the subgenus *Viannia* are autochthones of the Americas, while species of the subgenus *Leishmania* are found in the Americas, Europe and Asia.

Infection of the vertebrate host is established when an infected phlebotomine female regurgitates the metacyclic promastigotes of the parasite into the mammalian skin during blood feeding, together with part of the vector salivary gland contents. At this time, the current understanding is that the majority of the parasites are eliminated by the lithic action of the complement system and by neutrophils and eosinophils present in the host blood that flow to the site of the skin punctured by the vector proboscis[8]. However, promastigote forms that escape the nonspecific host defense mechanisms are phagocytized by skin macrophages, housed within the parasitophorous vacuoles, where they transform into the amastigote forms. After successive binary divisions, the mass of parasites provokes increased intracytoplasmic pressure inside the macrophage, disrupting the host cell and the free amastigotes are phagocytized by other macrophages, establishing the infectious cycle in the host[7].

The role of saliva on the exacerbation of experimental *leishmania* infections

Not so far, hematophagous vectors were regarded as simple delivery tools of pathogens they carry. However, they all share one important feature: during probing and feeding, they salivate at the injured site facilitating blood sucking, and the establishment of the infecting pathogen by the action of active components found in saliva[9,10,11,12].

The first report concerning the effect of vector saliva on *Leishmania* infection dates from 1988, when Titus & Ribeiro[13] showed a markedly enhancement of the cutaneous lesions and parasite load in mice infected with *L. major* in the presence of salivary gland lysate (SGL) of *Lutzomyia longipalpis* compared to animals inoculated only with parasites. Also, they further observed that infection could be established with only 10 to 100 parasites using SGL in the inoculum[14]. Together, these two studies resulted in the establishment of experimental models of *Leishmania* infection under closer conditions to the natural transmission.

Later studies showed that the effect of *Lu. longipalpis* SGL on the evolution of experimental infection was not dependant on the mice lineage used, since both resistant and susceptible mice to *L. major* produced larger lesions in the presence of saliva compounds[15].

Other reports corroborated the effect of *Lu. longipalpis* SGL on the increased severity of infections caused by *L. major*[16], as well as by other species of the parasite. *L. (V.) braziliensis*, which produces spontaneously regressing

lesions even in BALB/c mice that are highly susceptible to other species of *Leishmania*, induced larger and longer-lasting lesions with intense parasitism if co-injected with *Lu. longipalpis* SGL[17,18,19]. Lesion severity and the number of parasites also increased in infections with *L. amazonensis*[15,20,21].

One important issue was still required to be checked: the effect of saliva in the natural vector/parasite combination. Only recently, *Lu. longipalpis* SGL was tested in *L. chagasi* infections. Surprisingly, the addition of SGL in the inoculum did not alter the evolution of infection in dogs, did not lead to early amastigote detection and did not increase the parasite load in the organs compared to control animals[22]. Similar results were obtained for experimental infection in hamsters[23]. In another natural vector/parasite combination, *Lu. whitmani* SGL promoted larger cutaneous lesions in mice infected with *L. braziliensis*[24]. Nonetheless, *in vitro* experiments showed that pretreatment of human monocytes with *Lu. intermedia* SGL did not change the parasite burden, as determined by the number of monocytes infected by *L. braziliensis* or by the number of amastigotes per infected cell, compared to monocytes not exposed to SGL[25]. However, it should be highlighted that the exacerbation of cutaneous lesions and increased parasitism have been consistently verified in *L. major* infections in the presence of SGL from *Phlebotomus papatasi*, the natural parasite vector in the Old World[15,26].

As a whole, vector saliva is accepted as a crucial element for the establishment of *Leishmania* sp based on evidences that it : a- minimizes host hemostatic processes by vasodilatory and antiplatelet actions[10,27,28],b- modulates the host immune response, and c- enhances parasite infectivity. Concerning this issue, Charlab and collaborators[29,30] verified cytostatic effect of *Lu. longipalpis* SGL on *Leishmania*, indicating a role in the generation of infective parasites .

Regarding both the suppressive and immunogenic modulatory effects, *in vitro* experiments show that mice macrophages pretreated with *Lu. longipalpis* SGL are incapable of presenting antigens, which compromises the activation of specific T lymphocytes, are refractory to IFN-γ activation, and drastically reduce the production of hydrogen peroxide and nitric oxide, the main molecules responsible for lysing the parasite[14,31,32].

Similar data have also been observed concerning SGL of Old World vectors, as human mononuclear cells when exposed to *P. papatasi* SGL produce lower levels of IFN-γ and higher levels of IL-6[33]. Moreover, SGL of *Phlebotomus duboscqi*, the vector of *L. major* in Kenya, is chemotatic for monocytes and this attraction could represent one of the mechanisms by which the saliva ensures the successful parasitism of macrophages in susceptible hosts[34].

The *in vivo* effects of *Lu. longipalpis* or *P. papatasi* SGL on the immune response in experimental infections show a much more complex picture and heterogenous results, which are dependent on the parasite species and the genetic background of the animals used. Resistance of C57BL/6, CBA and C3H mice to *L. major* infection is a result of the production of cytokines associated with Th1, especially IFN-γ and TNF-a, while those associated with Th2, particularly IL-4, determine disease progression in BALB/c mice[35].

