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International Journal for Parasitology 31 (2001) 810–814

INTERNATIONAL Journal for PARASITOLOGY

www.parasitology-online.com

Effect of sand fly saliva on *Leishmania* uptake by murine macrophages

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Received 18 December 2000; received in revised form 2 March 2001; accepted 3 March 2001

Abstract

Leishmania promastigotes are introduced into the skin by blood-sucking phlebotomine sand flies. In the vertebrate host, promastigotes invade macrophages, transform into amastigotes and multiply intracellularly. Sand fly saliva was shown to enhance the development of cutaneous leishmaniasis lesions by inhibiting some immune functions of the host macrophages. This study demonstrates that sand fly saliva promotes parasite survival and proliferation. First, macrophages gravitated towards increasing concentrations of sand fly saliva in vitro. Secondly, saliva increased the percentage of macrophages that became infected with *Leishmania* promastigotes and exacerbated the parasite load in these cells. Thus, during natural transmission, saliva probably reduces the exposure of promastigotes to the immune system by attracting macrophages to the parasite inoculation site and by accelerating the entry of promastigotes into macrophages. Saliva may also enhance lesion development by shortening the generation time of dividing intracellular amastigotes.

Keywords: Chemotaxis; *Leishmania*; Macrophage; Sand fly saliva

1. Introduction

The leishmaniases are sand fly-borne parasitic diseases that affect large populations in vast areas of the world (Ashford et al., 1992). Once inoculated into the skin, *Leishmania* promastigotes rapidly invade macrophages (Mϕ) at the bite site of the vector sand fly and replicate as intracellular amastigotes. Parasite entry into Mϕ and survival inside the phagolysosomes, is facilitated by the parasites' ability to subvert the Mϕ's scavenger functions (reviewed by Liew and O'Donnel, 1993). Despite these capabilities, experimental inoculations with low doses of promastigotes fail to initiate infections in susceptible mouse strains and promote immunity instead (Doherty and Coffman, 1996; Menon and Bretscher, 1996). However, when similarly small numbers are inoculated by vector sand flies, infections flourish (Warburg and Schlein, 1986).

During natural transmission, *Leishmania* promastigotes are inoculated into the vertebrate skin together with saliva. Sand fly saliva has been shown to exacerbate experimental cutaneous lesions caused by several different *Leishmania* species (Samuelson et al., 1991; Theodos et al., 1991; Titus and Ribeiro, 1988; Warburg et al., 1994). Different mechanisms probably contribute to this phenomenon, including inhibition of antigen presentation, reduction in nitric oxide production in *Leishmania*-infected Mϕ and enhancement of interleukin-4 (IL-4) secretion by T-lymphocytes (Lima and Titus, 1996; Hall and Titus, 1995; Theodos and Titus, 1993; Waitumbi and Warburg, 1998).

Here we describe additional means by which saliva exacerbates infections. First, in order to survive and proliferate in the skin, *Leishmania* parasites require an adequate supply of Mϕ. In our experiments, sand fly saliva significantly enhanced Mϕ-positive chemotaxis. Secondly, promastigotes need to invade Mϕ with high efficiency and replicate within them as intracellular amastigotes. We show that, in vitro, sand fly saliva facilitates both of these processes.

2. Materials and methods

2.1. Mice

Female BALB/c and C3H/HeN mice (6–8 weeks) were purchased from Harlan laboratories, Israel. Mice were maintained in a sterile pathogen-free animal facility.

2.2. Parasites

Leishmania donovani infantum (strain: HN-194) promas-

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0020-7519/01/$20.00
PII: S0020-7519(01)00190-4

tigotes were cultured in RPMI 1640 supplemented with 15% foetal calf serum (FCS) and gentamycin (50 μg/ml) under 5% CO_2 at 26°C.

2.3. Sand flies

Phlebotomus papatasi (originating in the Jordan Valley) and *Lutzomyia longipalpis* (from the Island of Marajo, Brazil) were reared in the laboratory as described by Modi and Tesh (1983). Salivary glands were dissected from 4–6-day-old female flies using fine watchmaker's forceps. Glands were stored in a small volume of PBS containing 0.1% BSA at −70°C. Before use, glands were thawed and 90 μl of water was added. Glands were then sonicated in order to achieve total disruption and the solution was made isotonic using 10× PBS. For experimental use, concentrations were adjusted using RPMI 1640. The solution was filter sterilised using syringe filters (0.2 μm).

