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Analysis of Enhancing Effect of Sand Fly Saliva on *Leishmania* Infection in Mice

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Salivary gland lysates of the sand fly *Lutzomyia longipalpis* markedly enhance the course of infection with *Leishmania major* in mice. Here we examine various parameters of this phenomenon. The exacerbative effect of *L. longipalpis* salivary gland lysates occurred in five different mouse strains; however, the character of the effect varied from one strain to another. Consistent exacerbation of infection was achieved with as little as 1/10 of a gland. The exacerbative effect applied to more than one *Leishmania* species and to more than one species of sand fly, since salivary gland lysates of *L. longipalpis* enhanced infection with *L. mexicana amazonensis* and salivary gland lysates of *Phlebotomus papatasi* enhanced infection with *L. major*. A synthetic rat calcitonin gene-related peptide was also found to exacerbate infection with *L. major* but was found to be approximately 100-fold less potent than saliva in mediating this effect. In addition, lesions induced at skin sites at which *L. longipalpis* had probed for a blood meal exhibited an exacerbated course of infection similar to that seen when parasites were injected with sand fly salivary gland lysates.

Members of the genus *Leishmania* are protozoan parasites which are transmitted to the mammalian host by phlebotomine sand fly vectors when the fly probes the skin of the mammalian host for a blood meal. Within the mammalian host, the parasites reside within the phagolysosomes of macrophages. Resistance to the parasites is thought to be mediated by parasite-specific T cells which secrete gamma interferon. Gamma interferon activates infected macrophages in vitro to destroy intracellular *Leishmania* parasites, a phenomenon which is largely due to H_2O_2 production by the activated macrophages (1).

We have previously reported that salivary gland lysates of the sand fly *Lutzomyia longipalpis* markedly enhance the infectivity of *Leishmania major* for mice (11). In fact, when mice were injected with the number of *L. major* parasites that the sand fly injects (10 to 100 parasites [13]), the parasites did not survive in the animals unless they were injected with sand fly saliva (11). This saliva effect was not due to a direct effect of the saliva on the parasite, but rather to an effect on the host (12). Sand fly saliva contains a potent vasodilator which, pharmacologically, is very similar to the mammalian neuropeptide calcitonin gene-related peptide (CGRP [6]). Both sand fly saliva and CGRP inhibit the ability of macrophages to produce H_2O_2 in response to stimulation with gamma interferon (4, 9, 12). In addition, saliva and CGRP inhibit the ability of macrophages to present antigens (4, 9, 12). Thus, it is possible that sand fly saliva contains a CGRP-like substance which exacerbates infection by initially enhancing parasite survival through its ability to prevent H_2O_2 production by macrophages. In addition, sand fly saliva could further promote infection by preventing the presentation of leishmanial antigens by macrophages and thus the effective activation of host-specific T-cell immunity.

Since sand fly saliva is a potent immunosuppressive and since it determines whether *L. major* survives in mice injected with the low numbers of parasites that the sand fly injects, it is possible that leishmaniasis could be prevented in human beings by vaccinating against vector saliva. Specifically, if vaccinated individuals were able to neutralize the immunosuppressive effect of sand fly saliva, the parasite might be prevented from establishing infection in the host. However, if such a vector saliva-based vaccine were to be feasible in humans, it is important to first determine whether the exacerbative effect of sand fly saliva is a generalizable phenomenon in mice. For example, the entire spectrum of clinical manifestations of leishmaniasis seen in humans can be mimicked by infecting different strains of mice with the parasite (2). It is therefore important to determine whether the exacerbative effect of sand fly saliva occurs in several strains of mice which range from strains that are susceptible to infection with *L. major* to strains that are resistant. In addition, it is important to determine whether the exacerbative effect of sand fly saliva applies to vector-parasite combinations which are both coindigenous and noncoindigenous as well as whether the sand fly injects the material responsible for exacerbation when it probes the skin for a blood meal. If the material in sand fly saliva responsible for disease exacerbation is conserved among many species of sand flies, it may be possible to vaccinate human beings against the saliva of one species of sand fly and protect those individuals against subsequent infections with several different *Leishmania* species delivered by several different species of sand fly.

