Effects of Sand Fly Vector Saliva on Development of Cutaneous Lesions and the Immune Response to *Leishmania braziliensis* in BALB/c Mice

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The saliva of the sand fly vector of leishmaniasis significantly enhanced infection with doses of $>10^4$ *Leishmania braziliensis* parasites in BALB/c mice. With a dose of 10^7 *L. braziliensis* parasites, 60 to 70% of the mice developed lifetime infections. Mice infected with *L. braziliensis* plus saliva produced two- to threefold more interleukin-4 than did mice infected with *L. braziliensis* alone. Treatment with a neutralizing anti-interleukin-4 antibody abrogated the effects of the saliva.

Leishmania spp. are protozoan parasites that exist as promastigotes in the sand fly vector for the parasite. The vertebrate host becomes infected when the sand fly probes the host's skin for a blood meal and injects the parasite admixed with its saliva (33). Extensive work with *Leishmania major* in a murine model for leishmaniasis has revealed that Th1 T cells and the Th1-associated cytokines gamma interferon and tumor necrosis factor alpha mediate healing, while Th2 T cells and the Th2-associated cytokine interleukin 4 (IL-4) cause the progression of leishmaniasis (3, 7, 13, 22; for a review, see reference 32). Comparatively little work with *Leishmania braziliensis* has been done, probably because *L. braziliensis* does not readily infect laboratory mice. However, inoculation with *L. braziliensis* by needle does not mimic natural transmission since the sand fly injects *L. braziliensis* within its saliva. We have reported previously that sand fly saliva enhances *L. braziliensis* infection in mice (19). In this work, we analyze the phenomenon further and attempt to elucidate the mechanism underlying the effects of the saliva.

Sand fly saliva exacerbates infection with *L. braziliensis* in BALB/c mice and can change the outcome of infection. The *L. braziliensis* strain (LTB 111) used in these studies was isolated from a Brazilian patient with cutaneous leishmaniasis (10). We maintained the parasite in Schneider's insect medium (Sigma, St. Louis, Mo.) plus supplements (2 mM L-glutamine, penicillin [100 U/ml], streptomycin [100 µg/ml] [all from Gibco BRL, Grand Island, N.Y.], gentamicin [50 µg/ml; Sigma], and 25 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [Gibco BRL])–10% (vol/vol) heat-inactivated fetal calf serum (Sigma)–2% (vol/vol) sterile normal human urine (8) at 26°C. The *L. major* strain used in these studies was a clone of LV39 (11). Stationary-phase promastigotes were used in all experiments. We confirmed that LTB 111 was an *L. braziliensis* strain (before use in experiments, and after isolation

from cutaneous lesions at the end of experiments) using a monoclonal antibody (XIII-3E6-B11 * B16 [kindly provided by Diane McMahon-Pratt, Yale University]) specific for *L. braziliensis* and indirect immunofluorescence (15, 24).

Sand fly salivary gland lysates were obtained from *Lutzomyia longipalpis* sand flies (Lapinha cave or Costa Rican isolates) and used as described previously (31).

Initially, we injected a broad range of doses of *L. braziliensis* (10^3 to 10^7 parasites) with or without saliva (the lysate of half of a sand fly salivary gland [31]) subcutaneously into the hind footpads of a panel of mouse strains that cover the full spectrum of susceptibility and resistance seen after *Leishmania* infection (BALB/c [National Cancer Institute, Frederick, Md., or Jackson Laboratory, Bar Harbor, Maine], C3H/HeJ, C57BL/6, DBA/2 [National Cancer Institute], and CBA/Ca [National Cancer Institute or our colony at Colorado State University]). Lesion progression was recorded as described previously (29). At various times after infection, three to six mice per group were killed for evaluation. Parasite numbers were determined as described previously (30) with one modification: the medium employed was Schneider's medium supplemented as described above.

