

IgG1/IgG2 antibody dichotomy in sera of vaccinated or naturally infected dogs with visceral leishmaniasis

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Abstract

Canine antibody IgG, IgG1 and IgG2 anti-FML responses were investigated in dogs vaccinated with the fucose–mannose ligand (FML)-vaccine of *Leishmania donovani* and in dogs with naturally acquired visceral leishmaniasis. While similar levels of total IgG antibodies were seen in the seropositive naturally infected dogs and in vaccinees, significant differences between the groups were found regarding their IgG1/IgG2 anti-FML antibody composition ($P < 0.005$). Higher IgG1 absorbencies were seen in infected dogs, while the IgG2 subtype was predominant in pre-immune sera, and in vaccinated animals, both after the first and the third dose ($P < 0.005$). The average ratio between IgG1/IgG2 was then 1.124 for infected animals and 0.733 for FML-vaccinees. Also, a significant increase in IgG2 antibodies was observed from the first to the third vaccine injection ($P < 0.005$). In the infected dogs, a high correlation between their IgG absorbance (Abs) values and the number of symptoms ($P = 0.017$) was disclosed. Thus, the analysis of IgG subclasses disclosed a dichotomous response to visceral leishmaniasis: IgG1 associated to natural infection and IgG2 associated to a humoral response subsequent to the FML-vaccine treatment. An IgG1/IgG2 ≥ 1 would characterize the sera of visceral leishmaniasis infected animals evolving towards the overt disease while ratios ≤ 1 would characterize the sera response of vaccinated protected dogs.

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1. Introduction

Zoonotic visceral leishmaniasis (ZVL) is one of the most important emerging diseases [1]. Peridomestic sand flies acquire the etiological agent (*Leishmania chagasi* or *Leishmania 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 of new human kala-azar cases are registered annually, most of them (90%) in India, Sudan, Bangladesh and Brazil. Anti-*Leishmania donovani*

IgG serum antibodies appear in dogs and human's blood, soon after infection reaching high levels during the development of the disease. Hypergammaglobulinemia is one of the typical signs of both human and canine disease.

The epidemiological prophylactic control of the disease, as recommended by WHO involves: the elimination of seropositive infected dogs, the insecticide treatment within domestic and peridomestic habitations and the systematic treatment of human cases [1]. Dog removal based on seropositivity, however, was pointed out as a low impact tool on the reduction of human and canine VL incidence [2], probably due to the relatively low sensitivity of the immunofluorescent assay, used for diagnosis [3]. In spite of this, a protective vaccine for dogs against kala-azar would represent a very effective control tool in eradication of disease [2].

We recently described the protective effect of the fucose–mannose ligand (FML)-vaccine on canine visceral

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leishmaniosis. This formulation had already shown to be safe, immunogenic and protective in Phase I–IIa trials in Balb/c (87.7%; $P < 0.01$), Swiss albino mice (85%, $P < 0.01$) and CB hamsters (84%; $P < 0.001$) [4–7]. In a Brazilian area endemic for both, human and dog visceral leishmaniosis, recent Phase III trials of efficacy using the FML-vaccine in dogs, induced 92 [8] and 95% [9] of protection in naturally exposed vaccinees (76 and 80% of vaccine efficacy, respectively). The FML-vaccine induced a significant, long-lasting and strong protective effect against canine kala-azar in the field [8,9].

As expected for the use of saponin adjuvants, the FML-QuilA canine vaccine induced a strong humoral and cellular immune response, soon after the complete vaccination [9]. Indeed, a 100% of seropositivity and 100% of intradermal reaction to the *L. donovani* promastigote lysate were detected 2 months after the complete vaccination [9]. Seropositivity was recorded by monitoring, total IgG antibodies, either by the ELISA or by the immunofluorescent assay. IgG seropositivity achieved after protective vaccination, however, was indistinct from that due to natural [8,9] or experimental [10] infection by *L. chagasi* and *L. donovani*, respectively. Therefore, in epidemiological control campaigns based on IgG-screening in dog sera, vaccinated protected dogs might be confused with the naturally infected ones, reservoirs of *Leishmania*, that are normally removed for sacrifice.

