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The FML-vaccine (Leishmune®) against canine visceral leishmaniasis: A transmission blocking vaccine

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Abstract

Transmission blocking vaccines are one of the control strategies for vector-transmitted protozoan diseases. Antibodies raised in the vaccinated host prevent the development of the parasite in the insect vector, interrupting the epidemiological cycle. The FML antigen of *Leishmania donovani* in combination with saponin (FML-vaccine and Leishmune®) induced 92–97% of protections against zoonotic visceral leishmaniasis. We assayed the ability of FML to inhibit *Leishmania donovani* and *Leishmania chagasi* procyclic promastigote-binding to dissected *Lutzomyia longipalpis* midguts. We found a dose-dependent inhibition, more pronounced on *L. donovani* (80%) than on *L. chagasi* promastigotes ($p < 0.001$). On the other hand, the Fab-IgG serum fraction of Leishmune® vaccinated dogs (IgG2 predominant), also inhibited parasite binding in a dose-response ($p < 0.0001$) with an equally potent effect against *L. donovani* or *L. chagasi* ($p = 0.061$). The transmission blocking properties of the Leishmune® vaccine was also assessed by an in vivo membrane assay, with sand flies fed with 1.5×10^7 amastigotes, human blood and, vaccinated or normal control dog sera. Significantly higher values were found in rate of infection ($p < 0.025$) and intensity of infection (number of parasites/insect) ($p < 0.05$) of control sand flies, making a very reduced infection index (20.7%) in the vaccine group. Our results disclosed that the Leishmune® vaccine is a TBV, and that the dog antibodies present in sera, even 12 months after vaccination, lead to a significant effective protection of 79.3%.

Keywords: Transmission blocking vaccines; IgG2; Efficacy; *Lutzomyia longipalpis*; Canine visceral leishmaniasis; Kala-azar; TBV; Sand fly; Membrane-feeding assay

1. Introduction

Human visceral leishmaniasis or kala-azar is a canid zoonosis. Peri-domestic sand flies acquire the etiological agent (*L. chagasi* or *L. infantum*) by feeding on infected fox's skin and transmit it to dogs. The subsequent transmission to humans by sand flies causes human visceral leishmaniasis (VL), which is a severe disease, fatal if not treated by the onset of the symptoms [1]. Five-hundred thousand new human kala-azar cases are registered annually, most of them (90%) in India, Sudan, Bangladesh and Brazil. A protective prophylactic vaccine against human disease is not yet available. The best performance was obtained with a first gen-

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eration vaccine that gave 12% protection among individuals that skin-tested positive for leishmanial antigen [2]. Furthermore, chemotherapy against kala-azar is highly toxic and not always effective [1]. Since the disease is a canid zoonosis, the reduction of dog infectivity to sand flies by prophylactic vaccination would reduce the human incidence of the disease [3]. We have described the development of a prophylactic vaccine against canine visceral leishmaniasis, based on saponin and the FML antigen (fucose mannose ligand) of *L. donovani* [4–7]. The FML-vaccine showed 92–95% specific protection (76–80% vaccine efficacy) in Phase III trials against natural visceral leishmaniasis in Brazil [5,6]. Vaccination reduced both morbidity and incidence of the canine disease [5,6]. This effect lasted for at least 3.5 years and was concomitant with the reduction of the human incidence of the disease in the area [6]. We also showed that the FML-vaccine also has an immunotherapeutic effect when administered to *Leishmania donovani* or *Leishmania chagasi* infected dogs while they were still asymptomatic [8]. The decrease in the canine and human incidence of visceral leishmaniasis in the vaccinated area [5,6], and the maintenance of normal proportions of CD4 and CD21 lymphocyte levels in the blood of vaccinated dogs [8] indicate that dog vaccination with the FML-vaccine reduces dog infectivity to sand-flies [9,10]. Recently, we proved that dogs treated with Leishmune® (FML-licensed vaccine) are not infectious [11], as indicated by a complete absence of clinical signs and of parasites in skin, lymph node and blood PCR amplified samples ($p < 0.01$). Exposed untreated controls on the other hand, were symptomatic (25%), lymph node (56.7%) and blood *Leishmania*-DNA PCR (15.7%) positive, showing also positive immunohystochemical reactions in skin (25%).