To address these and other issues, we further analyzed the exacerbative effect of sand fly saliva on *Leishmania* infections in mice. The enhancing effect of *L. longipalpis* salivary gland lysates on infection with *L. major* was seen in several mouse strains; however, the magnitude and character of the effect varied from one strain to another. The maximum effect of the saliva on infection was achieved when 1/10 of a gland was injected with *L. major*; however, amounts as low as 1/250 of a gland had an observable effect on the course of the infection. In addition to enhancing infection with *L. major* in mice, salivary gland lysates of *L. longipalpis* enhanced infection with *L. mexicana amazonensis*. Moreover, the same exacerbative phenomenon was observed when salivary

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gland lysates of a different sand fly vector, *Phlebotomus papatasi*, were examined.

Since the material in sand fly saliva that is responsible for exacerbating leishmaniasis may be related to mammalian CGRP, we determined the ability of synthetic rat CGRP to exacerbate infection. CGRP exacerbated infection but was found to be approximately 100-fold less potent than saliva in mediating this effect. Finally, we determined whether the material in saliva responsible for exacerbation was injected by the sand fly when it probed the skin for a blood meal. Lesions induced at murine skin sites at which *L. longipalpis* had probed for a blood meal exhibited an exacerbated course of infection similar to that seen when parasites were injected with sand fly salivary gland lysates.

MATERIALS AND METHODS

Mice. Young adult female BALB/c, C3H/HeN, C57BL/6, CBA/Ca, and DBA/2 mice were obtained from Taconic Farms (Germantown, N.Y.) or from Jackson Laboratory (Bar Harbor, Maine).

Parasites. The maintenance of *L. major* (LV39) has been described elsewhere (10). When used in experiments, parasites were always obtained from stationary-phase cultures of *L. major* (7). *L. mexicana amazonensis* (PH8) was maintained in a similar fashion.

Sand flies and sand fly salivary gland lysates. Sand flies were reared and salivary gland lysates were collected as described previously (11). In brief, salivary glands from mated, non-blood-fed, 5- to 7-day-old adult female sand flies were dissected, placed in 0.1% bovine serum albumin in water (pH 7.0), and frozen to achieve complete disruption. The glands were stored at −70°C until use, at which time they were brought to isotonicity by the addition of 10× phosphate-buffered saline.

Synthetic rat CGRP was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Infection of mice with *L. major*. Mice were inoculated subcutaneously (s.c.) in one hind footpad with *L. major* or *L. mexicana amazonensis* promastigotes or promastigotes mixed with either sand fly salivary lysate or CGRP. The development of lesions was monitored by measuring the increase in the thickness of the infected footpad with a vernier caliper in comparison with the thickness of the control, contralateral footpad.

In the case of mice on which *L. longipalpis* sand flies had probed for a blood meal, hair was shaved from the rumps of the animals. The mice were anesthetized with sodium pentobarbital, and female sand flies were allowed to feed on the animals. One hour later, *L. major* promastigotes were injected s.c. at the same skin sites at which the sand flies had fed. Lesion development was monitored by determining the mean diameter of the lesion with a vernier caliper.

RESULTS

We first determined whether the exacerbative effect of *L. longipalpis* salivary gland lysates on infection with *L. major* in mice could be observed in several different mouse strains and whether the magnitude and character of the exacerbative effect varied from one strain to another. Five different mouse strains were used: BALB/c (the prototype genetically susceptible mouse strain [2]), DBA/2 (intermediate susceptibility [2]), and C3H, C57BL/6, and CBA/Ca (genetically resistant [2]). Each mouse strain was challenged s.c. in the footpad with either 10^5 *L. major* or 10^5 *L. major* mixed with the lysate of 1/2 of one salivary gland of *L. longipalpis*. Exacerbation of disease occurred in all mouse strains, but the intensity and character of the effect varied (Fig. 1). Exacerbation of disease was most pronounced in C57BL/6 and CBA mice (Fig. 1); however, even in these two strains of mice, the character of the effect was different. In C57BL/6 mice, exacerbation of lesion size occurred primarily in the latter portion of the disease process, whereas in CBA mice, lesion size was enhanced throughout the course of the infection (Fig. 1). This difference between C57BL/6 and CBA mice was consistent, and the data presented in Fig. 1 are representative results of several experiments.