As observed for humans, the differential increase in several IgG immunoglobulin subclasses might be marker of overt kala-azar. Indeed, the IgG1 and IgG3 [11], or IgG1 and IgG2 subtypes [12] have shown to be increased in individuals with severe clinical kala-azar. In the kala-azar mouse model of infection, a mainly IgG1 increase would characterize the TH2 response of *L. donovani*-infected animals, while the combined IgG1, IgG2a and IgG2b production discloses a TH1/TH2 response characteristic of the vaccination with the highly protective QS21 [13,6] or QuilA saponin [6]. Two subtypes of IgG antibodies are described in dog serum: IgG1 and IgG2. Recent evidences obtained in naturally infected dogs with visceral leishmaniosis pointed out to the presence of a mainly IgG1 response being associated with disease (symptomatic dogs, non or low-responsive to chemotherapy) with IgG2 related with asymptomatic infections and natural resistance to infection [14]. In the present work, we aimed to analyze the possible dichotomous response to *L. donovani* antigens, comparing the anti-FML antibody subtypes in sera of FML-vaccinated dogs of a non-endemic area and of naturally infected dogs of a kala-azar endemic area.

2. Material and methods

2.1. Sera and vaccine procedure

A canine serological screening for the presence of anti-*L. chagasi* antibodies was performed in Jardim Progresso,

Natal, Rio Grande do Norte, a populated urban area of Natal, of low socioeconomic status, where human and canine kala-azar are highly endemic. A total of 419 samples of domiciliary dogs were collected from the cephalic vein by our personnel. Clinical symptoms of kala-azar (loss of weight, cachexia, alopecia, onychogryphosis, apathy, ulcerative skin lesions) were identified and recorded for each animal. Furthermore, 68 healthy dogs of the non-endemic area were vaccinated with three dose of the FML-vaccine (1.5 mg FML antigen) [8] through the subcutaneous route with a 21 days interval. Sera of the animals were collected 20 days after each vaccine dose. Isolation and chemical characterization of the fucose-mannose ligand obtained from stationary-growth phase promastigotes of *L. donovani* Sudan (LD 1S/MHOM/SD/00-strain 1S) was performed as previously described [15]. The FML-vaccine is registered as a Patent: INPI number: PI1100173-9 (18.3.97), Federal University of Rio de Janeiro, Brazil.

2.2. Serological analysis

The first available pre-vaccination and post-dose serum samples from the vaccinated and control dogs and the sera of naturally infected dogs from Jardim Progresso, RN, were tested for the presence of total anti-*L. donovani* antibodies by the FML-ELISA assay [10] using the FML antigen (2 µg per well) solubilized in carbonate buffer (pH 9.6) coated on flat-bottom 96-well plates (Corning 25805-96, cat. number 430480, highly absorbent). Total antibodies were detected by using peroxidase-labeled protein-A (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) at a 1:16,000 dilution, in blocking buffer. The cut-off of the FML-ELISA assay, as determined by the Youden test calculation [10] is absorbance (Abs) 492 nm: 0.450 (mean average of absorbance values of normal healthy serum plus two standard deviations). Furthermore, goat anti-dog IgG1 heavy chain specific (1:2000) or IgG2 (1:32,000) (Bethyl Laboratories Inc., Montgomery, TX, USA) conjugated with horseradish-peroxidase were used for the IgG subtype determination of each sera. We titrated the IgG1 and IgG2 conjugates, in a two-fold dilution, against 2 µg of FML antigen per well, using a pool of 28 sera of normal healthy dogs of non-endemic area (our kennel in UFRJ), a pool of 29 sera from clinically and parasitologically confirmed, symptomatic and asymptomatic kala-azar cases of endemic areas (Araçatuba, SP and Natal, RN) and a pool of 68 sera of FML vaccinated dogs described before. The reactions were developed as described elsewhere [16]. The absorbance values at 492 nm were compared using a 1:100 dilution of the individual serum samples. Results are expressed as mean values of triplicates.

2.3. Statistical analysis

Student's *t*-test was determined for the analysis of the differences in total IgG absorbance values. Correlation

coefficient analysis between IgG, IgG1, IgG2 absorbencies and increasing kala-azar symptoms was determined on a Pearson bivariate, two tailed test of significance (SPSS).