It became important then, to determine if the Leishmune®-FML-vaccine is actually a transmission blocking vaccine (TBV). The term "transmission blocking vaccine" (TBV) is used for the malaria vaccines that stimulate antibody production in humans against the sexual gamete forms of the parasite present in the midgut of the anopheles vector [12]. During a blood meal, these antibodies are acquired by mosquitoes and block the fertilization process and further development of the parasites in the vector, making the insect incapable of transmitting the disease [13]. Therefore, TBV are designed to raise antibodies against the gamete stage of the parasite present in the mosquito gut, and while they do not reduce disease in the infected person, prevent the spread of malaria through the community. Such antibodies would block further parasite development in rendering the vector non-infectious. There is a functional test to make the Phase I analysis of this kind of vaccines. Laboratory-raised mosquitoes are fed through a membrane with immune sera generated in vaccinated or infected animals. The mosquito gut can then be dissected to determine the number of infectious gametocytes that developed [12,14].

In the epidemiological cycle of the agents of visceral leishmaniasis (*Leishmania chagasi* and *Leishmania infantum*), the sand fly ingests amastigote-containing macrophages and monocytes from infected dogs during its blood meal. These amastigotes released into the sand fly midgut differentiate into flagellated, procyclic promastigotes and attach to the midgut epithelium [15]. The dividing procyclic promastigotes go into metacyclogenesis, acquiring virulence and transforming into non-dividing metacyclic promastigotes that detach from the midgut epithelium, migrate to the buccal cavity and infect a new vertebrate during the next blood meal [15]. The metacyclogenesis involves morphological changes of the parasite and biochemical transformation of its lypophosphoglycan (LPG) terminal exposed saccharide residues [16–18].

Being a surface antigen of *L. donovani* promastigotes and amastigotes [19,20], and a highly protective immunogen for canine vaccination [5,6,8], the FML antigen could also be specifically recognized by the midgut of the *Lutzomyia longipalpis* vector, acting as a parasite ligand to the midgut and the Leishmune® vaccine could be a TBV. In the present work, we assayed the possible transmission blocking vaccine potential of the Leishmune® vaccine by: (1) using the FML antigen to block the adhesion of *Leishmania donovani* and *Leishmania chagasi* procyclic promastigotes to dissected midguts of the *Lutzomyia longipalpis* sand fly vector; (2) testing the ability of antibodies raised in dogs after vaccination with the Leishmune® to block the adhesion of procyclic promastigotes to dissected sand flies midguts; and (3) assaying the reduction in the proportion of sand flies that become in vivo infected, when fed through a membrane with *Leishmania chagasi*, in the presence of sera taken from Leishmune® vaccinated dogs.

2. Material and methods

2.1. Leishmania promastigote culture

Promastigotes of *Leishmania (L.) donovani* (LD 1S/MHOM/SD/00-strain 1S) and *Leishmania (L.) chagasi* (IOC L-579) were grown in screw-capped tubes at 28 °C with a complex medium containing: brain heart infusion (37 g/l) (Difco, USA), hemin (0.01 g/l) and folic acid (0.02 g/l) (Sigma, SL, USA) supplemented with 10% heat inactivated fetal calf serum (FCS) (Nutricell, Campinas, Brazil). Procyclic promastigotes (exponential growth phase) were obtained after 24 h in culture. The parasites were washed twice in 0.01 M phosphate buffered 0.9% saline (PBS), centrifuged at $2760 \times g$ for 15 min and used for all interaction assays.

2.2. FML antigen

Isolation and chemical characterization of the fucose mannose ligand (FML) obtained from stationary-growth phase promastigotes of *Leishmania (L.) donovani* Sudan (LD 1S/MHOM/SD/00-strain 1S) were performed as previously

described [19]. Briefly, promastigotes were submitted to an aqueous extraction followed by heat inactivation and centrifugation. The aqueous supernatant was lyophilized and fractionated by gel filtration on a Bio-Gel P-10 column yielding the FML glycoproteic complex in void volume [19].

