



ELSEVIER

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Veterinary Parasitology 114 (2003) 97–111

veterinary
parasitology

www.elsevier.com/locate/vetpar

A follow-up of Beagle dogs intradermally infected with *Leishmania chagasi* in the presence or absence of sand fly saliva

Moacir Paranhos-Silva^a, Geraldo G.S. Oliveira^a, Eliana A. Reis^a,
Rejane M.C. de Menezes^a, Octávio Fernandes^b, Ítalo Sherlock^a,
Regis B.B. Gomes^a, Lain C. Pontes-de-Carvalho^{a,c},
Washington L.C. dos-Santos^{a,c,*}

^a Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão 121,
40295-001 Salvador, BA, Brazil

^b Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz,
Fundação Oswaldo Cruz, Av. Brasil 4365, Manguinhos, 21.045-900 Rio de Janeiro, RJ, Brazil

^c Escola Bahiana de Medicina e Saúde Pública, Av. Dom João VI No. 274,
Brotas, 40290-000 Salvador, BA, Brazil

Received 1 May 2002; received in revised form 12 February 2003; accepted 28 March 2003

Abstract

In this study, we compare the development of infection and/or disease in Beagle dogs intradermally infected with *Leishmania chagasi*, in the presence or absence of *Lutzomyia longipalpis* saliva, with those of intravenously infected animals.

Spleen samples of all the animals inoculated with parasites had positive polymerase chain reaction tests for *Leishmania* DNA. Positive spleen cultures for *Leishmania* were detected earlier ($P \leq 0.018$) and were more frequent (five out of the five animals) in intravenously infected animals than in the intradermally infected animals, in presence (two out of the six animals) or absence (three out of the five animals) of salivary gland lysate of *L. longipalpis*. Significant increase in serum antibodies against *Leishmania* was observed only in the intravenously infected group ($P = 0.004$). In addition, dogs with infection confirmed by isolation of amastigotes or detection of parasite DNA were, nevertheless, negative for anti-*Leishmania* antibodies up to 5 months or more after infection. Only animals of the intravenously infected group developed progressive decreases in hematocrit (Pearson $r = -0.8076$, $P = 0.0026$) and hemoglobin (Pearson $r = -0.8403$, $P = 0.0012$) during the infection period. No significant difference in the course of infection was observed between groups of intradermally infected animals. The data presented herein confirms that the intradermal

* Corresponding author. Present address: Centro de Pesquisas Gonçalo Moniz-Fiocruz, Fundação Oswaldo Cruz, Rua Waldemar Falcão 121, 40295-001 Salvador, BA, Brazil. Tel.: +55-71-356-8781x262; fax: +55-71-356-4292. E-mail address: wluas@cpunet.com.br (W.L.C. dos-Santos).

inoculation of dogs with *Leishmania* produces an asymptomatic form of infection. It also fails to show an advantage in using *L. longipalpis* saliva as an infection-enhancing agent in experimental canine leishmaniasis.

© 2003 Elsevier Science B.V. All rights reserved.

Keywords: *Leishmania chagasi*; Leishmaniasis; Dog; Experimental; Saliva

1. Introduction

Zoonotic visceral leishmaniasis is a disease caused by *Leishmania infantum/Leishmania chagasi* that affects dogs and human beings in the New World and in several European countries (Lainson and Shaw, 1978; Enserink, 2000). In fact, the dog is considered to be the main domestic reservoir of the disease (Deane and Deane, 1955; Dye, 1996).

A vaccine against canine visceral leishmaniasis (CVL) would be of great relevance both for epidemiological control of human disease and in veterinary medicine. The generation of such vaccine would be largely facilitated by the existence of adequate experimental models, so that initial trials of vaccine candidates could be carried out under safe and controlled laboratory conditions.

The current models of experimental canine leishmaniasis use large number of culture-derived promastigotes injected by the intravenous or the intraperitoneal routes. More than 80% of these parasites are promptly lysed by complement (Soares et al., 1993), releasing relatively large amount of antigen. There are also differences between the immune responses raised against antigens injected in the skin and blood stream (Cremer et al., 1983). Other authors (Abranches et al., 1991; Oliveira et al., 1993) used amastigotes, which are antigenically distinct from promastigotes, the naturally infective form of the parasite for mammals (Zhang and Matlashewski, 1997). Therefore, although these experiments reproduce some features of CVL, their differences with natural infection may hinder the interpretation of results obtained in experiments designed to assess the efficacy of vaccine candidates.

In nature, dogs are infected with promastigotes injected in the skin by sand flies. After infection some dogs remain asymptomatic, but many develop a progressive disease with lymphadenopathies, dermatitis, weigh loss, anemia and locomotion problems. The ideal model of canine leishmaniasis to be used in vaccine and immunotherapy studies would reproduce the immunological aspects of natural infection and disease, including the characteristics of an immune response generated to antigens injected in the dermis. It would, therefore, use the skin as the route of infection and low number of metacyclic promastigotes (Warburg and Schlein, 1986). In order to be cost-effective, the model should induce disease in a short observation period (2–4 months would be a reasonably short pre-patent period for laboratory work with dogs, a period also observed in natural infection; Moreno and Alvar, 2002). The model should also induce disease in all the animals, allowing for small groups to be used, fulfilling the ethical and economical requirements of reducing the number of animals used in experiments. Experiments performed using skin as a route of parasite inoculation in dogs, however, lead to the appearance of signs of infection only after a long period of follow-up, and in most of the animals disease is never evident (Paranhos et al., 1993; Killick-Kendrick et al., 1994).