3. Results

The initial serological screening in kala-azar endemic area disclosed 121/419 dogs that were seropositive in the FML-ELISA assay, making a 28.9% of canine seroprevalence in Jardim Progresso. Furthermore, the FML-ELISA assay for total IgG antibodies was negative in the 95 pre-immune sera samples of non-endemic area, and positive, in 48% of the dogs after the first (32/67), and in 70% of the animals, after the third FML-vaccine dose (46/65) (Fig. 1). The result analysis disclosed significant increases of mean absorbency values, for all groups, over the pre-immune sera ($P < 0.005$). Furthermore, the absorbencies increased from the first to the third vaccine injection ($P < 0.005$). Also, similar levels of total IgG antibodies were seen in the seropositive naturally infected dogs of the endemic area. Therefore, an indistinct total IgG response is observed in vaccinated or naturally infected dogs.

Aiming to determine the anti-FML IgG subtype composition of the sera samples, we titrated the IgG1 and IgG2 conjugates, on a two-fold dilution, against 2 μg per well FML antigen using pools of normal healthy, infected and FML vaccinated dog's sera. The results obtained are summarized in Fig. 2. We observed that while similar levels of IgG1 and IgG2 were seen in infected animals, a predominance of IgG2 was disclosed both in the pools of vaccinated and normal

pre-immune animal sera (lower values), indicating a different isotype of antibody composition in these groups. Using as reference the cut-off value of the FML-ELISA for total IgG (Abs 492 nm: 0.450), the first conjugate dilution showing negative values for normal sera were: 1:2000 for IgG1 and 1:8000 for IgG2. However, further experiments with individual sera showed that only the IgG2 1:32,000 dilution disclosed best differential values for infected or vaccinated dogs.

Each sera sample was then subsequently analyzed for its anti-FML IgG antibody subtype composition at these conjugate working dilutions. In this case, significant differences between the groups were found. Indeed, while higher absorbencies were seen in IgG1, in 65% of naturally infected dogs (Fig. 3), the IgG2 subtype was predominant in vaccinated animals, both after the first (78%) and the third (70% dose) (Fig. 4). With lower absorbance values, IgG2 was also higher in 60% of the pre-immune sera (Fig. 4) although no specific anti-*L. donovani* antibodies are expected in this group. Taken into account the whole sera sample (Table 1), we observed significant differences between IgG1 and IgG2 titers in all tested groups. While the IgG1 is significantly increased in infected animals, the IgG2 is predominant in pre-immune and vaccinated animals (Table 1). Also, the IgG2 mean absorbance values were significantly higher in sera of vaccinated than in pre-immune animals ($P < 0.005$) and a significant increase in IgG2 antibodies was observed from the first to the third vaccine injection ($P < 0.005$). On the other hand, the IgG1 response of infected animals was significantly higher than that of pre-immune sera ($P < 0.005$). Finally, the average