2.3. Fab purification from the IgG antibody fraction of Leishmune® vaccinated dogs

Four healthy adult mongrel dogs were vaccinated with the Leishmune® (FML vaccine commercial licensed formulation). The FML-vaccine is registered as Patent: *INPI number*: PI1100173-9 (18.3.97), Federal University of Rio de Janeiro, Brazil. The animals received three vaccine doses, with 20-day intervals, by the subcutaneous route on the flank. Dog serum was collected 20 days after the third Leishmune® injection. The IgG anti-FML antibody titers were determined by the FML-ELISA assay [21]. The IgG absorbencies of 1/100 diluted sera were: 0.818, 0.761, 0.887 and 0.776. All of them showed higher levels of IgG2 than of IgG1 (1.114/0.284, 0.836/0.235, 0.949/0.393, 1.067/0.574) anti-FML absorbencies, respectively. We also used for control, a pool of normal sera from eight healthy dogs that participated in the same Leishmune® vaccination assay, collected at day 0, before vaccination. The IgG absorbencies of 1/100 control diluted sera were: 0.141, 0.211, 0.189, 0.185, 0.198, 0.143, 0.123 and 0.186.

In order to purify the IgG fraction, all serum samples were pooled, diluted (1:1) in 0.01M Na_2PO_4H, pH 8.8 (Reagen) (PBS*) and incubated with ammonium sulphate (2.92 g) under agitation, for 1 h at 4 °C [22]. The precipitate was separated by centrifugation at $10{,}000 \times g \times 10$ min at 4 °C, ressuspended and dialyzed against PBS* for 12 h at 4 °C. The isolated fraction was applied to a Protein-A-Sepharose (Amersham Biosciences, Sweden) column (1.8 cm × 4.0 cm). Its non-linked fraction was eluted in 0.0 2M Na_2PO_4H, pH 8.8 while the IgG purified fraction was recovered with 0.1 M citric acid, pH 2.2, neutralized with 1.5 M Tris-base pH 8.8 (Sigma, Co.) and dialyzed against BBS buffer pH 8.4 (g/l: boric acid 6.184; Na tetra-borate 9.536; NaCl 4.38). Protein concentration was assayed by the Lowry et al. [23] method, and purified IgG was concentrated in a dialysis membrane against Polyethylene-Glycol 6000 (Vetec) [22]. The Fab and Fc IgG fragments were obtained using ImmunoPure Fab kit (Pierce, USA) and further chromatography on the Protein-A-Sepharose column. The Fab fragments were eluted in the first two volumes of the column while the Fc fraction was removed using Immunopure Elution Buffer (Pierce). Both fractions were dialyzed and assayed for protein content [23].

2.4. FML antigen inhibition of the Leishmania–midgut interaction

Adult female *Lutzomyia longipalpis* sand flies were reared and maintained at the Department of Entomology (Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil) at 26 °C on sucrose solution. Binding of protozoa to insect midguts was performed as previously described [16]. Briefly, 3–6 day-old females were dissected in 0.9% NaCl saline solution (Reagen). Heads, crops and Malpighian tubes were removed and the isolated midguts were opened along the length of the abdominal segment in RPMI* (Sigma Co.) medium supplemented with 1% bovine serum albumin (BSA), 1 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (Reagen). Opened midguts were incubated with the FML antigen at 40, 100, 200 or 400 μg/ml concentration, in 250 μl RPMI*, in a humid chamber for 15 min at 25 °C. *L. donovani or L. chagasi* procyclic promastigotes (10^6) were then added and incubated for further 45 min at 25 °C. The guts were washed by successive changes of saline solution and then individually transferred to microcentrifuge tubes containing 40 μl of saline solution and homogenized with a Teflon pestle [16,17,29]. The released *Leishmania* parasites were counted in a Neubauer chamber. Results are shown as the mean ± S.E. of three experiments with 7–10 midguts assayed for each antigen concentration.

2.5. Anti-Leishmune Fab antibody fraction inhibition of the Leishmania–midgut interaction

The Fab purified IgG fraction of sera from pre-immune controls and Leishmune® vaccinated dogs was incubated at 4, 40 and 400 μg/ml in 250 μl RPMI* with 10^6 procyclic promastigotes for 15 min at 25 °C. Midguts processed as above were then added and further incubated for 45 min. After this period, guts were individually washed and homogenized and the released promastigotes counted as described above. Results are shown as the mean ± S.E. of two experiments with 7–10 midguts for each Fab fraction concentration.