Titus and Ribeiro (1988) proposed that *Lutzomyia longipalpis* saliva enhances tegumentar infection with *Leishmania major* in mice. Since then, this observation has been confirmed by using different strains of mice and species of *Leishmania* that cause cutaneous lesions (Theodos et al., 1991; Lima and Titus, 1996) and *L. chagasi*-infected hamsters (Warburg et al., 1994). In a previous study, we used lysate of salivary glands from *L. longipalpis* in an attempt to enhance *L. chagasi* infection in mongrel dogs (Paranhos et al., 1993). No clinical, parasitological or serological signs of infection were observed in any of the nine dogs inoculated intradermally with *L. chagasi*, with the exception of a dog that received *Leishmania* in saline. Killick-Kendrick et al. (1994) infected Beagle dogs with *L. infantum* associated with *Phlebotomus perniciosus* salivary gland lysate (SGL). Their study, however, did not investigate a possible enhancing effect of sand fly saliva, since the same animals were subjected to injections of parasites with and without SGLs. The authors were unable to observe macroscopic or histological differences in sites of dermal infection with *L. infantum* in the presence or absence of SGL. Only 12 out of the 25 parasite-inoculated animals developed clinical signs of disease.

The aim of this work was to improve the experimental model of CVL to be used in future tests of vaccine candidates. In order to do that, the possible effect of *L. longipalpis* SGL on the enhancement of intra-cutaneous experimental *L. chagasi* infection in Beagle dogs was investigated. We chose to work on Beagle dogs because of a possible high resistance of mongrel dogs to *Leishmania* infection (Oliveira et al., 1993), which were used in our previous study with *L. longipalpis* salivary glands (Paranhos et al., 1993). Our hypothesis was that sand fly SGL would enhance the infection of dogs with *L. chagasi*, leading to an early viceralization and to the progression of infection to symptomatic disease. The course of intradermal *L. chagasi* infection in the presence or absence of *L. longipalpis* saliva was therefore followed up in Beagle dogs. As a reference we infected a group of animals according to a classical model, injecting large number of parasite by the intravenous route. In our (Paranhos et al., 1993) and in other groups' experience (Nieto et al., 1999) this intravenous inoculation of *Leishmania* leads to parasite dissemination to internal organs and positive serological tests for anti-*Leishmania* antibodies.

2. Material and methods

2.1. Parasite and antigens

L. chagasi promastigotes (8J96 strain) were isolated from the spleen of a dog from the endemic area of Jequié (Bahia State, Brazil) and characterized by reactivity with species-specific monoclonal antibodies (G. Grimaldi, Fundação Oswaldo Cruz, Rio de Janeiro). The parasites were grown in vitro using a modified liver infusion tryptose (LIT) medium containing 25 mg/l of hemin, 20% RPMI 1640 (Sadsgursky and Brodskyn, 1986) and 10% of fetal calf serum. Parasites were washed three times in 0.15 M phosphate buffered saline (PBS), pH 7.2, resuspended in PBS containing 1% bovine serum albumin (BSA) (PBS-BSA) and used for infection. The percentage of complement-resistant promastigotes present in this suspension was 26%, as determined according to the technique described by Soares et al. (1993). The *Leishmania* lysates used for ELISA were obtained from *L. chagasi*

(MHOM/BR2000/Merivaldo2 strain) promastigotes, by ultrasound treatment at 4 °C, and stored at –20 °C until used.

2.2. *Lutzomyia longipalpis* salivary glands

Sand flies (from Jacobina, Bahia, Brazil) were reared at the Gonçalo Moniz Research Center, as described previously (Sherlock and Sherlock, 1959). Salivary glands were isolated from non-blood-fed, 3-day old female sand flies. Each five pairs of salivary glands were placed in 10 µl of ice-cold distilled water containing 0.1% BSA. Glands were pooled, homogenized and made isotonic by addition of 10-fold concentrated PBS. As expected, the lysate produced cutaneous hyperemia when injected intradermally in rabbits, even in amounts corresponding to 1/20 of an acinus.

2.3. Dogs and experimental design

Four groups of 20 to 34-month old Beagle dogs (see Table 1) were subjected to the following infection protocols:

- Group A: Six animals inoculated with promastigotes of *L. chagasi* in stationary phase of culture growth, resuspended in PBS containing 1% BSA (PBS-BSA) and *L. longipalpis* SGL. Twenty-five microliters of parasite suspension, containing 4×10^4 promastigotes and an amount of SGL corresponding to 1/2 acinus, were injected intradermally in the internal face of the ear of each animal, using a 29G needle.
- Group B: Five animals injected with 4×10^4 stationary-phase *L. chagasi* promastigotes in 25 µl of PBS-BSA, without SGL, under conditions similar to those described for group A.
- Group C: Five dogs injected with 25 µl of PBS-BSA containing *L. longipalpis* SGL corresponding to 1/2 acinus, under conditions similar to those described for group A.
- Group D: Five animals injected intravenously with 10^8 stationary-phase *L. chagasi*, resuspended in 2 ml of PBS, using a 21G1/4 needle.

The parasites used for infection of the animals of the different groups were from the same growth batch. The number of complement-resistant metacyclic promastigotes injected intradermally and intravenously per dog were, therefore, 1.04×10^4 and 2.6×10^7 , respectively, as determined by the complement-lysis assay cited above. Viability of the parasites in each preparation was confirmed, by observation of motility of promastigotes under the microscope, in aliquots collected before and after injection in the animals.

Prior to inclusion in this study, the animals were vaccinated against rabies and coronavirus infection and treated with anti-helminthics. The animals were also examined according to clinical and laboratorial protocols described below. They were tested for *Leishmania* infection through serology for anti-*Leishmania* antibodies (ELISA) and culture of spleen aspirate. Animals to be injected with sand fly saliva were also tested for previous sensitization with *L. longipalpis* saliva, through ELISA (see Fig. 2). All tests produced negative results. Since mixed groups of male and female animals were used, male animals were

Table 1
Clinical and laboratory characteristics of the groups of beagle dogs experimentally infected with *L. chagasi*