The presence of *P. papatasi* SGL in the *L. major* inoculum promotes increased levels of IL-4, but not of IL-10 and TNF-a, and a reduction in IFN-γ, IL-12 and nitric oxide synthase production, a profile associated with the enhanced severity of cutaneous lesions induced by saliva[26,36]. In contrast, *Lu. longipalpis* SGL determines increased IL-10 production in BALB/c when inoculated with *L. major*[37].

Spontaneous control of experimental *L. braziliensis* infection is determined by IFN-γ and IL-12 generation and low production of IL-4[38,39]. *Lu. longipalpis* SGL promotes an increase of two to three-fold more IL-4 levels in BALB/c infected with *L. braziliensis* and lesions which persist for the lifetime of the mice[18].

Infection with *Leishmania amazonensis*, on the other hand, causes progressive cutaneous lesions in most inbred lineages of mice, with no evidence of a polarized Th1/Th2 response as observed in that caused by *L. major*[40]. Among others, IL-10, but not IL-4, has an important role in compromising the host immune response[41]. Addition of *Lu. longipalpis* SGL to the inoculum promotes a significant rise in IL-10 levels that is associated with increased parasite infectivity and larger lesions[20].

Questions have been raised regarding which saliva components could be related to the effects above mentioned, since a complex network of biologically and pharmacologically active molecules have been detected in the salivary secretion, although investigations are still ongoing to better characterize these compounds (to review see Kamhawi,

2000 and Andrade et al., 2007)[42,43]. The most studied saliva is that of *Lu. longipalpis* whose principal component is maxadilan (MAX), a potent vasodilator that also modulates cytokines production of human and mice macrophages by up-regulating those associated with Th2, (IL-10, IL-6 and TGF-b) and down-regulating cytokines associated with Th1 (IL-12 and TNF-a) and nitric oxide production[33,44]. As a consequence, the parasite load of peritoneal macrophages of mice infected with *L. major* is drastically increased in the presence of MAX[44]. *In vivo* experiments show that MAX can also exacerbate infection with *L. major* to the same degree as whole salivary gland[16,45]; nonetheless, another study reports dissociation between the vasodilator effect of MAX and lesion enhancement in infections caused by *L. major* or *L. braziliensis*[46].

Maxadilan has not been identified in the saliva of other phlebotomines. However, other components as adenosine and its precursor 5'-AMP are detected in *P. papatasi* saliva[28] and both present vasodilatory and antiplatelet-aggregation properties. In addition, they enhance the production of Th2 cytokines (IL-10, IL-6), but down-regulate those of Th1 (IL-2 and IFN-γ) and nitric oxide synthesis[47,48]. Adenosine and AMP have also been detected in *Lu. Longipalpis*, but their *in vivo* effects on *Leishmania* infection have not been performed yet.

Currently, the mechanisms by which salivary compounds act and which of them are involved in the modulation of host response and on parasite survival remain an unsolved question. Given the complexity of these molecules and their possible interactions, it seems unlikely that full comprehension of these mechanisms will be elucidated soon.

Despite the fact that the parasites are transmitted exclusively by sand flies, the establishment of infection in experimental models via sand fly bites is unusual and the scarce reports in literature have not addressed the host response to infective bites[49,50]. However, Kamhawi and collaborators[51] recently described a murine model of *L. major* infection transmitted by laboratory-reared *P. papatasi* that made it possible to compare the effects of real sand fly saliva with salivary glands used in all experiments concerning saliva effects. Surprisingly, the infection of C57BL/6 mice transmitted by vector bites always resolved over time, similarly to mice inoculated with parasite alone and in strong contrast to the results obtained when SGL is inoculated together with *L. major*[26]. In addition, the bites of infected *P. papatasi* did not elicit a potent IL-4 response at the inoculation site observed in studies involving needle inoculation of SGL, which was again more comparable to the inoculation with only parasites[26]. These data highlight a probable bias when using salivary glands instead of real saliva.

To the best of our knowledge, all experiments concerning vector saliva effects were performed with lab-colonized sand flies, which raised the question of whether SGL from lab-colonized vectors and that from sand flies recently captured in the field could exert different effects on host and parasite survival. Our first study using SDS-PAGE gel electrophoresis indicated diversity in the expression and concentration of proteins between *Lu. longipalpis* SGL from these distinct sources, which prompted us to investigate their effects on *Leishmania* infection. We verified that wild-caught *Lu. longipalpis* SGL induced smaller sized lesions and lower tissue parasitism, less inflammatory cells at the inoculation site and lower production of cytokines associated with a susceptible response, compared to that obtained from laboratory-reared vectors[52]. The results address another probable bias caused by the use of SGL from lab-colonized sand flies instead of wild-caught vector SGL in experiments concerning saliva effects.