2.6. Inhibition of Lutzomyia longipalpis in vivo infection by sera of dogs immunized with Leishmune® vaccine

Lutzomyia longipalpis colony bred females, 4–6 days old, were infected by feeding through a chick-skin membrane (obtained as protocol P0097-01, Committee of Ethics for Laboratory Animal Use of FIOCRUZ, Brazil) on a mixture composed of: 0.4 ml of defibrinated and complement-inactivated human blood [24], 0.1 ml of saline solution containing 1.5×10^7 amastigotes of *Leishmania (L.) chagasi* obtained from hamsters spleens [25] and 0.5 ml of pools of either pre-immune sera (day 0) or sera from dogs vaccinated with Leishmune®, 12 months before. A second similar experiment was done using 1×10^6 amastigotes. The sera were collected from dogs that belong to canine visceral leishmaniasis Brazilian highly endemic areas (Araçatuba, Andradina, Guararapes, SP, and Nova Lima, MG). The *Leishmania chagasi* strain was recently isolated from the spleen of a symptomatic infected dog from the same area (Araçatuba, SP, Brazil) and its infectivity maintained by six successive in vivo cycles in hamsters.

Engorged females were separated and maintained for 4–12 days at 25 ± 1 °C. They had free access to saturated sucrose solution and were examined after dissection in 0.9% NaCl saline solution (Reagen, Brazil). Heads were removed and the isolated midguts observed microscopically under 400× magnification, for the presence or absence of metacyclic promastigotes. Also, in a random sample, the phlebotomine infection was measured after disrupting each isolated midgut in 40 μl of saline solution, and counting the total promastigote contains in a haemocytometer chamber.

Protection induced by the vaccine antibodies was calculated using the infection index = the ratio of infected sand flies × mean average of the number of promastigotes [19,26] or the average intensity of infection recorded through a 0–4 scale in sand flies midguts [27].

2.7. Statistical analysis

Means were compared by a standard *t* test, ANOVA analysis, simple factorial test and by one way ANOVA, Student–Newman–Keuls method (SPSS for Windows). Proportions were compared by the χ^2 test.

3. Results

3.1. FML antigen inhibits the L. donovani and L. chagasi binding to sand fly midguts

We assayed the potential ability of FML antigen to compete for *Leishmania* procyclic binding to sand fly midguts, by pre-incubating midguts with increasing concentrations of FML. Our results, summarized in Fig. 1A and B, showed a dose-dependent inhibition on binding for both *Leishmania* species. The ANOVA analysis disclosed significant differences in the number of linked promastigotes among different FML concentrations, for both parasites ($p < 0.0001$). All concentrations of FML induced significantly higher binding inhibition levels than saline controls. Also, significant differences in inhibition were detected between different FML concentrations, except for 100 and 200 μg/ml for *L. dono-*