Since the exacerbative effect of sand fly saliva was most pronounced in either C57BL/6 or CBA/Ca mice, these mice offered the most sensitive experimental system. Thus, the remainder of the experiments presented in this paper which utilized *L. major* were performed with either one of these two strains. We next determined the amount of salivary gland material that was necessary to produce an observable effect on an infection with *L. major*. While as little as 1/50 to 1/250 of a gland had a demonstrable effect on the course of infection (Fig. 2), the sizes of the lesions did not differ significantly from the sizes of the lesions on control mice ($0.05 < P < 0.2$). Injection of parasites with 1/10 of a salivary gland consistently resulted in lesion sizes that were significantly larger than control lesion size ($0.005 < P < 0.05$). A very similar increase in lesion sizes could be induced with the lysate of 1/2 of a gland or 1 whole gland; for the sake of clarity in Fig. 2, these data have been omitted.

To determine whether sand fly salivary gland lysates enhanced infection in mice with a different species of *Leishmania*, *L. longipalpis* salivary gland lysates were injected into mice with *L. mexicana amazonensis*. The lysates enhanced infection with *L. mexicana amazonensis* in both BALB/c and CBA mice (Table 1). Thus, the exacerbative effect of sand fly salivary gland lysates on the course of cutaneous leishmaniasis applies not only to infection with *L. major* (Old World cutaneous leishmaniasis) but also to infection with a parasite causing cutaneous leishmaniasis in the New World, *L. mexicana*.

We also examined the effect of salivary gland lysates from the sand fly *P. papatasi* on the course of *L. major* infection in mice. *P. papatasi* is the natural vector for *L. major* in the Old World (3). Salivary gland lysates from *P. papatasi* enhanced infection with *L. major* in C57BL/6 mice to a degree equivalent to, if not somewhat superior to, the exacerbative effect of *L. longipalpis* salivary gland lysates on infection (Table 2). *P. papatasi* salivary gland lysates also enhanced infection with *L. major* in BALB/c mice and C3H mice (data not shown) but, as with the effect of *L. longipalpis* salivary gland lysates (Fig. 1), the degree of exacerbation of disease was less pronounced.

It was also important to determine whether the sand fly itself injects a material which exacerbates infection when it probes the skin for a blood meal. For these experiments, the rumps of C57BL/6 mice were shaved clean of hair, the animals were anesthetized, and female *L. longipalpis* sand flies were allowed to feed on the exposed skin. One hour later, the same skin sites at which sand flies had fed were challenged s.c. with *L. major*. Typical results of such an experiment are shown in Fig. 3. Cutaneous lesions that developed at sites at which sand flies had probed for a blood meal developed more rapidly (lesion onset at day 6 of infection, as compared with day 17 of infection in mice not exposed to sand flies; Fig. 3). In addition, the overall course of infection in these mice was more severe, resembling

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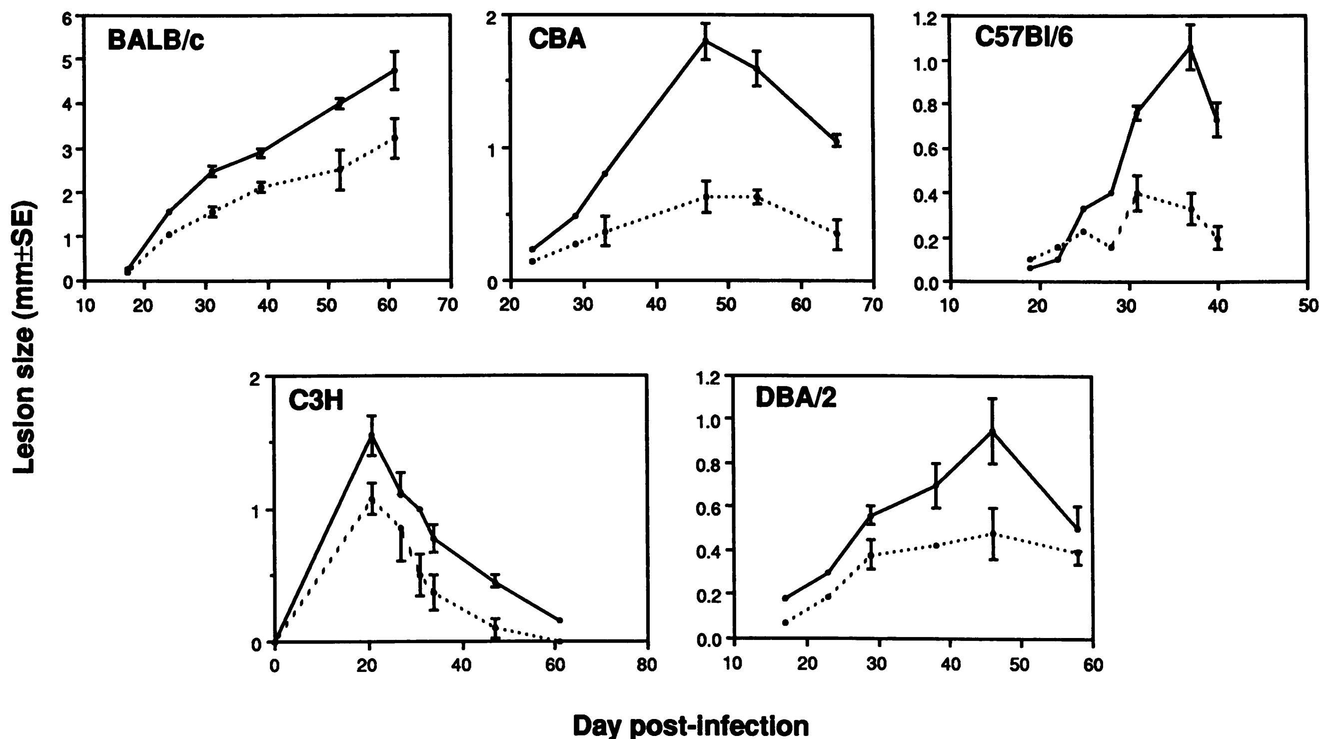