Groups	Infection age (months)	Sex	Positive spleen culture ^a	PCR (spleen) ^a				Body weight (%) ^b	Lymphoproliferative response to ^c Con-A
				9	10	11	13		
<i>L. chagasi</i> , 10 ⁴ + salivary gland lysate, intradermal									
A1	24	M	–	–	–	+	+	7.3	20.4
A2	26	M	9–10, 12	+	+	–	+	20.2	1.7
A3	33	F	–	–	–	–	+	15.4	29.4
A4	33	F	–	+	+	+	+	18.5	27.6
A5	33	F	10	+	+	ND	ND	ND	ND
A6	22	F	–	ND	ND	ND	ND	ND	ND
Mean and ratios	29 ± 5	M2:4F	2/6					15.4 ± 5.7	
<i>L. chagasi</i> , 10 ⁴ intradermal									
B1	32	F	9	+	+	+	+	12.1	5.4
B2	34	F	–	–	–	+	+	24.8	18.8
B3	33	M	10–13	+	+	+	+	7.1	1.4
B4	33	F	9, 12	+	+	+	+	14.7	5.8
B5	33	M	–	ND	ND	ND	ND	ND	ND
Mean and ratios	33 ± 1	M2:3F	3/5					14.7 ± 7.4	
Negative control									
C1	24	F	–	–	–	–	–	–15.7	ND
C2	33	F	–	–	–	–	–	13	5.5
C3	33	M	–	–	–	–	–	16.4	5.0
C4	22	M	–	–	–	–	–	19.2	0.3
C5	22	M	–	–	–	–	–	31.1	65.0
Mean and ratios	27 ± 6	M3:2F	0/5					12.8 ± 17.3	
<i>L. chagasi</i> , 10 ⁸ intravenous									
D1	20	M	2–13	+	+	+	+	4.9	44.8
D2	33	F	2–13	+	+	+	+	–45.5	ND
D3	33	M	2–7, 9–13	+	+	+	+	19.9	6.1
D4	33	F	2–4, 6–7, 9–13	+	+	+	+	–5.5	14.5
D5	24	F	1–4, 6–8	ND	ND	ND	ND	ND	ND
Mean and ratios	29 ± 6	M2:3F	5/5					–6.55 ± 24.2	

^a Month after infection.

^b Expressed as relative (percentual) body weight gain relative to that observed at the beginning of the experiment.

^c Stimulation index. Polymerase chain reactions are expressed as negative (–) or positive (+) for *Leishmania*. ND: not determined.

subjected to vasectomy in order to prevent fertilization of female animals during the experiments. All animals were kept in the kennel of the Gonçalo Moniz Research Center in downtown Salvador, a non-endemic area of leishmaniasis. Periodical surveys for the presence of vectors of *Leishmania* were performed with the use of light traps, with negative

results. All the animals in all groups were identically followed up according to clinical, parasitological and immunological parameters, as described below. All experiments were conducted in accordance with the Oswaldo Cruz Foundation guidelines for experimentation with animals.

2.4. *Clinical follow-up of animals*

Clinical examinations of the animals were carried out fortnightly by a veterinarian, in accordance with a previously established protocol. This included measure of body weight and rectal temperature, as well as inspection of skin and eyes. Five milliliters of peripheral blood were collected monthly for hematological tests (hematocrit and hemoglobin quantification, erythrocyte and leukocyte counts). The animals were followed up, during all the course of the experiment, by a veterinarian (RMCM), which provided all necessary medical support for the animals. In the pre-experiment phase of observation we noticed that some animals developed a slight anemia. Therefore, supplements of vitamins and iron were provided for short periods for these animals. One of the animals had cystitis, two others developed gingivitis and one developed a bacterial abscess in the pad. These animals were treated with penicillin.

2.5. *Parasitological follow-up of the animals*

Spleen samples were collected each month by aspiration under anesthesia and cultured in biphasic (agar–blood–Schneider) medium. Cultures were examined weekly for at least 2 months. From 9 to 13 months after infection, spleen aspirates were also processed by PCR for identification of *L. chagasi* DNA (Lopes and Wirth, 1986; Fernandes et al., 1999). Briefly, DNA from spleen aspirates was prepared by incubation at 65 °C for 3 hours in 100 µl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 100 µg ml⁻¹ of proteinase K. The protease was then inactivated at 95 °C for 15 min. The mixture was centrifuged at 12,000 × g, at 4 °C, for 15 min, and the supernatant containing DNA stored at –20 °C until use.

The target DNA for amplification was a 120 bp fragment in the constant region of the kDNA minicircle. The primers used were: 5'-GGG GAG GGG CGT TCT GCG AA (forward) and a mixture of 5'-GGC CCA CTA TAT TAC ACC AAC CCC and 5'-CCG CCC CTA TTT TAC ACC AAC CCC (reverse). The reaction conditions were optimized, using mimic spleen samples, in order to obtain a sensitivity of less than or equal to one parasite per reaction tube. Such optimized conditions were the following: 0.5 pmol of each forward or reverse primer mixture, deoxynucleoside triphosphate at a concentration of 0.2 mM each, 2 mM of MgCl₂, 1.25 U of *Taq* DNA polymerase (Perkin-Elmer) and pirogen and RNase-free H₂O (Biotech) in a total volume of 50 µl, including 1–4 µl of sample DNA. The reactions were cycled in a Perkin-Elmer (Cetus, CT-2400) thermal cycler by using the following conditions: 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 10 min. One positive control (culture promastigote kDNA) and two negative controls (H₂O and spleen samples from normal dogs) were included in each reaction.

The product of the reactions was visualized under UV light after electrophoresis of the reaction solution in an agarose gel. After denaturation in 0.4 N NaOH, for 1 h at room

temperature, samples of 200 μ l were spotted onto a positively charged nylon membrane (Hybond-N, Amersham), using a dot blot apparatus (Hybri dot, Gibco), and fixed by UV exposure for 1 min.

The α^{32} P-labeled probe used for hybridization was IPAN V (GeneBank accession number U19811), corresponding to molecules cloned from the minicircle of *Leishmania (Viannia) panamensis*. Filters were hybridized at 65 °C, washed with saline-sodium-citrate solution containing 1% of sodium dodecyl sulfate and left overnight over an X-ray film at –70 °C.