In BALB/c mice, saliva enhanced the progression of *L. braziliensis*-induced lesions with all doses of the parasite injected. At doses of 10^5 , 10^6 , or 10^7 *L. braziliensis* parasites, this enhancement was significant (by analysis of variance for repeated measures) (Fig. 1). Moreover, when the number of *L. braziliensis* parasites in the lesions was assessed at days 21, 42, and 70 of infection, it was found that *L. braziliensis* was not destroyed in saliva-treated mice (Fig. 1, lower panel). Finally, in mice infected with 10^7 *L. braziliensis* parasites plus saliva, a majority (typically 60 to 70%) of the animals developed nodular, non-ulcerated lesions which persisted for the lifetime of the mice. Importantly, *L. braziliensis* also induces chronic cutaneous lesions in humans infected with the parasite by being bitten by infected sand flies (12). Therefore, sand fly saliva may also markedly affect *L. braziliensis* infection in humans.

It is interesting that saliva did not significantly enhance infection with *L. braziliensis* when a dose of 10^4 or fewer parasites was used. This is in contrast to previous results from this laboratory that showed that saliva markedly enhanced infection with *L. major* even with the low dose of 10 *L. major* parasites (31). The reason for the differences between the effects of saliva on infection with *L. major* and *L. braziliensis*

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FIG. 1. Sand fly saliva exacerbates infection with *L. braziliensis* in BALB/c mice and can change the outcome of infection. Groups of five BALB/c mice each were injected with the indicated doses of *L. braziliensis* (*Lb*) with or without the lysate of half of a salivary gland of *L. longipalpis*. The numbers next to symbols in the lower panel indicate the numbers of parasites in lesions at days 21, 42, and 70 of infection. The results are representative of two to five independent experiments. Bars show standard errors (SE).

are unknown. However, the sand fly injects the metacyclic form of *Leishmania* spp. Moreover, only the metacyclic form of *Leishmania* spp. survives and efficiently infects macrophages (M ϕ) in the vertebrate host (5, 16). As mentioned above, *L. braziliensis* is poorly infective for most laboratory animals. Therefore, although little is known about metacyclogenesis in *L. braziliensis*, it is probable that the conversion of *L. braziliensis* parasites to the metacyclic form under standard culture conditions such as the Schneider's medium used in this study is very inefficient. If this is the case, it would explain (i) the poor infectivity of *L. braziliensis* for laboratory animals and (ii) the need for large doses of *L. braziliensis* to produce a significant effect of sand fly saliva on the infectivity of the parasite for mice. To date, the only immune cell shown to be affected by saliva is the M ϕ . Saliva inhibits NO (6) and H₂O₂ (32) production, the secretion of several monokines (3a), and antigen presentation (28) by M ϕ . All of these effects of saliva would enhance the survival of *Leishmania* spp. within M ϕ . However, for *L. braziliensis* to benefit from these effects of saliva, the parasite would have to find its way inside the M ϕ . If *L. braziliensis* forms few metacyclic parasites (the form of the parasite that efficiently enters M ϕ), mice would have to be injected with a large dose of *L. braziliensis* to successfully infect the M ϕ within the mice. In other words, if *L. major* forms metacyclic parasites in culture with an efficiency of 10% (an efficiency that is easily obtained with *L. major* [5, 16]), this preparation would still infect the M ϕ of a mouse if only 10 parasites were injected. On the other hand, if *L. braziliensis* forms metacyclic parasites in culture with an efficiency of only 0.01%, then a dose of 10^4 or more *L. braziliensis* parasites would be required for the parasite to successfully infect the M ϕ of a mouse.

The fact that *L. braziliensis* is poorly infectious for mice

might also explain why saliva had its major effect on survival of *L. braziliensis* in mice at later time points of infection (Fig. 1). When one injects *L. major* at a dose of 10^7 parasites into BALB/c mice, there are 1.7×10^6 , 123×10^6 , and 421×10^6 parasites in the resulting lesion at 3, 8, and 14 days of infection, respectively (30). In contrast, when 10^7 *L. braziliensis* parasites are injected into BALB/c mice, the rate of parasite replication is quite slow (Fig. 1). Therefore, it is possible that the ability of saliva to enhance the survival of *L. braziliensis* would take a relatively long time to become evident.