FIG. 1. Effect of *L. longipalpis* salivary gland lysates on infection with *L. major* in five mouse strains. Groups of four mice of each of the mouse strains indicated were infected s.c. in one hind footpad with 10^5 *L. major* promastigotes (dotted lines) or 10^5 promastigotes mixed with the lysate of 1/2 of one salivary gland of *L. longipalpis* (solid lines). Lesion development was monitored as detailed in Materials and Methods. The data represent the increase (mean ± standard error, in millimeters) in the thickness of the infected footpad as compared with the thickness of the uninfected, contralateral footpad.

lesion development seen in mice injected with sand fly salivary gland lysates and parasites (compare Fig. 3 with Fig. 1 or 2). Finally, in addition to being larger, the lesions on these mice ulcerated far in advance of lesions developing on mice not exposed to sand flies (day 11 versus day 45). Thus, while probing the skin for a blood meal, the sand fly appears to inject a material which has an exacerbative effect on infection with *L. major* that is similar to the effect seen when parasites are injected with salivary gland lysates.

Finally, since *L. longipalpis* saliva has been shown to contain a substance that is related to mammalian CGRP, we examined the ability of synthetic rat CGRP to exacerbate infection with *L. major* in mice. Compared with infecting mice with *L. major* alone, infecting mice with either parasites plus CGRP (100 ng) or parasites plus *L. longipalpis* salivary gland lysates (1/2 of a gland) markedly enhanced and greatly prolonged infection with the parasite (Fig. 4). Doses smaller than 100 ng of CGRP had little effect on the course of disease (data not shown).

DISCUSSION

We have previously reported that lysates of the salivary glands of *L. longipalpis* enhance infection with *L. major* in mice, as exhibited by an increase in lesion size as well as a marked increase in parasite numbers in the lesion (11, 12). To determine whether this was a general phenomenon, we examined the effect of salivary gland lysates in mice of several different strains (Fig. 1). Although the lysates exacerbated disease in all mouse strains tested, the intensity and character of the effect varied. This variation did not appear to correlate with either the *H-2* haplotype of the mouse or the relative susceptibility of the mouse to infection with *L. major*. For example, the mouse strain in which the greatest degree of exacerbation was observed was CBA/Ca, while the mouse strain in which the smallest effect was observed was C3H (Fig. 1). CBA and C3H mice are both of the *k* haplotype and are both genetically resistant to infection with *L. major* (2). Therefore, the basis for the variation in the degree of exacerbation of sand fly saliva in different strains of mice is currently unknown. However, the fact that the intensity and character of the effect varied even among genetically resistant mice implies that the mechanism by which sand fly saliva exacerbates cutaneous leishmaniasis is complex.