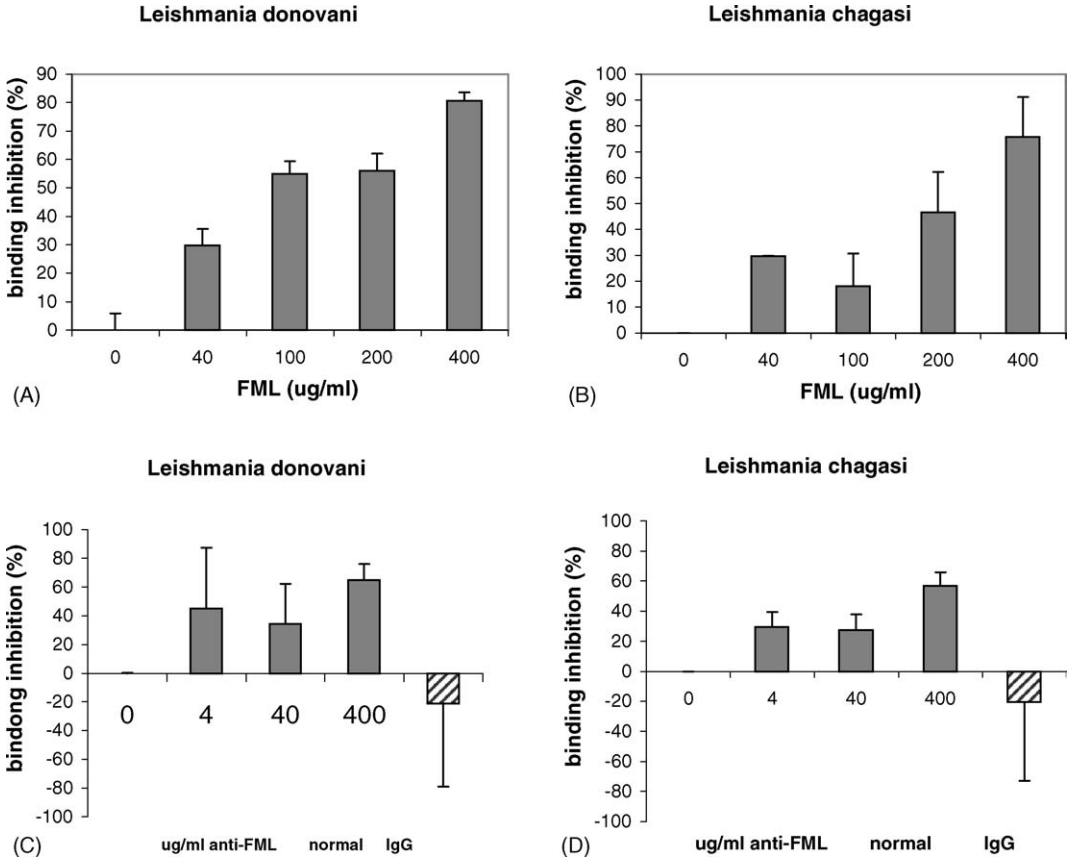

Fig. 1. *Leishmania donovani* and *Leishmania chagasi* binding inhibition by FML antigen and Fab-IgG antibodies of Leishmune® vaccinated dogs. *Lutzomyia longipalpis* midguts were pre-incubated with increasing concentrations of FML antigen and further incubated with 10^6 procyclic promastigotes of *L. donovani* (A) and *L. chagasi* (B). Alternatively, increasing concentrations of the Fab fraction purified from IgG antibodies of Leishmune® treated dogs were incubated with 10^6 procyclic promastigotes of *L. donovani* (C) and *L. chagasi* (D) and further added to insect midguts. As controls we used 400 μg/ml of the Fab purified IgG fraction obtained from normal untreated dogs (hatched bars). After 45 min incubation of any of these systems, guts were individually washed and homogenized. The *Leishmania* parasites released were counted in a Neubauer chamber. Results are shown as the mean ± S.E. of 2 (FML) or 3 (Fab fraction) experiments with 7–10 midgut for each ligand concentration.

vani and 40 μg/ml for *L. chagasi* (Student–Newman–Keuls test with significance level 0.05). Although the FML antigen isolated from *L. donovani* inhibited the binding of both parasites (Fig. 1A and B), the inhibition was more pronounced for *L. donovani* than for *L. chagasi* promastigotes ($p < 0.001$), indicating a degree of species-specificity in the recognition of the homologous antigen.

3.2. Fab fragment from Leishmune® vaccinated dog IgG inhibits binding to sand fly midguts

The transmission blocking potential of the Leishmune® vaccine on canine visceral leishmaniasis was investigated by assaying the inhibition of promastigote binding to sand fly midguts, after incubation with the IgG-Fab moieties purified from vaccinated and control dog sera. The results are summarized on Fig. 1C and D. The ANOVA analysis disclosed significant differences among treatments for the inhibition of either *L. donovani* or *L. chagasi* binding ($p < 0.0001$). For both parasites, inhibition with 400 μg/ml IgG obtained from Leishmune® treated dog's was significantly higher than with all other concentrations (Student–Newman–Keuls test with significance level 0.05). The same maximal concentration of IgG obtained from normal dogs before vaccination, did not inhibit but instead promoted 21.2% of promastigote binding for *L. donovani* and 20.5% for *L. chagasi*, respectively ($p < 0.05$ to all treatments). No differences are found between the two different parasite species ($p = 0.061$) indicating that the antibodies raised in vaccinated dogs prevent the binding of procyclic promastigotes to the sand fly midgut, blocking the transmission of the disease.