In contrast to results obtained with BALB/c mice, saliva did not affect *L. braziliensis* infection in any of the other mouse strains tested (data not shown).

Treatment with a neutralizing anti-IL-4 antibody abrogates the exacerbative effects of sand fly saliva on *L. braziliensis* infection in BALB/c mice. We next attempted to determine the mechanism by which saliva exacerbates *L. braziliensis* infection in BALB/c mice. We prepared single-cell suspensions from the lymph nodes (inguinal and popliteal) draining the lesion site at 3, 7, 21, 42, and 70 days of infection with 10^7 *L. braziliensis* parasites with or without saliva. The cells were restimulated with *L. braziliensis* by published protocols (27), and at 6, 24, 48, and 72 h of culture aliquots of the culture supernatants were harvested and tested for the presence of various cytokines by enzyme-linked immunosorbent assay (4, 23). Little or no difference in the secretion of IL-2, IL-10, gamma interferon or tumor necrosis factor alpha by cells from mice infected with *L. braziliensis* alone or *L. braziliensis* plus saliva was seen (data not shown). However, cells from BALB/c mice infected with *L. braziliensis* plus saliva secreted two- to threefold more IL-4 at days 7 and 21 of infection than did cells from BALB/c mice infected with *L. braziliensis* alone (e.g., at 21 days of infection, cells from *L. braziliensis*-infected mice produced 38.8 ± 4.3 pg of IL-4 per ml [mean $\pm$ standard deviation], while cells from animals infected with *L. braziliensis* plus saliva produced 83.0 ± 10.7 pg of IL-4 per ml [this difference was significant; $P \leq 0.05$, nonpaired *t* test]).

To test whether the increase in IL-4 secretion seen in the first 3 weeks of infection in BALB/c mice infected with *L. braziliensis* plus saliva was necessary for the exacerbative effects of saliva, we treated mice with a neutralizing anti-IL-4 monoclonal antibody (11B11 [14]). As an internal control for these experiments, we first tested whether our 11B11 preparation would block IL-4 production and thus induce a cure in BALB/c mice infected with *L. major*, a phenomenon reported by Sadick et al. (18) and later reproduced in several laboratories. The inset in Fig. 2 shows that our 11B11 preparation was fully capable of blocking IL-4 production and inducing a rapid cure in BALB/c mice infected with *L. major*. BALB/c mice infected with *L. major* produce 8.4 to 13.9 times more IL-4 than BALB/c mice infected with *L. braziliensis* (10a). Therefore, our preparation of 11B11 would be more than capable of blocking IL-4 production in BALB/c mice infected with *L. braziliensis*. When we treated mice infected with *L. braziliensis* plus saliva with 11B11, this treatment completely abrogated the effects of the saliva (Fig. 2). Moreover, treatment with 11B11 allowed the mice to destroy *L. braziliensis* rapidly in their lesions (data not shown).

The simplest interpretation of this result is that enhanced IL-4 production is necessary for the disease exacerbation caused by saliva. Alternatively, another factor that leads to exacerbation of infection could be induced by saliva, which, in turn, results in increased secretion of IL-4. IL-10 is a candidate for another factor that might enhance *L. braziliensis* replication; however, saliva did not enhance its production. Transforming growth factor β (TGF- β) is another candidate. Pro-