Sand fly salivary gland lysates were found to be very potent in their ability to enhance infection, since as little as 1/10 of a salivary gland exacerbated infection with *L. major* (Fig. 2). In addition, the exacerbative effect of sand fly salivary gland lysates on the course of leishmaniasis appeared to be a generalized phenomenon. Infections with *L. major* or *L. mexicana amazonensis* were enhanced by *L. longipalpis* saliva (Fig. 1 and Table 1). In addition, *L. major* infection was enhanced by saliva from either *L. longipalpis* or *P. papatasi* (Fig. 1 and Table 2). This latter observation is important since, unlike *L. longipalpis*, *P. papatasi* is the primary vector for *L. major* in nature (3). Thus, the enhancing effect of saliva on infection with *Leishmania* species is operative in at least one coindigenous combination of vector and parasite. In addition, we have recently observed that *L. longipalpis* salivary gland lysates make it possible to infect mice with *L. braziliensis braziliensis*, a parasite which, in the absence of saliva, is poorly infective even for hamsters, the

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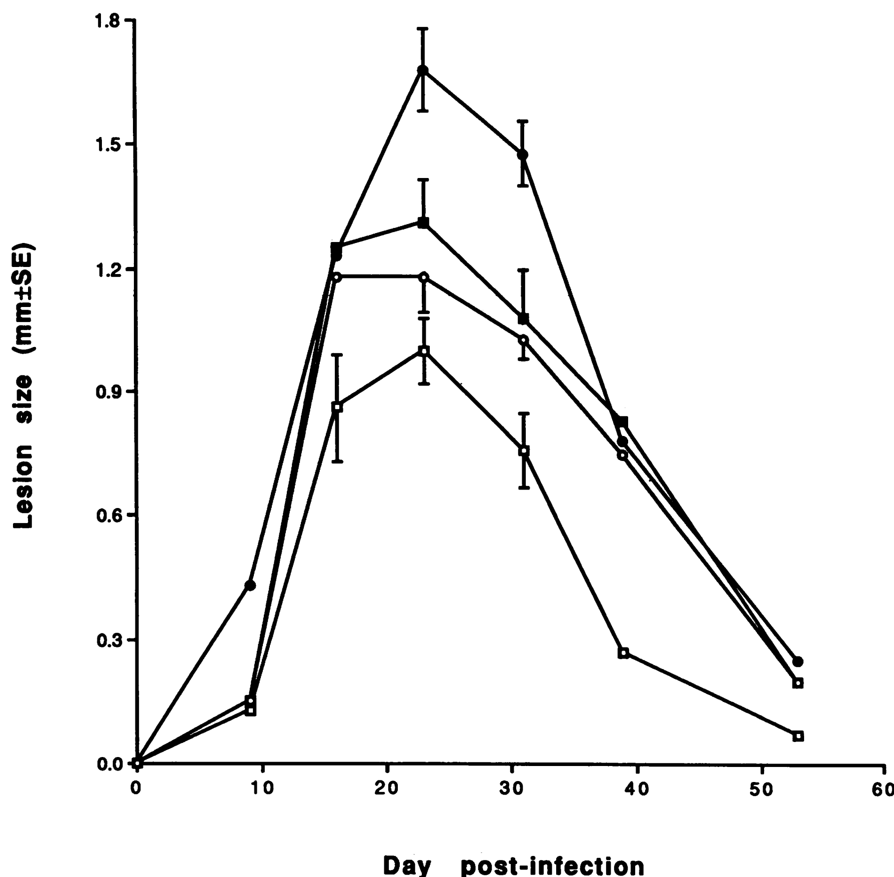

FIG. 2. Effect of low doses of *L. longipalpis* salivary gland lysates on *L. major* infection in mice. Groups of four CBA/Ca mice each were injected s.c. in the hind footpad with 10^5 *L. major* promastigotes (□), 10^5 *L. major* promastigotes plus the lysate of 1/250 of a salivary gland of *L. longipalpis* (○), 10^5 *L. major* promastigotes plus the lysate of 1/50 of a salivary gland of *L. longipalpis* (■), or 10^5 *L. major* promastigotes plus the lysate of 1/10 of a salivary gland of *L. longipalpis* (●). Lesion development was monitored as detailed in Materials and Methods. The degrees of exacerbation of disease obtained with 1/10, 1/2, or 1 gland per mouse were almost identical; for the sake of clarity, only results obtained with 1/10 gland per mouse are shown. The data are as defined in the legend to Fig. 1.

model of choice for infection with *L. braziliensis braziliensis* (8). Currently, we are examining the ability of the saliva of *L. whitmani*, the vector for *L. braziliensis braziliensis*, to enhance infection with the parasite.