2.6. Detection of anti-*Leishmania* and anti-*L. longipalpis* saliva antibodies in serum

These were measured in serum samples by ELISA (Paranhos-Silva et al., 1996; Barral et al., 2000). Briefly, 96-well plates were sensitized with crude sonicated antigen obtained from a mixture of logarithmic- and stationary-phase *L. chagasi* promastigotes or from *L. longipalpis* salivary glands. The plates were washed, blocked with PBS containing 5% of skimmed milk, and serum of each animal was applied in duplicate at the dilution of 1:400 (for detecting anti-*Leishmania* antibodies) or 1:100 (for detecting anti-salivary gland antibodies), followed by an anti-dog IgG-peroxidase conjugate (Sigma Chemical, St. Louis, USA). The enzymatic reaction was developed and measured as described elsewhere (Paranhos-Silva et al., 1996). All measurements were performed at least twice. In order to allow a more accurate comparison among groups and among blood samples collected at different periods, the tests were performed with the samples randomized in different plates. The results are expressed as mean \pm standard error of the mean (S.E.M.) of the OD_{450 nm} obtained by testing sera from different animals in each group.

2.7. Non-specific lymphoproliferative response to mitogen

Peripheral blood leukocytes were separated in a gradient of Ficol-Hypaque (Histopaque 1077-Sigma, USA). Viable mononuclear cells were adjusted to 4×10^6 ml⁻¹ in RPMI 1640 medium (Gibco), supplemented with 10% of normal canine serum, 50 μ g/ml of gentamycin, 10 mM of 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, Sigma) and 2 mM of L-glutamine. Cells (4×10^5 per well) were distributed in flat-bottom microtitre plates in a volume of 100 μ l, and an equal volume of medium alone or containing 10 μ g of concanavain A (Con-A) was added. Each treatment was done in triplicate. Cultures were incubated for 5 days at 37 °C, in a humidified atmosphere with 5% CO₂. The optimal concentration of Con-A and time of stimulation used in this study was established in preliminary experiments. Cell proliferation was quantified by measuring the incorporation of ³H thymidine (Amersham). Twelve hours before harvesting 1 μ Ci was added per well. Cells were harvested on fiber filters utilizing a cell harvester (both from Skatron). The radioactive uptake was determined in a liquid scintillation beta counter. The intensity of the proliferative response was expressed by the stimulation index, calculated by dividing the mean of counts obtained in Con-A containing wells, by the mean counts of control wells. Stimulation indices equal or higher than 3 were considered to be indicative of proliferation. This threshold for positive lymphoproliferative response was based on data from a previous experiment performed in our laboratory using 38 animals. In this study, the mean value of the proliferative response of cells in presence of medium alone plus three standard deviation of the

mean (encompassing 99.85% of the values) corresponded to a stimulation index lower than 3 (dos-Santos et al., unpublished).

2.8. Expression and statistical significance of results

The data shown in the graphs are numerical, with dots representing individual animals and lines representing the mean \pm S.E.M. of results obtained from each group. In order to correct for the natural heterogeneity in body weights in the studied groups, this variable is expressed as relative (percentual) body weight gain relative to that observed at the beginning of the experiment. Lymphoproliferative response is expressed as stimulation index: the ratio between the mean CPM obtained from cells incubated with the mitogen and from non-stimulated cells. The significance of the differences observed among the groups was tested using one-way analysis of variance (ANOVA). When the *F*-test was found significant, the difference between two groups was identified using the Student–Newman–Kuel's test. Trends were measured using Person's correlation coefficient (*r*), followed by comparison of slope and intercept of two lines when appropriate. For some cross-table comparisons, Fisher's exact probability test was used. The critical level of significance admitted was $\alpha = 0.05$ (Glantz, 1996).

3. Results

3.1. Group A

In this group, injected intradermally with *L. chagasi* associated with *L. longipalpis* SGL, two out of the six animals had parasites cultured from the spleen. Parasites were identified in culture only after 9 and 10 months of infection. PCR was positive for *Leishmania* DNA in all five examined animals. In A1, A2 and A3 animals, however, the PCR varied from negative to positive in different samples. No change was observed in the intensities of anti-*Leishmania* antibody activities in the sera (Fig. 1). Significant lymphoproliferative response upon stimulation with Con-A was observed in three out of the four examined animals: SI of 20.4, 29.4 and 27.6. In one of the animals the SI was 1.7. The other clinical–laboratorial features studied did not change significantly in this group during the experiment. Two animals, A5 and A6, died during the experiment: the A6 animal died subtly on the eighth month of infection after topic treatment with Triatox (amitraz), for *Sarcoptes scabiei* infestation. The A5 animal died 11 months after infection with an asthenia that developed suddenly. White blood cell count revealed 14,200 leukocytes, with 64.7% neutrophils. Of these two animals, only the A5 animal had *Leishmania* amastigotes detected in the spleen (see Table 1).

3.2. Group B

Three out of the five animals in this group, injected intradermally with *L. chagasi* alone, had spleen cultures positive for *Leishmania*. Parasites were isolated in the cultures after 9 (two animals) and 10 (one animal) months of infection. PCR was positive for *Leishmania* DNA in spleen samples from all four examined animals. In the B2 animal, however, the PCR