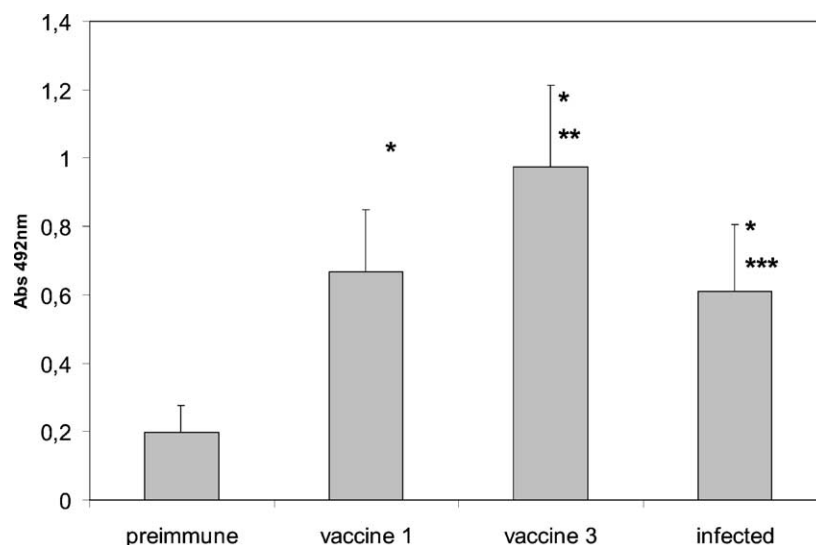


Fig. 1. Anti-FML total antibody absorbance values in naturally exposed dogs from a visceral leishmaniasis endemic area and in dogs vaccinated with FML-vaccine. The bars represent the mean average of absorbance and standard deviation values in sera of: pre-immune controls ($n = 31$); vaccinated dogs: 20 days after the first dose (vaccine 1) ($n = 68$) and 20 days after the third dose (vaccine 3) ($n = 68$) and naturally infected dogs from Jardim Progresso, RN ($n = 121$) (infected). Each sera sample was tested in triplicates. The y-axis represents the FML-ELISA absorbance values at 492 nm of the sera samples diluted 1:100. The 0.450 value (Abs 492 nm) represents the cut-off of the FML-ELISA assay. (*) significantly different from pre-immune sera ($P < 0.005$); (**) significantly different from vaccine 1 sera ($P < 0.005$) and (***) not significantly different from vaccine (1 or 3) sera ($P > 0.05$) (Student's *t*-test).