2.4. Peritoneal Mϕ

Mouse peritoneal exudate cells were obtained by peritoneal lavage of unstimulated mice as previously described (Waitumbi and Warburg, 1998). Cells adhering to plastic after washing are referred to as Mϕ.

2.5. Mϕ chemotaxis assays

Peritoneal exudate cells harvested from BALB/c female mice were washed in serum-free RPMI 1640. Cells were treated with 168 mM fresh, sterile NH_4Cl for 10 min at room temperature to lyse erythrocytes and washed again as above. Mϕ were counted using a Neubauer chamber and 10^5 Mϕ were suspended in 150 μl RPMI 1640 and dispensed into 6 mm diameter microporous-bottomed inserts with 8 μm pore diameter (Costar's Transwell System™ for 24-well plates). The wells in which the inserts were placed were filled with 250 μl of RPMI 1640 with or without saliva (one gland/well). After 3 h incubation at 37°C, inserts were washed in serum-free RPMI 1640. Polycarbonate microporous membranes with adherent Mϕ were removed using cotton-tipped watchmaker's forceps. The filters were fixed in methanol and stained with Giemsa. Membranes were removed from inserts and mounted on microscope slides bottom side up using Permount. Mϕ adhering to the bottom face of the membranes, having passed through the pores, were counted in 25 microscope fields representing 13.3% of the total area of the membrane. Since pore-size is smaller than the typical size of Mϕ, cells on the bottom face had to adhere to the membrane and move actively in order to pass through its micropores. Mϕ could not have fallen freely through these pores while smaller remaining cells, if any, either fell through the holes and sank to the bottom of the wells or remained on top of the membrane and were washed and wiped away during preparation. As a negative control we used RPMI 1640 without saliva. As a positive control we used 1% Zymosan-activated mouse serum in RPMI 1640.

2.6. Amastigote survival and replication assay

Peritoneal exudate cells were suspended in RPMI 1640 with 10% FCS at a concentration of 4×10^5/ml and 500 μl suspension was seeded into each chamber of a multichamber slide (Nunc). Slides were incubated for 24 h at 37°C. Non-adhering cells were removed by washing and 200 μl of RPMI 1640 containing stationary-phase promastigotes (5:1 promastigotes/Mϕ ratio), with or without saliva, was added to the chambers. Cultures were incubated for 3 h at 37°C to enable phagocytosis by Mϕ. Parasites that had not been internalised were washed off and the cultures were incubated for a further 24 h in RPMI 1640 with or without saliva. This time frame was selected since salivary components maintain activity at the bite site for at least that long (Warburg et al., 1994). Following incubation, the plastic chamber frames were removed, and slides were washed three times with PBS, fixed with methanol and stained with Giemsa. Infection rates and parasite burdens were scored microscopically. Mϕ were separated into five categories: uninfected, and infected with one, two, three and four or more amastigotes. Scoring slides microscopically allowed remaining clumps of extracellular promastigotes to be distinguished from true dividing intracellular amastigotes.

2.7. Statistical methods

The results of the Mϕ chemotaxis assays were analysed using the paired *t*-test. Analysis of parasite internalisation was complicated because quantitative differences were smaller so meta-analysis was used (Inglefinger et al., 1994). Parasite load was treated as a quantitative variable with possible values of 1, 2, 3, and ≥4. Mϕ internalisation and parasite load were both analysed separately for mouse strains BALB/c and C3H/HeN. An additional mixed model analysis of variance was run to test differences between mouse strains. SAS Version 6.12 was used to run these analyses (SAS Institute, 1992).