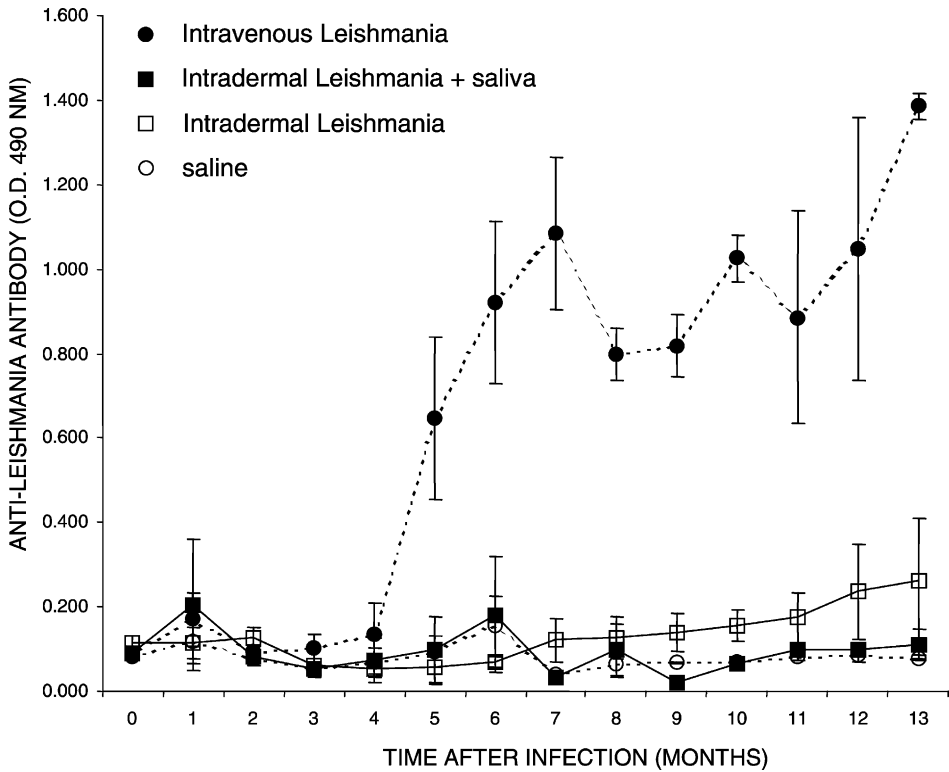


Fig. 1. Serum anti-*Leishmania* antibodies in dogs infected with *L. chagasi*. Curves correspond to the mean values of results obtained from the serum of dogs infected intravenously (intravenous *Leishmania*), infected intradermally in the presence (intradermal *Leishmania* + saliva) or in the absence (intradermal *Leishmania*) of *L. longipalpis* SGL, or uninfected (saline). Vertical bars represent the standard error of means.

varied from negative to positive in different samples. Circulating anti-*Leishmania* antibody levels remained low during all the experiments, and only a small increase in these levels was observed after 11 months of infection (Fig. 1). Positive lymphoproliferative response to Con-A was observed in three out of the four examined animals: SI of 5.4, 18.8, and 5.8. In one of the animals the SI was 1.4. The other clinical-laboratorial features did not significantly change in the group during the experiment. One animal, B5, was sacrificed in the 10th month of infection after developing gingivitis with abscess and loss of one tooth. All the spleen cultures performed during the experiment were negative for *Leishmania* in this animal.

3.3. Group C

Culture, PCR and ELISA for detection of anti-*Leishmania* antibodies in the serum were consistently negative in this group of uninfected animals, during all the experiments. The lymphoproliferative response to Con-A was significantly higher than in cells treated with medium alone in three out of the four examined animals: SI of 5.5, 5.0, and 65.8. In one of

the animals the SI was 0.3. The other investigations of the clinical–laboratorial parameters did not show significant changes during the experiment.

3.4. Group D

All the animals in this group, inoculated with *L. chagasi* by the intravenous route, had positive spleen culture. Parasites were detected in the spleen in the first month after parasite inoculation in one animal and from the second month in all others. The spleen cultures remained positive throughout the 13 months of observation (Table 1). All intravenously infected animals examined between the 9th and 13th month of infection had positive PCR for *Leishmania* DNA in all investigated time points. All the animals infected by the intravenous route had a significant increase in the levels of anti-*Leishmania* antibodies in the serum 5 months after infection (Fig. 1). The levels of anti-*Leishmania* antibodies reached a plateau on the seventh month after infection, and remained high until the end of the experiment. Positive lymphoproliferative response to Con-A was observed in all three examined animals: SI of 44.8, 6.1 and 14.5. In the animals of this group, the levels of hematocrit and hemoglobin decreased during the experiment. The average levels of hematocrit varied from 41.1 ± 4.6 at the beginning to 29.4 ± 5.9 at the end of the experiment (Pearson $r = -0.8076$, $P = 0.0026$). The average levels of hemoglobin varied from 14.3 ± 1.7 in the beginning to 10.4 ± 2.3 at the end of the experiment (Pearson $r = -0.8403$, $P = 0.0012$). The investigation of the other clinical–laboratorial parameters did not show significant changes during the experiment. One animal (D5) died suddenly in the ninth month of infection. Although the immediate event that caused the death of this animal was not identified, this animal had a papular dermatitis, anemia and low leukocyte count (3700) on the day before death, and also positive spleen culture for *Leishmania*.

3.5. Comparison among groups

The difference in the rate of positive spleen culture between intravenously and intradermally infected groups was statistically significant from the second to the eighth month of infection ($P = 0.0001$ – 0.018 , Fisher's exact probability test) and was no longer significant after the ninth month of infection ($P = 0.070$, Fisher's exact probability test). There was no difference in the incidences of positive spleen culture for *Leishmania* between the groups of intradermally infected animals (groups A and B, $P = 0.564$, Fisher's test). There was no difference in incidences of positive PCRs among the groups infected with *Leishmania* ($P = 0.497$, Fisher's exact probability test). In comparison with the intradermally infected animals (groups A and B), the animals infected by the intravenous route had significant increase in serum levels of anti-*Leishmania* antibodies ($P = 0.004$, ANOVA followed by Student–Newman–Kleus' test). No statistically significant difference in serum anti-*Leishmania* antibody was observed between the groups of animals intradermally infected with *L. chagasi* with (group A) or without (group B) sand fly saliva; or between the intradermally infected (groups A and B) and control non-infected group. Antibodies against *L. longipalpis* salivary gland were not detected in any of the animals tested before or after infection (Fig. 2). Among the other clinical–laboratorial parameters, only hematocrit and hemoglobin were significantly decreased in the intravenously infected group. Compared

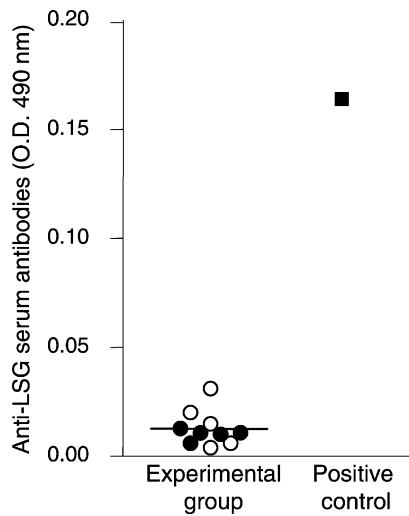


Fig. 2. Anti-*L. longipalpis* salivary gland serum antibodies in dogs before injection with *L. chagasi* in presence (●) or absence (○) of *L. longipalpis* SGL. None of the animals had significant levels of antibody in ELISA. The closed square represents the result obtained with a serum from a dog living in an endemic area of leishmaniasis.

with the control group such decrease in levels of hematocrit and hemoglobin was statistically significant (t test for comparison of the slope of two lines: for hematocrit, $P \leq 0.0001$; for hemoglobin $P \leq 0.0001$). Body weight loss appeared to be higher in the intravenously infected animals, although such difference was not statistically significant ($P = 0.259$).