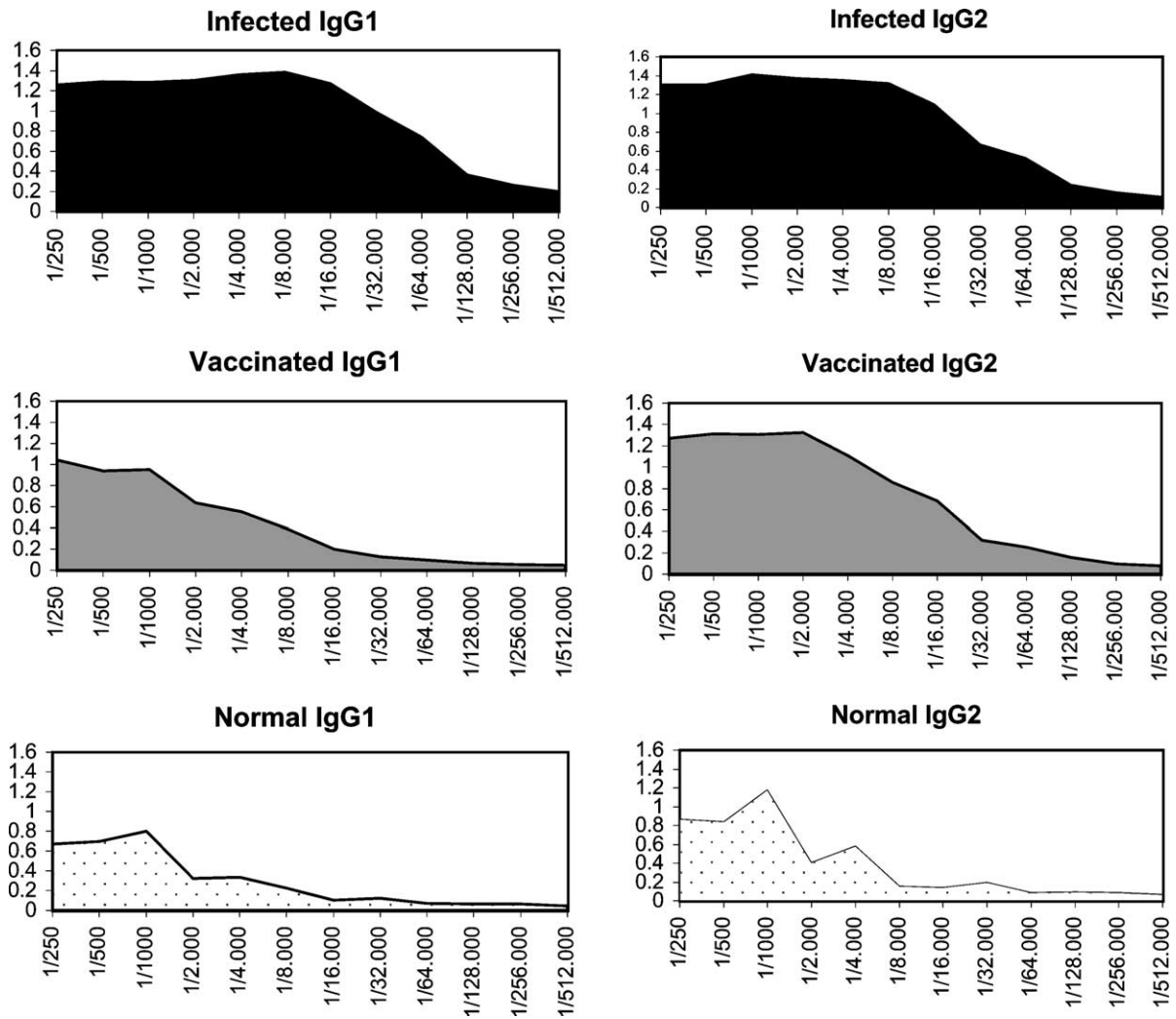


Fig. 2. Goat anti-dog IgG1 and anti-dog IgG2 horseradish-peroxidase conjugate titration against the FML antigen ($2 \mu\text{g}$ per well), using pools of normal healthy ($n = 28$) and FML-vaccinated canine sera ($n = 68$) from a visceral leishmaniasis non-endemic area and sera from clinically and parasitologically confirmed *L. chagasi*-infected dogs of an endemic area ($n = 29$). All the pools were used in a 1/100 dilution.

ratio between IgG1/IgG2 was then 1.129 for infected animals and 0.585 for FML-vaccinees. As previously disclosed for the total IgG antibody response, a significant IgG1/IgG2 enhancement is detected already after a unique dose of the FML-vaccine (Table 1). Our results indicate that an IgG1/IgG2 ≥ 1 would characterize the sera of vis-

ceral leishmaniasis infected animals evolving towards the overt disease while ratios ≤ 1 would characterize the sera response of vaccinated protected dogs. The levels of IgG, IgG1 and IgG2 antibodies were highly correlated, both in vaccinated ($P < 0.0005$) and in naturally infected animals ($P < 0.001$).

Table 1
Anti-FML absorbance values in the ELISA assay with anti-IgG1 and IgG2 conjugates

	<i>n</i>	IgG1		IgG2		Significance (<i>P</i>)	IgG1/IgG2 ratio
		Average	S.E.	Average	S.E.		
Infected	121	0.693	0.022	0.614	0.027	<0.025	1.124
Vaccinees 1st dose	68	0.387	0.032	0.661	0.032	<0.005	0.585
Vaccinees 3rd dose	58	0.906	0.066	1.236	0.052	<0.005	0.733
Pre-immune	95	0.141	0.006	0.203	0.014	<0.005	0.695

Results express the average and standard error of the anti-FML IgG1 and IgG2 absorbance values at 492 nm of the individual sera samples analyzed through the ELISA assay against FML antigen. Significance of the differences was analyzed by a standard Student's *t*-test.