3.3. In vivo inhibition of Lutzomyia longipalpis infection by Leishmune® dog hyperimmune serum

The transmission blocking properties of the Leishmune® vaccine were assessed by an in vivo Phase 1 assay, which tested the potential of the Leishmune® vaccinated dog sera antibodies to inhibit the promastigote infection of membrane-feed sand flies. In a first experiment, the parasite inoculum was 1.5×10^7 amastigotes. The initial quantification of parasite infection is summarized in Table 1. Midgut dissection was performed on day 6 after blood meal, when all blood was already digested and promastigote infection was already established. No differences between control and Leishmune® sera treated sand flies were detected, in numbers of engorged or spontaneously dead insects ($p > 0.05$). On the other hand, while infection in controls reached 60.7% of the population only 30.6% of the insects fed with immune sera showed parasites in their midguts ($p < 0.025$). Concomitantly, the intensity of infection was also higher in controls than in Leishmune® sera fed sand flies ($p < 0.05$), thus making a very reduced infection index (20.7%) in the vaccine group. These results indicate that the Leishmune® vaccine is a TBV, and that the antibodies induced even 12 months after vaccination, lead to a significant effective protection of 79.3% (Table 1). An overall picture of the high intensity of *Leishmania chagasi* infection in sand flies fed on normal pre-immune serum can be obtained in Fig. 2. As expected for the development of *Leishmania chagasi*, the intense infection advanced towards the anterior midgut.

Detailed count of promastigotes was performed in a randomly selected sample of sand flies ($n = 10$ for each group) (Table 2). Here again, the infection index was higher in controls than in Leishmune® sera fed sand flies due to both, the higher ratio of infection and the higher average number of recorded promastigotes. Compared to controls then, the sand flies fed on the immune sera showed an infection index of only 25.7% which point out 74.3% of sand fly protection by the TBV vaccine.

A second identical experiment performed with a lower inoculum (1×10^6 promastigotes) disclosed lower percents of infection (results not shown). While 14.3% of the controls showed promastigotes, the infection was null in the sand flies fed with the same pool of Leishmune® vaccine sera.

4. Discussion

The reduction of the in vivo parasite infection due to Leishmune®-FML vaccine sera, was predicted by our in vitro experiments that disclosed an 80% of maximal binding inhibition after incubation with FML. This value however, pointed that also other parasite ligands are involved in parasite vector interaction. LPG complex [16–18,28] and galactose containing glycoconjugates of *L. major* were pointed out as responsible [29]. Adhesion of *Leishmania* promastigotes to the midgut epithelial cells was found to be a property of the non-infective procyclic promastigote stage which was lost during transformation to metacyclic forms, on day 5 after meal [30] allowing the selective release of infective stage parasites [16]. This cycle is controlled by stage-specific modifications of the terminal exposed saccharide on LPG [16]. The *L. chagasi* LPG for instance, shows β 1->3 glucose

Table 1
Survival, infection and protection of *Lutzmoyia longipalpis,* after in vivo feeding with *Leishmania (L.) chagasi* amastigotes and sera of dogs vaccinated with Leishmune®

Sera	T[a]	Engorged	Death	Death %	Dissected	Infected	Infection %	Intensity of infection (mean)	Infection index[b]	Protection%
C	97	83	28	33.7	28	17	60.7	1.821	111	0
L®	92	72	19	26.6	36	11	30.6	0.750	23	79.3

[a] Total number of phlebotomines at the beginning of the experiment.
[b] Infection index = percent of infected phlebotomines × average intensity (0–4) of promastigote infection in sand flies midguts.

Table 2
Percent of infection and parasite counts in *L chagasi* infected phlebotomine fed on pre-immune control or Leishmune® vaccinated dog sera

Treatment	Number of insects	Infected	Number of promatigotes/insect	Infection index[a]	Protection %
Control serum	1	−	0		
	2	+	22000		
	3	−	0		
	4	+	54000		
	5	+	10000		
	6	+	34000		
	7	+	10800		
	8	−	0		
	9	+	6000		
	10	−	0		
Total	10	6/10 = 0.6	22800 ± 18382	13680	0
Leishmune® serum	1	−	0		
	2	−	0		
	3	−	0		
	4	−	0		
	5	+	18400		
	6	−	0		
	7	−	0		
	8	−	0		
	9	+	8800		
	10	+	8000		
Total	10	3/10 = 0.3	11733 ± 5787	3520	74.3

[a] Infection index = ratio of infected phlebotomines × average number of promastigotes in insect's midguts.