4. Discussion

As shown in this paper, both intravenous and intradermal injections of *L. chagasi* promastigotes are effective in terms of infecting Beagle dogs. Infection by the intradermal route, however, develops slowly and the animals remain asymptomatic for a long period. Indeed, some clinical and laboratorial parameters tended to progress less favorably in intravenously than in intradermally infected animals. Even with complete medical support provided, excluding the use of anti-*Leishmania* drugs, the animals in the former group developed anemia and persistently high serum levels of anti-*Leishmania* antibodies and spleen parasitism. Infection signs were less prominent in intradermally infected animals, since parasites were detected only sporadically in their spleen, both by parasite cultivation and PCR.

PCR and spleen cultures were always positive in intravenously infected animals, and were observed sporadically in intradermally infected animals. Mathis and Deplazes (1995) found similar sensitivities for both lymph node cultures and PCR. In our experiment, however, PCR was capable of detecting *Leishmania* DNA even in samples that were negative for parasites in culture. Even so, some intradermally infected animals had negative PCR results in some tests. One of the possible reasons for the variable detection of *Leishmania* in spleen aspirates of the intradermally infected animals, both in PCR and culture, could be a non-continuous

(multifocal) distribution of parasites in the tissue, usually present in animals with low parasite burden. Although a control of DNA integrity, through the amplification of constitutive canine genes, was not performed in the small samples obtained by spleen puncture, the fact that the material from all the animal groups were handled strictly under the same conditions makes unlikely that the inter-group differences in PCR result from poor DNA preservation.

The differences in infection efficiencies between intravenous and intradermal routes, as observed in this work, may not be completely ascribed to the larger amount of parasites injected intravenously, since infection and disease have been induced with similar efficiency by intravenous promastigote doses varying from 10^5 to 10^9 (Abranches et al., 1991; Paranhos et al., 1993; Nieto et al., 1999). On the other hand, the intradermal injections of 10^8 or more promastigotes only produced mild signs of disease in one out of the five infected animals in a recent study (Santos-Gomes et al., 2001). Tolerance induced by intravenous release of antigen (Cremer et al., 1983), provided it happened in the intravenous model of CVL, could theoretically increase the infectivity of *Leishmania* injected by this route. Tolerance induced against *Leishmania* antigens would, however, limit the use of the intravenous model of infection, particularly in those experiments designed to assess the efficacy of vaccine candidates. It is possible that under such tolerogenic conditions, the efficacy of some vaccine candidates could be underestimated. In intradermal models of infection, both killed and live parasites are promptly phagocyted by macrophages and Langerhans cells, and a presumed tolerogenic effect would be less likely to occur (Schwartz, 1998).

This work confirms previous observations from ours and other groups (Paranhos et al., 1993; Killick-Kendrick et al., 1994; Santos-Gomes et al., 2001) that evidence of infection takes a long period to develop in animals infected by dermal injection of *Leishmania*, and signs of disease may never be present in these animals. The clinical course of experimental CVL may be influenced by a number of factors, including strain virulence, parasite dose, source and developmental stage of promastigotes, dog breed (Solano-Gallego et al., 2000) and other less easily controllable events (reviewed by Moreno and Alvar (2002)). The unpredictable nature of the experimental infection may also reflect the spectrum of clinical responses seen in natural infection (Hommel et al., 1995). In fact, little is known about the dynamics of natural *Leishmania* infection in dogs. Low number of parasites are injected intradermally during one or, probably, repeated inoculations. Nutritional status and concomitant diseases may affect the course of infection in different animals. Under such variable conditions, a pre-patent period of about 110 days until the appearance of positive serology against *Leishmania* has been estimated (Quinnel et al., 1997). Some naturally infected animals may never seroconvert, even after 20 months of infection (Hommel et al., 1995), and only half serologically positive animals may develop clinical signs of disease (Hommel et al., 1995). Hence, the single exposure to the parasite, and the selection, in the experimental model of healthy dogs, which may be able to mount an effective cellular immune response, may additionally reduce the success of the intradermal models for CVL.

In this work we tried to improve the experimental model of CVL by associating salivary gland of *L. longipalpis* (the natural vector of *L. chagasi*) with a low number of infective parasites inoculated into the dermis. The addition of *L. longipalpis* SGL to the infective inoculum did not change the course of *L. chagasi* infection; it did not lead to early amastigotes detection in the spleen or to an increase in parasite burden in the organ. Therefore, it had no advantage in terms of producing an experimental model for CVL. In order to exclude the

possibility that a putative previous contact with sand flies might have immunized the animals against salivary gland components, and thus interfered with a possible infection-enhancing effect of saliva (Belkaid et al., 1998), animal sera were tested, before infection, for the presence of anti-*L. longipalpis* salivary gland antibodies. The lack of enhancing effect of the SGL cannot be explained by anti-saliva antibody activity, since the animals were negative for that activity before the cutaneous injection of SGL. In effect, our results are in agreement with the observations that *L. longipalpis* SGLs with high vasodilation activity did not enhance experimental *L. chagasi* infection in mongrel dogs (Paranhos et al., 1993) and may vary in their ability to enhance infection in murine models of tegumentar leishmaniasis (Castro-Sousa et al., 2001; Warburg et al., 1994).