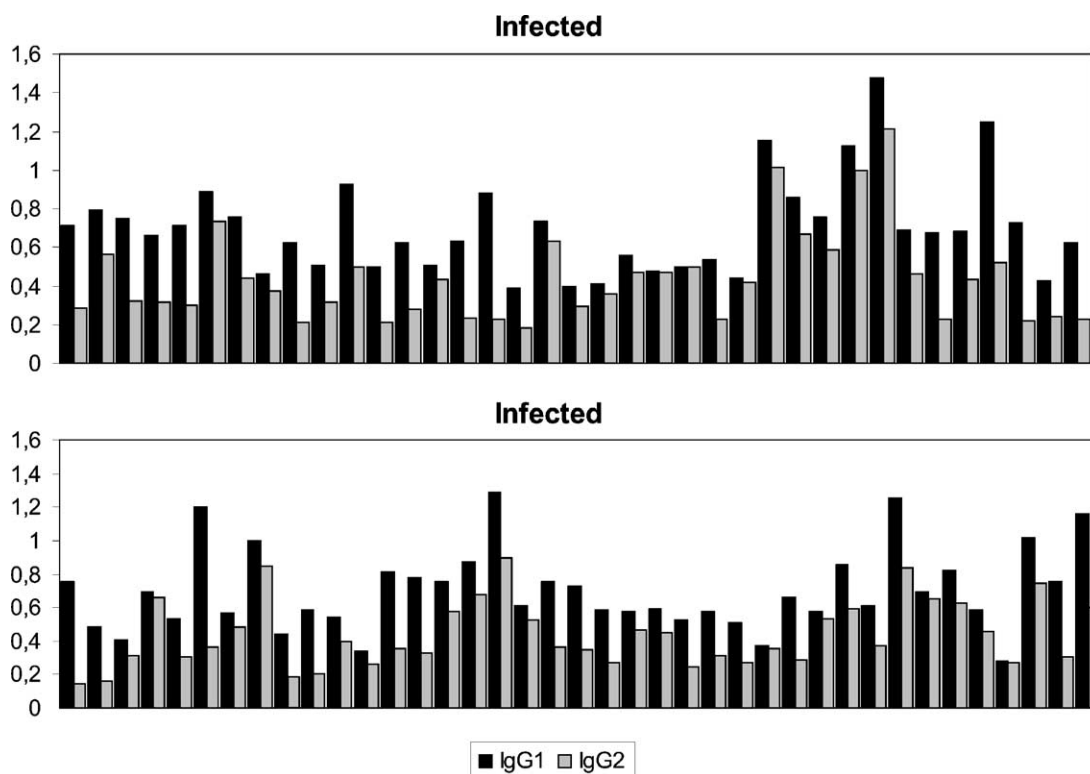


Fig. 3. Anti-IgG1 and IgG2 antibody absorbance values in naturally exposed dogs from a visceral leishmaniasis endemic area (37 individuals on the top, 39 on the bottom). The bars represent the individual absorbance values in sera of infected dogs from Jardim Progresso, RN (infected). Each sera sample was tested in triplicates. The y-axis represents the FML-ELISA absorbance values at 492 nm of the sera samples diluted 1:100.

Monitoring of the visceral leishmaniasis clinical symptoms was performed in 89 among the 121 naturally infected dogs from Jardim Progresso. The clinical signs, as well as their individual IgG anti-FML absorbances are summarized in Table 2. A strong correlation between increasing IgG absorbencies and increasing number of symptoms was found ($P = 0.017$) suggesting that the FML-ELISA assay might point out the evolution of the disease.

4. Discussion

A 28.9% of canine anti-*L. donovani* seroprevalence was detected in this investigation, using the FML-ELISA assay. Previous work done in Brazil, using Elisa assays, either performed with crude or purified antigens showed similar seroprevalences in dog sera: 23.5 [10], 23.5 [17], 28.7 [18] and 30.5% [19]. These values might indicate then, the proportion of canine populations susceptible to canine visceral leishmaniasis in the different endemic areas. The epidemiological control based on the results of an ELISA assay could then, show more impact in reduction of the incidence of the disease [3]. Cabral et al. [20] and Pinelli et al. [21] described a stronger antibody synthesis only in symptomatic dogs both naturally or experimentally infected. Solano Gallegos et al. [22] found higher titers in symptomatic than in asymptomatic dogs. In previous reports, we showed that

the FML-ELISA assay has a prognostic value on the development of human [16] and canine visceral leishmaniasis [10]. We also disclosed high correlation values between the absorbencies in the FML-ELISA assay and the increase in number of canine symptoms ([23], and this investigation) and of the intensity of *Leishmania* PCR products in bone marrow of seropositive human blood donors [24]. Since a positive correlation was also found between the increase in canine clinical signs and infectiousness to sandflies [25], the use of the FML-ELISA assay in epidemiological control campaigns could be helpful in the reduction of the parasite reservoir of endemic areas.

In previous work with the FML-vaccine, the humoral response was significantly higher in vaccinees than in controls at all tested times (ANOVA analysis, $P < 0.0001$ differences for treatment and for time) [8,9]. Absorbance values were maximal by the second month [9] or 7 months after vaccination [8] with no decline detected until the 3.5 years after vaccination [9]. In this investigation we showed that the absorbencies increased from the first to the third vaccine injection ($P < 0.005$), indicating that, while a strong IgG response against *L. donovani* is obtained already after a unique dose of the FML-vaccine, the complete immunization schedule improves the antibody response. In this work, as expected, similar levels of total IgG antibodies were seen in the seropositive naturally infected dogs of the endemic area, confirming our previous observations [8–10] of an