units that branch from the repetitive disaccharide units and from the "cap". In the metacyclic form, glucose substitutions disappear, probably impeding its binding [18]. FML isolated from culture metacyclic promastigotes shows a glycidic *N*-linked oligosaccharide fraction composed of: (1) a branched chain alternating Manp 4-*O*, 3-*O* and Glc Nac 4-*O*-linked units; *N*-acetyl-glucosamine as branching point and fucopyranose, galactopyranose and mannopyranose as terminal residues and (2) linear short oligossacharides composed of 4-*O*, 3-*O* and 2-*O*-linked mannopyranose and fucopyranose and galactopyranose as terminal units [31]. We could have detected probably different inhibition indexes for the FML, alternatively isolated from procyclic promastigotes.

Also predicting the high TBV potential of the Leishmune® antibodies, the in vitro incubation with canine anti-*L. donovani* Fab FML antibodies diminished the binding of both *L. chagasi* and *L. donovani*. These results indicate that although, *L. donovani* causes human visceral leishmaniasis in the Old World, and *L. longipalpis* is the specific vector for *Leishmania chagasi* in America, the antibodies raised in dogs treated with Leishmune®, containing *L. donovani*-FML, could equally protect against *L chagasi* or *L. donovani* infection, showing cross-reactivity. These results are in agreement with the strong cellular and humoral IgG2 protection detected against visceral leishmaniasis, induced by the vaccine in dogs exposed to or infected with *L.(L.) chagasi* [5,6,8,32].

Although much work has been done in malaria TBVs, only a few studies deal with TBV on leishmaniasis. All of them refer to tegumentar leishmaniasis [33–36] and, to our knowledge, the present investigation is the first report on a Phase I trial of a TBV vaccine against visceral leishmaniasis. Indeed, sand flies previously fed on *Leishmania major* crude antigens, LPG alone or LPG plus recombinant GP63 immunized mice, showed reduced rates of infection as compared to controls [35]. Reduction was significant in sand flies fed upon LPG immunized mice. The inhibition by immune sera is probably caused by avoiding the exflagellation of the parasite or by degeneration of the sand fly midgut [35]. Furthermore [36], sand flies fed on immunized mice and further, on *L. major* infected mice were used to infect naive Balb/c mice. The animals infected by sand flies fed on crude lysate or rGP63 immunized mice showed exacerbation of the disease, whereas those infected by sand flies fed on LPG treated mice had the smallest lesion sizes [36]. These studies confirm the presence of *L. major* LPG-specific receptors on the insect midgut epithelium, and their relevance on recognition and interaction with *Leishmania* during its biological cycle.

The first level of evaluation of the efficacy of a malaria TBV vaccine is the reduction in the proportion of mosquitoes that become infected when fed through a membrane upon gametocytes in the presence of sera taken from TBV-vaccinated individuals [14]. Since malaria TBV antigens belong to the sexual stage of parasite that only infects the vector, they generate no protection for the vaccinated individual. Their impact is then, mainly epidemiological, and the basic reproduction number (Ro) would be reduced only in cases of proportionally high vaccine coverage [14].

Instead of that, the main antigen of the FML complex is the nucleoside hydrolase of *L. donovani* [37], a crucial enzyme in the early steps of the establishment of infection that breaks nucleosides, releasing free DNA purine bases which are further used in the synthesis of the DNA allowing