3. Results

3.1. Sand fly saliva is chemotactic for murine Mϕ

Sand fly saliva enhanced positive chemotaxis of BALB/c peritoneal Mϕ. A mean of 225 Mϕ passed through the microporous membrane when saliva was present, while only 174 Mϕ passed in control wells with medium alone (Fig. 1). Differences were significant using the two-tailed paired *t*-test ($P = 0.0062$). Consistently, a much higher number of Mϕ (mean of 560) passed through the membranes when 1% Zymosan-activated mouse serum was added to the medium as a positive control (data not

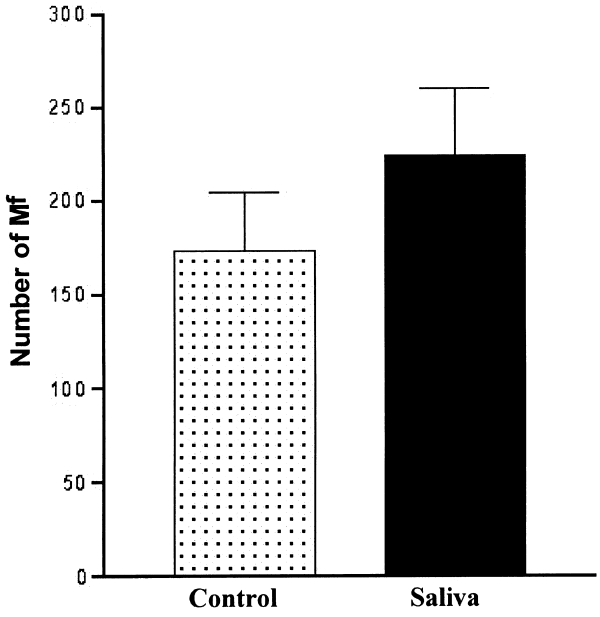

Fig. 1. Chemotaxis of murine peritoneal macrophages. Peritoneal exudate cells from BALB/c mice were seeded in Costar Transwell System™ inserts with a microporous (8 μm pore diameter) polycarbonate bottom. Inserts were placed inside wells of a 24-well plate containing 250 μl of culture medium with or without sand fly saliva (one gland/well). Macrophages adhering to the bottom of the membrane having migrated through it were counted microscopically. Bars represent the standard deviation from the mean.

shown). Chemotaxis assays were performed using salivary gland extracts from *L. longipalpis* (eight times) and *P. papatasi* (five times) with similar results.

3.2. Phlebotomus papatasi saliva enhances uptake and proliferation of Leishmania parasites in murine Mϕ

A higher percentage of Mϕ phagocytosed *Leishmania* parasites when saliva was present. The frequency of infection, in both BALB/c and C3H/HeN, was increased by the presence of saliva ($P < 0.05$). C3H/HeN Mϕ were significantly more susceptible to infection than Mϕ from BALB/c ($P < 0.0001$) (Table 1).

The effect of saliva on infection intensity was analysed using a mixed model analysis of variance. A significantly higher number of amastigotes were found in infected Mϕ cultured in the presence of saliva than in Mϕ cultured in medium alone. This was true of Mϕ extracted from BALB/c and C3H/HeN mice (Table 1, Figs. 2 and 3).

4. Discussion

The chemotactic effect of sand fly saliva on murine Mϕ has been documented previously. *Phlebotomus duboscqi* saliva was found to be 1.7 times more chemotactic than control medium in assays performed under agarose for 18 h (Anjili et al., 1995). We tested distinct saliva types using microporous membranes and limited the length of assays to 3 h. In our experiments, saliva enhanced chemotaxis by 30% over control medium. These studies show that the propensity of saliva to induce positive Mϕ chemotaxis is common to several phlebotomine species.

Although the chemotactic effects of sand fly saliva in vitro were not dramatic, in vivo the outcome may be of greater consequence for a number of reasons. First, in the confined space of the feeding pool, effective concentrations of saliva are much higher than in our experiments. Under these circumstances, chemotaxis is probably affected over extremely short distances, attracting Mϕ from areas adjacent to the bite site. Second, in vivo saliva may elicit the secretion of additional chemotactic factors that attract Mϕ. For example, sand fly saliva triggered expression of the IL-4 gene in cultured murine peritoneal exudate cells (probably mast cells; Waitumbi and Warburg, unpublished data). IL-4 was shown to be chemotactic for Mϕ (Heister et al., 1992). Such an indirect mechanism may continue to attract Mϕ even after the saliva itself is no longer active. Availability of Mϕ at the site of inoculation is a crucial factor for the preliminary establishment of *Leishmania* infections in the skin. Hence, the chemotactic effect of saliva on Mϕ could be one way by which saliva exacerbates cutaneous leishmaniasis.