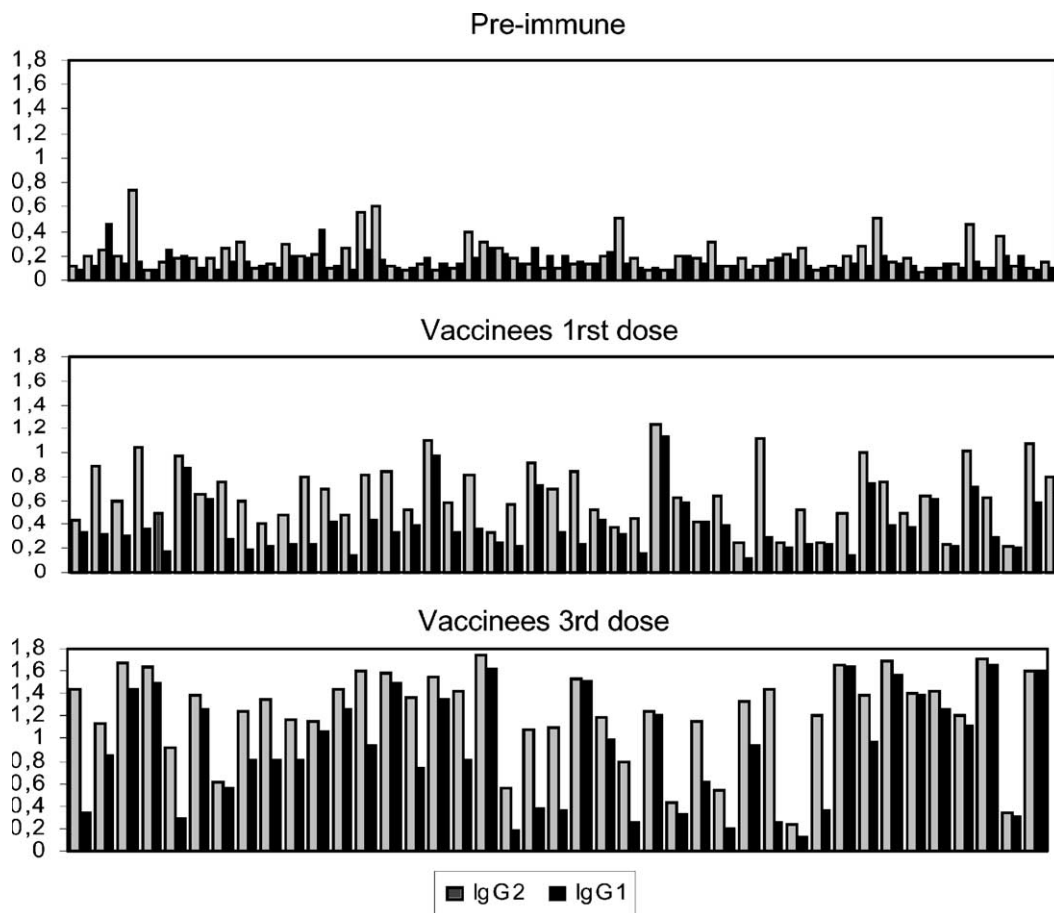


Fig. 4. Anti-IgG1 and IgG2 antibody absorbance values in dogs vaccinated with FML-vaccine. The bars represent the individual absorbance values in sera of: pre-immune controls ($n = 65$); vaccinated dogs: 20 days after the first dose (vaccine 1) ($n = 48$) and 20 days after the third dose (vaccine 3) ($n = 41$). Each sera sample was tested in triplicates. The y-axis represents the FML-ELISA absorbance values at 492 nm of the sera samples diluted 1:100.

indistinct total IgG response in vaccinated or naturally infected dogs.

Human [26] and canine visceral leishmaniasis [21] provoke a cellular immunosuppressive status. Asymptomatic or resistant dogs show a lymphoproliferative antigen-specific in vitro response and an in vivo cellular immune response expressed by a positive IDR to leishmanial antigens [20,27] indicating a degree of natural protection against the disease. A statistical association between infection of resistant animals and positive DTH response was found [27]. In these dogs, high levels of IL2 and TNF were observed [20]. Conversely, susceptible or symptomatic dogs showed suppression of the cellular immune response in vivo or in vitro and a fail in IL2 and TNF production [21]. These results suggest a different kind of response of the TH1 or TH2 effector T lymphocytes (CD4), capable of determining resistance or susceptibility to the canine disease. Apparently, the absence of an in vitro response observed in the susceptible dogs is antigen-specific [21]. CD4 lymphocytes are predominant in Type 4 of delayed-type hypersensitivity reactions. CD4 lymphocytes from the TH1 subset are involved in intradermal response to leishmanial antigens in