The kinetics of appearance of anti-*Leishmania* antibody in the sera of intravenously or intradermally infected animals disclosed an important characteristic of the humoral immune response to *Leishmania* in dogs: in spite of the infection being confirmed in animals infected both by intravenous and intradermal route, only those with intravenous infection had relatively high titers of anti-*Leishmania* antibodies. Even in this group, increase in antibody levels only occurred after the fifth month of infection, at least 3 months after parasites had been detected in the spleen. These findings are consistent with observations in naturally infected animals; in serological surveys for canine leishmaniasis in endemic areas, about 20% of animals had parasites in the spleen and low or undetectable anti-*Leishmania* antibodies by ELISA (Ashford et al., 1995). Such population of infected dogs with negative serology may be constituted by animals which (1) are asymptomatic, with the infection under control and, therefore, with low parasite burden, as described herein for animals infected intradermally or (2) undergo disease progression, with high parasite burdens, but still with low antibody response. This last course of infection could suggest a deficiency of both Th1 and Th2 responses, whereas the more commonly observed situation, namely high intensity of tissue parasitism, disease progression and high levels of specific antibody, would occur in animals in which a Th2-biased immune response to *Leishmania* had evolved (Pinelli et al., 1994; Cabral et al., 1998).

Finally, suppression of lymphoproliferative response to non-specific mitogens has been reported in symptomatic and apparently healthy *L. infantum*-infected animals (De Luna et al., 1999; Moreno et al., 1999; Rhalem et al., 1999) even in the early stages of infection (De Luna et al., 1999). Such observations were not confirmed in our study. Peripheral mononuclear cells, even from those animals intravenously infected with *L. chagasi*, with relatively persistent parasite burden and positive ELISA, proliferated as well as cells from control uninfected dogs in the presence of Con-A. This data agrees with the previously reported observation (Pinelli et al., 1994, 1995) that non-specific immunosuppression does not necessarily occur in *L. chagasi*/*L. infantum*-infected dogs. The possibility that the development of non-specific immunosuppression in CVL could depend on yet unidentified parasite- and/or host-derived factors cannot, however, be excluded.

Acknowledgements

We are indebted to Dr. Mitermayer G. dos Reis (Oswaldo Cruz Foundation) for his assistance in the experiment of *Leishmania* DNA identification by PCR. This work was

supported by the Ministry of Science and Technology, Brazil (Brazilian National Research Council, CNPq, grant no. 521260/98-1 and Pronex). All experiments were conducted in accordance with the current Brazilian laws and with Oswaldo Cruz Foundation guidelines for biosafety and experimentation with animals.

References

- Abranches, P., Santos-Gomes, G., Rachamim, N., Campino, L., Schnur, L.F., Jaffe, C.L., 1991. An experimental model for canine visceral leishmaniasis. *Parasite Immunol.* 13, 537–550.
- Ashford, D.A., Bozza, M., Freire, M., Miranda, J.C., Sherlock, I., Eulalio, C., Lopes, U., Fernandes, O., Degraeve, W., Barker Jr., R.H., Badaró, R., David, J.R., 1995. Comparison of the polymerase chain reaction and serology for the detection of canine visceral leishmaniasis. *Am. J. Trop. Med. Hyg.* 53, 251–255.
- Barral, A., Honda, E., Caldas, A., Costa, J., Vinhas, V., Rowton, E.D., Valenzuela, J.G., Charlab, R., Barral-Netto, M., Ribeiro, J.M.C., 2000. Human immune response to sand fly salivary gland antigens: a useful epidemiological marker? *Am. J. Trop. Med. Hyg.* 62, 740–745.
- Belkaid, Y., Kamhawi, S., Modi, G., Valenzuela, J., Noben-Trauth, N., Rowton, E., Ribeiro, J., Sacks, D., 1998. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. *J. Exp. Méd.* 188, 1941–1953.
- Cabral, M., O'Grady, J.E., Gomes, S., Sousa, J.C., Thompson, H., Alexander, J., 1998. The immunology of canine leishmaniasis: strong evidence for a developing disease spectrum from asymptomatic dogs. *Vet. Parasitol.* 76, 173–180.
- Castro-Sousa, F., Paranhos-Silva, M., Sherlock, I., Paixão, M.S., Pontes-de-Carvalho, L.C., dos-Santos, W.L.C., 2001. Dissociation between vasodilation and *Leishmania* infection-enhancing effects of sand fly saliva and maxadilan. *Mem. Inst. Oswaldo Cruz* 96, 997–999.
- Cremer, M.A., Hernandez, A.D., Townes, A.S., Stuart, J.M., Kang, A.H., 1983. Collagen-induced arthritis in rats: antigen-specific suppression of arthritis and immunity by intravenously injected native type II collagen. *J. Immunol.* 131, 2995–3000.
- De Luna, R., Vuotto, M.L., Ielpo, M.T., Ambrosio, R., Piantedosi, D., Moscaticello, V., Ciaramella, P., Scalone, A., Gradoni, L., Mancino, D., 1999. Early suppression of lymphoproliferative response in dogs with natural infection by *Leishmania infantum*. *Vet. Immunol. Immunopathol.* 70, 95–103.
- Deane, L.M., Deane, M.P., 1955. Leishmaniose visceral urbana (no cão e no homem) em sobral, Ceará. *Hospital* 47, 75–87.
- Dye, C., 1996. The logic of visceral leishmaniasis control. *Am. J. Trop. Med. Hyg.* 55, 125–130.
- Enserink, M., 2000. Infectious diseases. Has leishmaniasis become endemic in the U.S.? *Science* 290, 1881–1883.
- Fernandes, O., Catanho, M.P., Segura, I., Labrada, L.A., Derre, R., Saravia, N., Degraeve, W., 1999. Minicircle variable region probes for characterization of *Leishmania* (*Viannia*) species. *J. Parasitol.* 85, 563–568.
- Glantz, S.A., 1996. *Primer of Biostatistics*. McGraw-Hill, New York, 473 pp.
- Hommel, M., Jaffe, C.L., Travi, B., Milon, G., 1995. Experimental models for leishmaniasis and for testing anti-leishmanial vaccines. *Ann. Trop. Med. Pathol.* 89, 55–73.
- Killick-Kendrick, R., Killick-Kendrick, M., Pinelli, E., Del Real, G., Molina, R., Vitutia, M.M., Canavate, M.C., Nieto, J.A., 1994. Laboratory model of canine leishmaniasis: the inoculation of dogs with *Leishmania infantum* promastigotes from midguts of experimentally infected phlebotomine sandflies. *Parasite* 1, 311–318.
- Lainson, R., Shaw, J.J., 1978. Epidemiology and ecology of leishmaniasis in Latin-America. *Nature* 273, 595–600.
- Lima, H.C., Titus, R.G., 1996. Effects of sand fly vector saliva on development of cutaneous lesions and the immune response to *Leishmania braziliensis* in BALB/c mice. *Infect. Immun.* 64, 5442–5445.
- Lopes, U.G., Wirth, D.F., 1986. Identification of visceral *Leishmania* species with cloned sequences of kinetoplast. *DNA Mol. Biochem. Parasitol.* 20, 77–84.
- Mathis, A., Deplazes, P., 1995. PCR and in vitro cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. *J. Clin. Microbiol.* 33, 1145–1149.
- Moreno, J., Alvar, J., 2002. Canine leishmaniasis: epidemiological risk and the experimental model. *Trends Parasitol.* 18, 399–405.