Leishmania promastigotes that fail to invade Mϕ are quickly eliminated by the cytotoxic activity of natural killer cells, neutrophils and eosinophils in the vertebrate host (Pimenta et al., 1987). Therefore, it is crucial for *Leishmania* promastigotes to enter Mϕ as quickly and as efficiently as possible. In our assays sand fly saliva caused a marked increase in the numbers of promastigotes entering cultured Mϕ (Table 1). The direct outcome of such an effect in vivo

Table 1
Infection prevalence and intensity for BALB/c (experiment repeated seven times) and C3H/HeN (experiment repeated five times) macrophages with *Leishmania* amastigotes[a]

	BALB/c		C3H/HeN	
	+ saliva	Control	+ saliva	Control
Percentage of infected macrophages ±standard error	40.5 ± 0.44%	37.9 ± 0.42%	61.2 ± 0.69%	53.1 ± 0.71%
Mean number of amastigotes/macrophages	0.99	0.88	1.85	1.54
Total number of macrophages counted	12432	13191	4937	4988

[a] Macrophages were cultured either with or without sand fly salivary gland lysate (one gland/ml).

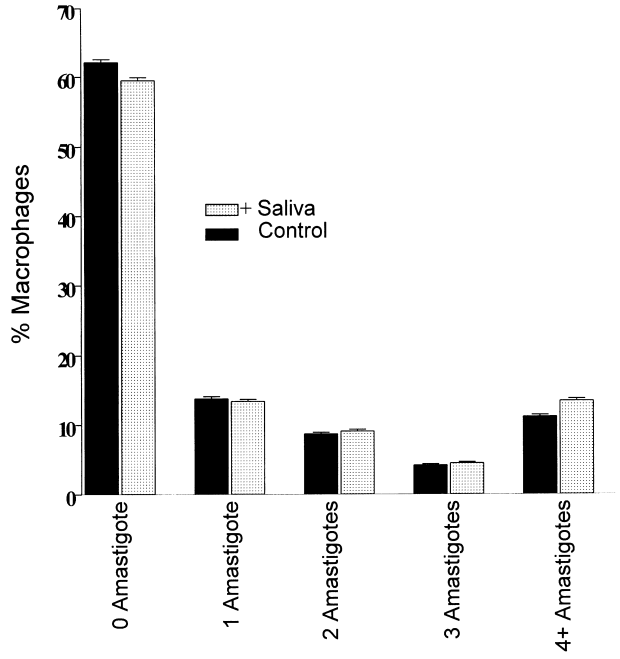

Fig. 2. Effect of sand fly saliva on multiplication of amastigotes (parasite load) in cultured murine (BALB/c) macrophages. Peritoneal exudate cells were seeded into multichamber slides, non-adhering cells were removed and medium containing stationary-phase promastigotes with or without saliva was added to the chambers. Following incubation for 24 h, the plastic chamber frames were removed and parasite burdens were scored microscopically. Macrophages were separated into five categories: uninfected, and infected with one amastigote, two amastigotes, three amastigotes and four or more (4+) amastigotes. See Section 2 for details. Bars represent standard errors from the mean.

would be to reduce the exposure of promastigotes to cytotoxic action and improve their prospects for survival.

In a previous study, Hall and Titus (1995) published a figure that indicates enhancement of parasite uptake in the presence of saliva. However, the differences were not statistically significant, perhaps because *Leishmania major* was used at a 10:1 parasite/Mϕ ratio and the overall rate of phagocytosis was very high. In our study exceptionally high numbers of Mϕ were counted in a large number of separate experiments and the results were significantly different.