We have previously shown that a peptide, termed erythema-inducing factor (EIF), which exists in the saliva of *L. longipalpis*, elicits intense long-lasting erythema in the skin of rabbits and human beings (6). This erythematous

TABLE 1. Enhancement of infection with *L. mexicana amazonensis* in mice by *L. longipalpis* salivary gland lysates[a]

Day of infection	Lesion size (mm ± SE) after the indicated treatment of the following mice:			
	BALB/c		CBA/Ca	
	LMA	LMA + SG	LMA	LMA + SG
21	0.2 ± 0.03	0.7 ± 0.08	0.8 ± 0	0.8 ± 0
40	0.5 ± 0.12	1.0 ± 0.15	1.1 ± 0.11	1.1 ± 0.07
70	0.6 ± 0.39	1.4 ± 0.16	1.5 ± 0.20	1.7 ± 0.25
91	0.7 ± 0.24	1.8 ± 0.21	1.2 ± 0.10	1.7 ± 0.17
127	2.7 ± 0.28	3.9 ± 0.33	0.4 ± 0.40	1.4 ± 0.05
153	3.8 ± 0.42	5.3 ± 0.24	0 ± 0	0.7 ± 0.15

[a] Groups of four BALB/c or CBA/Ca mice each were challenged with 10^6 *L. mexicana amazonensis* parasites (LMA) with or without the lysate of 1/2 of one salivary gland (SG) of *L. longipalpis*. Lesion development was monitored as detailed in Materials and Methods. The data represent the increase in the thickness of the infected footpad as compared with the thickness of the uninfected, contralateral footpad.

TABLE 2. Enhancement of infection with *L. major* in mice by *P. papatasi* salivary gland lysates[a]

Day of infection	Lesion size (mm ± SE) after the following treatment:		
	L. major	*L. major* + *L. longipalpis* SG	*L. major* + *P. papatasi* SG
19	0.10 ± 0	0.06 ± 0.05	0.06 ± 0.05
22	0.16 ± 0.03	0.10 ± 0	0.13 ± 0.03
25	0.23 ± 0.03	0.33 ± 0.05	0.53 ± 0.16
28	0.16 ± 0.03	0.40 ± 0.10	0.70 ± 0.08
31	0.40 ± 0.08	0.76 ± 0.14	0.90 ± 0.13
37	0.33 ± 0.07	1.06 ± 0.10	1.13 ± 0.12
40	0.20 ± 0.05	0.73 ± 0.08	0.90 ± 0.13

[a] Groups of four C57BL/6 mice each were challenged with 10^5 *L. major* parasites with or without the lysate of 1/2 of one salivary gland (SG) of either *L. longipalpis* or *P. papatasi*. Lesion development was monitored as detailed in Materials and Methods. The data represent the increase in the thickness of the infected footpad as compared with the thickness of the uninfected, contralateral footpad.

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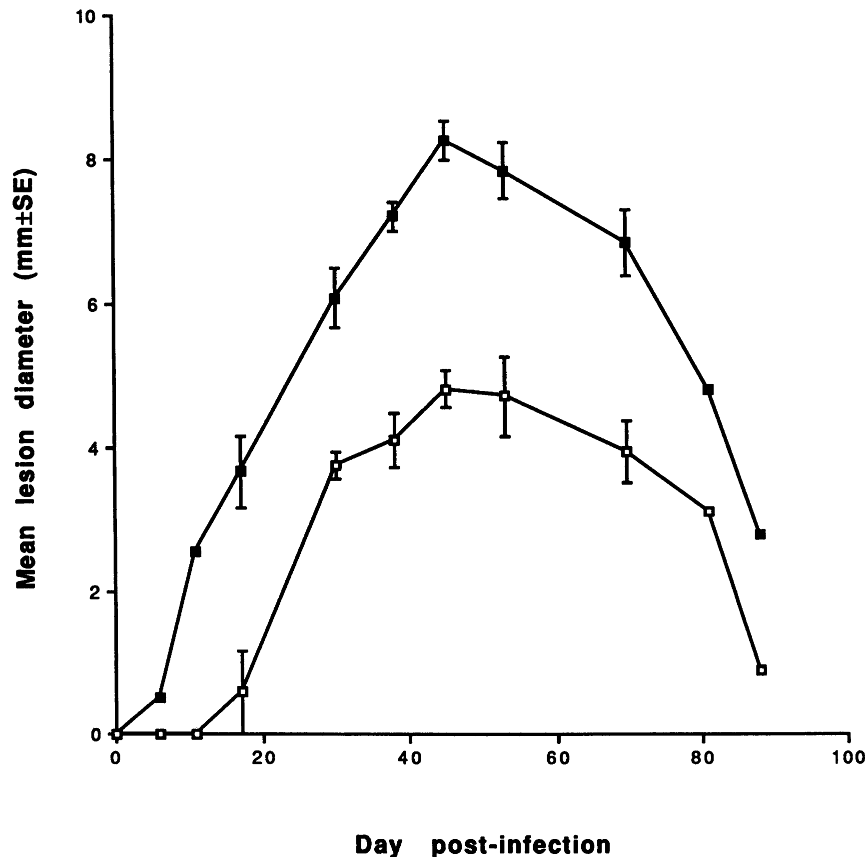