- Moreno, J., Nieto, J., Chamizo, C., Gonzalez, F., Blanco, F., Barker, D.C., Alva, J., 1999. The immune response and PBMC subsets in canine visceral leishmaniasis before, and after, chemotherapy. *Vet. Immunol. Immunopathol.* 71, 181–195.
- Nieto, G.G., Garcia-Alonso, M., Requena, J.M., Miron, C., Soto, M., Alonso, C., Navarrete, I., 1999. Analysis of the humoral immune response against total and recombinant antigens of *Leishmania infantum*: correlation with disease progression in canine experimental leishmaniasis. *Vet. Immunol. Immunopathol.* 67, 117–130.
- Oliveira, G.G., Santoro, F., Sadigursky, M., 1993. The subclinical form of experimental visceral leishmaniasis in dogs. *Mem. Inst. Oswaldo Cruz* 88, 243–248.
- Paranhos, M., dos-Santos, W.C., Sherlock, I., Oliveira, G.G., Pontes-de-Carvalho, L.C., 1993. Development of eosinophilia in dogs intradermally inoculated with sand fly saliva and *Leishmania (Leishmania) chagasi* stationary-phase promastigotes. *Mem. Inst. Oswaldo Cruz* 88, 249–251.
- Paranhos-Silva, M., Freitas, L.A.R., Santos, W.L.C., Grimaldi Jr., G., Pontes-de-Carvalho, L.C., Oliveira-dos-Santos, A.J., 1996. A cross-sectional serodiagnostic survey of canine leishmaniasis due to *Leishmania chagasi*. *Am. J. Trop. Med. Hyg.* 55, 39–44.
- Pinelli, E., Killick-Kendrick, R., Wagenaar, J., Bernadina, W., del Real, G., Ruitenber, J., 1994. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. *Infect. Immun.* 62, 229–235.
- Pinelli, E., Gonzalo, R.M., Boog, C.J., Rutten, V.P., Gebhard, D., del Real, G., Ruitenber, E.J., 1995. *Leishmania infantum*-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. *Eur. J. Immunol.* 25, 1594–1600.
- Quinnel, R.J., Courtney, O., Garcez, L., Dye, C., 1997. The epidemiology of canine leishmaniasis: transmission rates estimated from a cohort study in Amazonian Brazil. *Parasitology* 115 (Pt 2), 143–156.
- Rhalem, A., Sahibi, H., Guessous-Idrissi, N., Lasri, S., Natami, A., Riyad, M., Berrag, B., 1999. Immune response against *Leishmania* antigens in dogs naturally and experimentally infected with *Leishmania infantum*. *Vet. Parasitol.* 81, 173–184.
- Sadgursky, M., Brodskyn, C.I., 1986. A new liquid medium without blood and serum for culture of hemoflagelates. *Am. J. Trop. Med. Hyg.* 35, 942–944.
- Santos-Gomes, G.M., Campino, L., Abranches, P., 2001. Canine experimental infection: intradermal inoculation of *Leishmania infantum* promastigotes. *Mem. Inst. Oswaldo Cruz* 95, 193–198.
- Schwartz, R.H., 1998. Immunological tolerance. In: Paul, W.E. (Ed.), *Fundamental Immunology*, 4th ed. Lippincott-Raven, Philadelphia, 1589 pp.
- Sherlock, I.A., Sherlock, V.A., 1959. Criação e biologia, em laboratório, dos *Phlebotomus longipalpia*, Lutz, utz & Neiva, 1912 (Diptera, Psychodidae). *Rev. Brasil. Biol.* 19, 229–250.
- Soares, N.M., Carvalho, E.M., Pinho, R.T., Pontes de Carvalho, L.C., 1993. Induction of complement-sensitivity in *Leishmania amazonensis* metacyclicpromastigotes by protease treatment but not by specific antibodies. *Parasitol. Res.* 79, 340–342.
- Solano-Gallego, L., Llull, J., Ramos, G., Riera, C., Arboix, M., Alberola, J., Ferrer, L., 2000. The Ibizaian hound presents a predominantly cellular immune response against natural *Leishmania* infection. *Vet. Parasitol.* 90, 37–45.
- Theodos, C.M., Ribeiro, J.M., Titus, R.G., 1991. Analysis of enhancing effect of sand fly saliva on *Leishmania* infection in mice. *Infect. Immun.* 59, 1592–1598.
- Titus, R.G., Ribeiro, J.M.C., 1988. Salivary gland lysates from the sand fly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. *Science* 239, 1306–1308.
- Warburg, A., Schlein, Y., 1986. The effect of post-bloodmeal nutrition of *Phlebotomus papatasi* on the transmission of *Leishmania major*. *Am. J. Trop. Med. Hyg.* 36, 926–930.
- Warburg, A., Saraiva, E., Lanzaro, G.C., Titus, R.G., Neva, F., 1994. Saliva of *Lutzomyia longipalpis* sibling species differs in its composition and capacity to enhance leishmaniasis. *Phil. Trans. Roy. Soc. Lond. B* 345, 223–230.
- Zhang, W.W., Matlashewski, G., 1997. Loss of virulence in *Leishmania donovani* deficient in an amastigote-specific protein, A2. *Proc. Natl. Acad. Sci. USA* 94, 8807–8811.