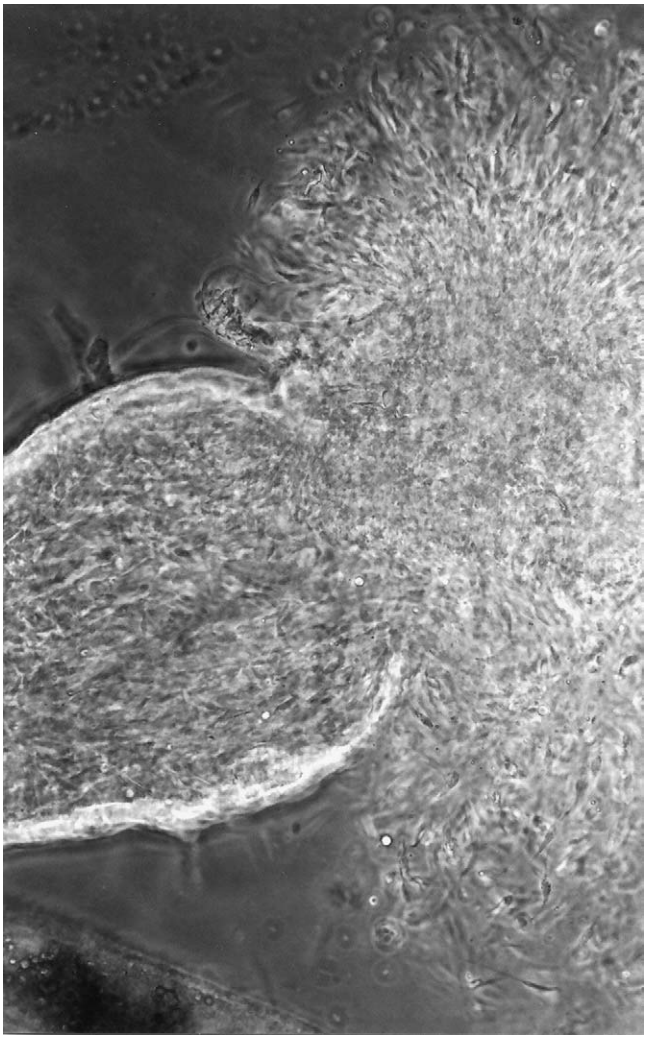

Fig. 2. *Lutzomyia longipalpis* experimentally infected with *Leishmania chagasi* and normal dog serum. Large number of flagellates are seen exuding from the cardia. Fresh preparation, phase contrast microscopy, 400× magnification.

the parasite multiplication. This explains why the vaccine containing FML and its nucleoside hydrolase was efficient in immunoprophylaxy and immunotherapy, protecting dogs from the early stage of infection [5–8,38]. This effect reduces the reservoir condition of the Leishmune® vaccinated animals in nature rendering dogs not infectious [11]. In this investigation, we demonstrate that the humoral response, also generated by the Leishmune® FML-vaccine, protects sand flies from *Leishmania chagasi* infection. The TBV potential described here for Leishmune® vaccine, certainly contributes to the observed decrease of canine and human incidence of visceral leishmaniasis in the field [6]. The high IgG2 protective antibody titers generated by the Leishmune® vaccine in dogs [32] were expected for a formulation containing the QS21-*Quillaja saponaria* saponin adjuvant [39].

The detection of 79.3% of protection by Leishmune® dog-sera is very impressive, considering the artificially high inoculum used in this investigation. Indeed, while most in vivo studies report inocula of $1–2 \times 10^6$ promastigotes/ml [28,40] in our investigation we used a 15-fold higher inoculum (1.5×10^7). If as deduced from Warburg's and Schlein's results [40], sand flies ingest 200 promastigotes in 0.1 μl of blood, in our study, 1500 parasites were ingested in each blood meal. The parasite load might have been even higher, since we fed the insects with amastigotes, the smallest parasite stage, which is also the parasite form ingested by the sand fly in nature after feeding on humans or canids.

Also, the TBV potential of Leishmune® vaccine was tested against an artificially high ratio of sand fly infection. Indeed, while 60.7% of the controls in our investigation, were infected with the 1.5×10^7 amastigotes/ml inoculum, only 14.3% showed parasites after being fed with 10^6 amastigotes inoculum. Infection rates seem to be lower in nature. In Brazilian epidemic and endemic regions of human and canine visceral leishmaniasis, only 0.2, 0.5 and 7.14% of natural infection was detected, after dissection of 3734, 1500 and 491 captured *Lutzomyia longipalpis*, respectively [41–43]. Therefore, in our in vivo model of infection we stressed much more severe conditions of infection than the ones used by other models [28,40] or detected in nature [41–43]. This means that the TBV potential of Leishmune® vaccines in nature might be even higher than 79.3%.

In this investigation, we demonstrate the presence of FML-specific receptors on *Lutzomyia longipalpis* midgut, its involvement in the binding of *L. donovani* or *L. chagasi* in vitro procyclic promastigotes to the insect vector and the transmission blocking potential of the Leishmune® vaccine against canine visceral leishmaniasis. Our results indicate that the Leishmune® vaccine might be considered an effective tool to be used in the epidemiological control of visceral leishmaniasis.

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