FIG. 3. Lesion development at skin sites at which *L. longipalpis* had probed for a blood meal. Hair was shaved from the rumps of four C57BL/6 mice, and the animals were anesthetized with sodium pentobarbital. Female *L. longipalpis* sand flies were allowed to feed on the skin of the animals, and the sites at which the flies had probed were marked. One hour later, these sites were injected s.c. with 10^5 *L. major* promastigotes (■). Control mice were shaved but not exposed to sand flies (□). Lesion development was monitored as detailed in Materials and Methods. The data represent the diameters (mean ± standard error, in millimeters) of skin lesions which developed at the sites of injection of *L. major*.

response is identical to that induced by CGRP, a neuropeptide which is found in mammals and which is the most potent inducer of skin erythema known in humans (6). This result led us to hypothesize that the EIF in sand fly saliva is a member of the CGRP family of erythema-inducing peptides. However, EIF and CGRP must somehow differ, since the potency of EIF in inducing erythema was approximately 100-fold greater than that of CGRP (6).

We have also reported that mammalian CGRP is a potent immunosuppressive substance which inhibits the ability of macrophages to produce H_2O_2 and to present antigens to T cells (4). This result led us to hypothesize (12) that the CGRP-like substance (EIF) in *L. longipalpis* saliva may also be responsible for the ability of the saliva to exacerbate infection with *L. major*. Thus, it is interesting that rat CGRP was found to exacerbate infection with *L. major* in mice (Fig. 4). However, the ability of saliva to exacerbate infection with *L. major* was far greater than the ability of rat CGRP to do so. The total protein content of the salivary glands is 1 μg, and no more than 10 ng of the sand fly CGRP-like substance (EIF) is present in the pair of salivary glands of each fly (6). Thus, if the CGRP-like substance (EIF) present in saliva is responsible for the exacerbation of infection, it must be highly efficient, since as little as 1/10 of one salivary gland of *L. longipalpis* (equal to 0.5 ng of sand fly CGRP-like substance) can exacerbate infection with *L. major* (Fig. 2) to a degree equivalent to that resulting from injection of 100 ng of rat CGRP (Fig. 4). Thus, in accordance with the 100-fold greater potency of sand fly saliva over mammalian CGRP in eliciting skin erythema (6), saliva also appears to be approximately 100-fold more efficient than CGRP in exacerbating infection with *L. major* in mice.

It is important to mention that, in contrast to *L. longipalpis*, *P. papatasi* salivary gland lysates contain a level of EIF activity that is only slightly above the threshold of detection in the assay systems used (skin erythema and vasodilatory capacity [5]). However, we report here that *P. papatasi* salivary gland lysates are as efficient as *L. longipalpis* salivary gland lysates in exacerbating infection with *L. major* in mice (Table 2). This result suggests that the substance in *P. papatasi* saliva responsible for disease exacerbation may not be related to CGRP. To clarify this point, we are currently attempting to establish the structure of the sand fly CGRP-like material by biochemical as well as molecular methods. These studies should allow us to determine the degree of homology between sand fly CGRP-like material and mammalian CGRP as well as whether a CGRP-like substance exists in *P. papatasi* salivary glands. If a CGRP-like substance proves not to be present in *P. papatasi* saliva, it will be interesting to determine the nature of the material responsible for the exacerbative effect of the saliva on infection with *L. major* in mice.