On average, both BALB/c and C3H/HeN Mϕ cultured in the presence of sand fly saliva were infected with more amastigotes than those cultured in medium alone. We interpret this observation to indicate that saliva accelerated the rate of amastigote division during the 24 h of the experiment. Data on the generation time of *Leishmania* amastigotes in cultured Mϕ are very limited. However, it has been previously demonstrated that *Leishmania donovani donovani* amastigotes cultured in human monocyte-derived Mϕ divide rapidly enough to present an average increase in amastigote load after only 24 h in culture (Pearson et al., 1981). Our results suggest that the generation time for *Leishmania infantum* amastigotes in murine peritoneal Mϕ is less than 24 h and somewhat accelerated in the presence of sand fly saliva.

To rule out the possibility that disparity in infection loads was a result of saliva causing a clumped distribution of promastigotes, we compared stained microscope preparations of infective inocula with and without saliva. Agglutination of promastigotes was not observed in either preparation. We also counted a sample of 325 Mϕ (with and without saliva) that were infected with four parasites or more. Seventy-one percent of these had four or five amastigotes and only 9% had eight to 10 amastigotes. There was no difference between Mϕ incubated with saliva and Mϕ incubated without it (data not shown). This supports our assumption that promastigotes were uniformly distributed in the culture medium and contact with Mϕ was random. Therefore, the most logical explanation for the observed results is that sand fly saliva enhanced the division of parasites within Mϕ.

The pattern of infection with or without saliva was similar for Mϕ derived from either BALB/c or C3H/HeN mice. However, BALB/c Mϕ were less suitable for invasion and replication of *L. infantum* parasites than peritoneal Mϕ from C3H/HeN mice. This is a somewhat surprising finding because BALB/c mice are highly susceptible to *L. major* infections while C3H/HeN mice are resistant and self-healing. Moreover, in experiments conducted in vitro it was shown that Mϕ derived from resistant mice are more adept at killing intracellular amastigotes than those derived from non-healer strains (Buchmuller-Rouiller and Mauel,

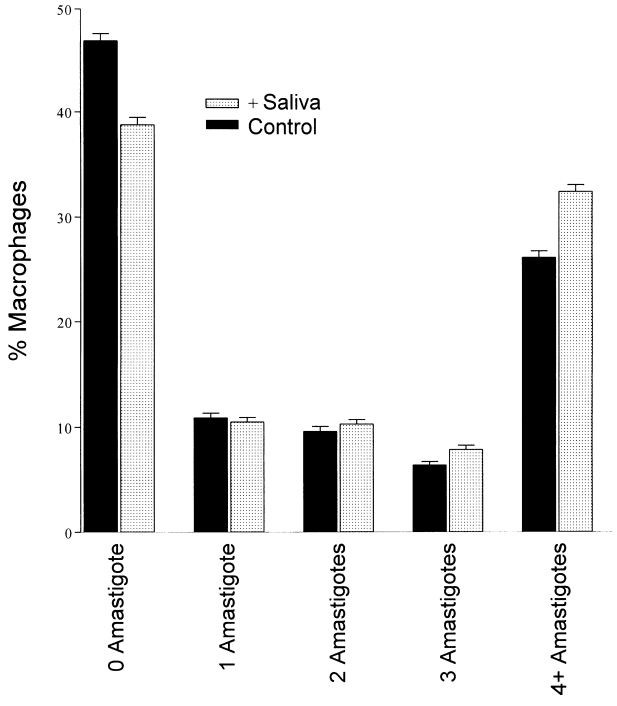

Fig. 3. Effect of sand fly saliva on multiplication of amastigotes in cultured murine peritoneal (C3H/HeN) macrophages. Methodology and legend as for Fig. 2.

1986). On the other hand it was also observed that leishmaniacidal activity is largely dependent on the parasite strain and on the level of activation of the cultured Mϕ (Scott and Sher, 1986). The significance of the present observation awaits elucidation.

Acknowledgements

This research was supported by grant number 235/99-2 from The Israel Science Foundation founded by the Israeli Academy of Sciences and Humanities, and the Deutsche Forschungsgemeinschaft (DFG) project on Leishmaniosis in Israel and the West Bank.

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