FIG. 2. Treatment with a neutralizing anti-IL-4 antibody abrogates the exacerbative effects of sand fly saliva on *L. braziliensis* infection in BALB/c mice. BALB/c mice were infected with *L. braziliensis* (Lb) and treated as indicated. The inset shows the results of experiments in which BALB/c mice were infected with 10^6 *L. major* parasites and treated as indicated. Mice that received anti-IL-4 were given intraperitoneal injections of 0.5 mg of the antibody (11B11; Biological Response Modifiers Program, National Cancer Institute [14]) at the time of infection and at weekly intervals thereafter through 6 weeks of infection. The results are representative of two independent experiments. Bars show standard errors (SE).

duction of TGF- β correlates with susceptibility to infection with both *L. braziliensis* (2; for a review, see reference 17) and *L. major* (25). However, we have been unable thus far to detect TGF- β (protein or mRNA, in vitro or in vivo, with or without saliva) following infection with *L. braziliensis*. This is not due to a technical failure, since we have detected substantial amounts of the cytokine in other settings. It should also be noted that different isolates of *L. braziliensis* vary in their ability to induce TGF- β production (1), and thus, the *L. braziliensis* strain used in these studies (LTB 111) could be a poor inducer of TGF- β .

It is interesting that saliva had little effect on infection with *L. braziliensis* in mice of strains other than BALB/c. The explanation for these observations could be related to the levels of IL-4 produced following infection with *L. braziliensis* plus saliva. That is, whereas BALB/c mice produce IL-4 when infected with *L. braziliensis* or *L. braziliensis* plus saliva, C3H mice do not produce detectable levels of IL-4 (10a). Moreover, unlike BALB/c mice, which are susceptible to infection with *L. major* and which produce large amounts of IL-4 in response to infection, C3H, C57, and CBA mice are all resistant to infection with *L. major* (20) and C57 (7) and C3H (21) mice produce little or no IL-4 when infected with this parasite. Thus, it is probable that only BALB/c mice are capable of making substantial amounts of IL-4 when infected with *L. braziliensis* or perhaps any *Leishmania* sp. If this is the case, it would suggest that saliva exacerbates infection with *L. braziliensis* in BALB/c mice but not other strains of mice, since the BALB/c mouse is the only mouse in which sand fly saliva can upregulate the production of IL-4. Indeed, Hsieh et al. (9) proposed that BALB/c mice naturally default towards the development of Th2 responses.

We initially proposed that the immunosuppressive or disease-enhancing effects of sand fly and other bloodsucking-arthropod salivas were due to proteins whose original purpose was to prevent the vertebrate host from becoming sensitized to vasomodulatory proteins in saliva (32). Inadvertently, the same immunosuppressive proteins promote the transmission of *Leishmania* spp. and may be required for the parasite to successfully infect the vertebrate host because (i) low doses of

L. major (equivalent to the number of parasites that the sand fly injects per bite) do not infect mice unless they are coinjected with sand fly saliva (31) and (ii) mice normally cure an infection with *L. braziliensis* but are unable to do so if the *L. braziliensis* parasites are coinjected with saliva (Fig. 1) (also see reference 19).

If salivary proteins are required for the successful transmission of pathogens by arthropod vectors, it may be possible to block the transmission of these pathogens by immunizing against the relevant components of saliva to block the activity of these molecules and, as a result, block the transmission of the pathogens. In the case of leishmaniasis, a vector-based vaccine approach might be particularly attractive. The disease-enhancing effects of sand fly saliva occur in several experimental systems using any of the medically important *Leishmania* spp. (*L. major*, *L. donovani*, *L. mexicana*, and *L. braziliensis*) and using the saliva of all sand flies tested. In addition, this observation has held true for coindigenous as well as noncoindigenous combinations of vectors and parasites (references 19, 26, 31, 32, and 34 and this work). Taken together, these observations represent a strong argument for the usefulness of developing a vaccine against leishmaniasis using the saliva of the sand fly vector. Since the saliva of most (probably all) sand flies exacerbates disease with all *Leishmania* spp., the host vaccinated against sand fly salivary gland proteins would become resistant to any *Leishmania* sp. transmitted by any sand fly.

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