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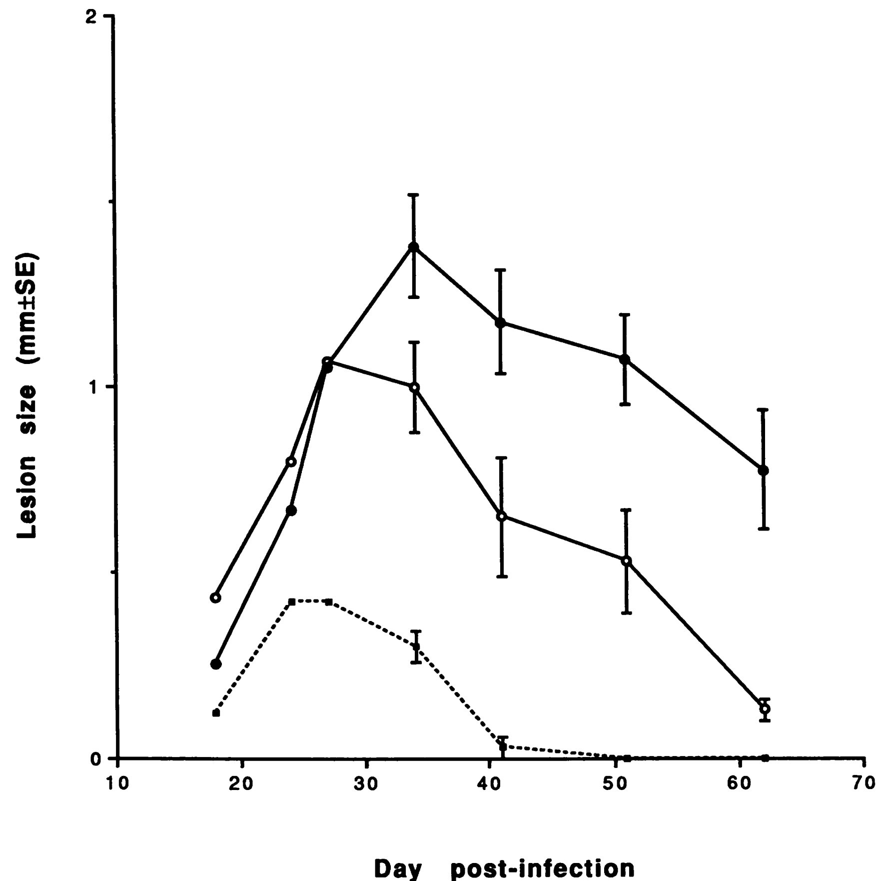

FIG. 4. Ability of rat CGRP to exacerbate infection with *L. major* in mice. Groups of four CBA/Ca mice each were injected s.c. in the hind footpad with 10^5 *L. major* promastigotes (dotted line), 10^5 *L. major* promastigotes plus 100 ng of rat CGRP (○), or 10^5 *L. major* promastigotes plus the lysate of 1/2 of a salivary gland of *L. longipalpis* (●). Lesion development was monitored as detailed in Materials and Methods. The data are as defined in the legend to Fig. 1.

It is important that exacerbation of infection with *L. major* in mice occurred as a result of challenging the animals with the parasite at skin sites at which *L. longipalpis* had probed for a blood meal (Fig. 3). This result suggests that the sand fly delivers the same substance to the skin that is administered when mice are injected with sand fly salivary gland lysates and *L. major* (Fig. 1 and 2 and Tables 1 and 2).

In conclusion, we have previously reported that when low numbers of parasites (10 to 100 promastigotes) are injected into mice, the parasites do not survive at the site of injection unless the parasites are injected with sand fly salivary gland lysates (11). Since the sand fly also injects 10 to 100 parasites when it probes for a blood meal on the mammalian host (13), it is possible that these parasites would also not survive were it not for the immunosuppressive effect of the sand fly's saliva. If this is the case, it may be possible to vaccinate human beings against leishmaniasis by vaccinating them against the component(s) of sand fly saliva that is critical to the initial survival of *Leishmania* parasites in the human host (12). The studies presented here lend support to the feasibility of this vaccine approach. The exacerbative effect of saliva was a rather generalized phenomenon. Salivary gland lysates of at least two species of sand fly exacerbated infection with at least three *Leishmania* species in several strains of mice (Fig. 1 and Tables 1 and 2) (8). Disease exacerbation was seen whether the vector saliva-*Leishmania* combinations were coindigenous or noncoindigenous (Fig. 1 and Tables 1 and 2). In addition, the material in saliva responsible for the effect was very potent and was delivered to the skin when the sand fly probed for a blood meal (Fig. 2 and 3). Therefore, it is possible that a related substance which markedly enhances the infectivity of several *Leishmania* species for the mammalian host is present in the saliva of many phlebotomine sand flies. Since vaccination against parasites themselves is proving to be very difficult, alternative approaches, such as vaccinating against the vector, may offer important new methods for controlling human disease.

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