The role of saliva in protection from infections by *leishmania*

Recent studies have shown that components of insect vector saliva could be candidates for vaccines against leishmaniases[53,54,55]. Belkaid and collaborators[26] were the first group to demonstrate that preexposure to the saliva could result in protection. In their work, the exacerbation effect of infection by *L. major* in mice in the presence of *P. papatasi* SGL was abolished when the mice were preexposed to vector saliva. In experiments involving the transmission of *L. major* by *P. papatasi* bite, observation determined a significant reduction in pathology in mice previously exposed to uninfected phlebotomine bite and in the transmission of parasites by other phlebotomines[51]. The protection conferred by presensitization with vector saliva appears to be associated with the delayed hypersensitivity (DTH) response, since inhabitants from endemic areas exhibit a strong DTH response to vector bites[42,56]. This diverse and intense dermatological reaction has also been observed in volunteers exposed several times to *Lu. Longipalpis* vector bites[57].

In mice preexposed to SGL, protection is also associated with reactive antibody generation that neutralizes the enhancing effect of the saliva in *Leishmania* infections[26].

Studies in endemic areas suggest that natural exposure to uninfected phlebotomine bite could influence the epidemiology of the disease. In residents from an endemic area for visceral leishmaniasis, the presence of class IgG antibodies against the saliva of *Lu. Longipalpis* was detected[58]. High levels of IgG1, IgG4 and IgE were detected in the sera of volunteers exposed to *Lu. Longipalpis* bites[57]. Simultaneous to the development of humoral immune response to saliva, immunity mediated by cells against *Leishmania* is also observed in residents from endemic areas[59]. Individual response to phlebotomine saliva can vary depending on genetic factors. In this way, individuals that develop positive DTH for *Leishmania* antigens together with anti-saliva IgG antibodies could be protected against visceral leishmaniasis; whereas individuals showing low anti-saliva antibody titers and negative DTH would not be[43].

The protective response triggered by components of insect vector saliva appears to be parasite/vector specific, since the exposure of BALB/c mice to bites from Old World vectors *P. papatasi* and *P. sergenti* and New World vector *Lu. longipalpis*, led to the production of specific antibodies against the different saliva sources used. Moreover, challenge with an infection specific to the New World, *L. amazonensis*, led to partial protection of mice preexposed to *Lu. longipalpis* bites and the absence of protection for the other two phlebotomine species used[21].

A recent study showed that the immunization of hamsters with *Lu. longipalpis* salivary protein conferred protection against the fatal evolution of experimental visceral leishmaniasis, such that the low parasite load was correlated with an increase in the IFN-γ/TGF-b ratio and an increase in iNOS expression in the spleen and liver; thus reinforcing the concept of using phlebotomine saliva components in vaccine strategies[23].

In contrast, immunization of BALB/c mice with *Lu. intermedia* salivary gland sonicate followed by challenge with an inoculum containing *L. braziliensis* promastigotes or *L. braziliensis* with added *Lu. intermedia* salivary gland sonicate, did not lead to protection, rather to a prolonged infection evolution[60]. Immunization with *Lu. longipalpis* followed by the same challenge, showed infection evolution similar to controls, with increase in lesion size up to six weeks postinfection, followed by spontaneous cure around 10 weeks postinfection. Besides vector/parasite specificity in the development of an effective immunity against leishmaniasis, the different proteins that exist in phlebotomine saliva must also be considered, since distinct proteins, such as PpSP15 and PpSP44 from *P. papatasi*, induce different immunological profiles that are correlated with resistance and susceptibility, respectively[61].

Concluding remarks

- Insect vector saliva contains pharmacologically active components that block vertebrate host hemostatic processes, facilitating blood feeding and the establishment of *Leishmania* infection.
- Extracts or lysates of *Lu. longipalpis* salivary gland lead to the exacerbation of infection caused by different *Leishmania* species in different mice strains.
- Insect vector saliva plays a crucial role in establishing infection by helping the generation of infective metacyclic promastigote forms in the vector gut and by modulating the host immune response, compromising the presentation of antigens by macrophages, as well as by down-regulating Th1 and up-regulating Th2 responses.
- Regarding parasite/vector specificity, *P. papatasi* salivary gland extract promotes the exacerbation of infection by *L. major*; whereas *Lu. longipalpis* and *Lu. intermedia* salivary gland extracts do not lead to the exacerbation of infection by *L. (L.) chagasi* and *L. (V.) braziliensis*, respectively.
- *Leishmania* infection in the presence of wild-captured *Lu. longipalpis* salivary gland extract induces smaller sized lesions, less inflammatory response in the skin lesion site and lower levels of cytokines associated with susceptibility compared to infection in the presence of lab-colonized vector salivary gland extract.
- Presensitization of vertebrate host with salivary gland extract or by vector bite protects the host against infection caused by coinoculation of parasites and vector saliva. Such protection is related to delayed hypersensitivity response and appears to be species-specific, although contradictory results can be found in the literature.
- Different proteic fractions of vector saliva used in host presensitization may or may not induce protection.

Taking all together, discrepancies were verified while comparing the available data regarding the effects of vector saliva, which clearly demonstrate the need for further studies to fully understand the high complexity of the vector- parasite- host interactions and the need to carefully investigate the role of saliva in the context of actual transmission in nature.

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