the murine model [28]. Furthermore, reduced levels of CD4 T cell [29–31] and CD21 B cells [30] were characterized in canine kala-azar. The reduction in proportion of CD4 cells was also correlated to an increase in the infectiousness of kala-azar dogs to phlebotomous [29]. In previous work, we achieved 76–80% of vaccine efficacy and 100% of positive IDR in dogs treated with FML vaccine [8,9]. Enhanced symptomatology and suppressed IDR were found in saline treated animals and the opposite in vaccinees ($\chi^2 = 11.59$; $P < 0.005$) [23]. Therefore, the maintenance of a strong and positive intradermal reaction to *Leishmania* antigen subsequent to FML-vaccination could possibly indicate the presence of normal levels of CD4 cells in vaccinated dogs. Preliminary results of normal CD4 and CD21 cell levels in experimentally infected and further vaccinated dogs confirm this hypothesis [32]. The differential isotype humoral immune response in FML vaccinated dogs might be then a consequence of the cellular immune reactions related to a protective response. Monitoring the IgG2 increased response in vaccinees might be an indirect and easy way of following the protective and active cellular immune response achieved in dogs treated with the FML-vaccine in large scale studies

Table 2
Incidence of clinical signs and IgG absorbance values in FML-ELISA assay of endemic area dog sera

Dog	Total IgG (Abs 492 nm)	Symptoms	Number of symptoms	Dog	Total IgG (Abs 492 nm)	Symptoms	Number of symptoms
1	0.559	O, L, AP, U	4	46	0.729	O, L	2
2	1.037	O, L, AP, U	4	47	0.798	O, L	2
3	1.155	O, L, AP, U	4	48	0.595	O, L	2
4	1.027	O, L, AP, U	4	49	0.470	O, U	2
5	0.894	O, L, AP, U	4	50	0.849	L	1
6	0.728	O, L, AP, U	4	51	0.753	O	1
7	0.534	O, L, U	3	52	0.817	L	1
8	0.962	O, L, U	3	53	0.714	O	1
9	0.857	O, AP, U	3	54	0.771	O	1
10	0.443	O, L, U	3	55	0.627	O	1
11	0.504	O, L, AP	3	56	0.463	O	1
12	0.765	O, L, U	3	57	0.575	O	1
13	0.524	O, L, AP	3	58	0.544	O	1
14	0.557	O, L, U	3	59	0.937	U	1
15	0.485	O, L, AP	3	60	0.705	U	1
16	0.839	O, L, U	3	61	0.438	O	1
17	0.780	O, L, U	3	62	0.600	O	1
18	0.922	O, L, U	3	63	0.438	O	1
19	0.722	O, L, U	3	64	0.461	O	1
20	0.402	O, L, U	3	65	0.507	O	1
21	0.479	O, L, U	3	66	0.565	O	1
22	0.700	L, U	2	67	0.576	L	1
23	0.444	O, L	2	68	0.695	O	1
24	0.544	O, U	2	69	0.471	O	1
25	0.723	O, U	2	70	0.583	O	1
26	0.871	O, U	2	71	0.596	U	1
27	1.054	O, AP	2	72	0.455	O	1
28	0.472	O, U	2	73	0.752	Asymptomatic	0
29	0.637	O, AP	2	74	0.823	Asymptomatic	0
30	0.439	O, L	2	75	0.802	Asymptomatic	0
31	0.608	L, U	2	76	0.656	Asymptomatic	0
32	0.450	O, U	2	77	0.444	Asymptomatic	0
33	0.988	O, L	2	78	0.507	Asymptomatic	0
34	0.450	O, L	2	79	0.542	Asymptomatic	0
35	0.449	O, L	2	80	0.639	Asymptomatic	0
36	0.593	O, L	2	81	0.457	Asymptomatic	0
37	0.530	O, U	2	82	0.836	Asymptomatic	0
38	0.663	O, L	2	83	0.594	Asymptomatic	0
39	0.882	L, AP	2	84	0.465	Asymptomatic	0
40	0.631	O, L	2	85	0.486	Asymptomatic	0
41	0.492	O, L	2	86	0.607	Asymptomatic	0
42	0.615	O, U	2	87	0.474	Asymptomatic	0
43	0.504	O, L	2	88	0.859	Asymptomatic	0
44	0.470	O, L	2	89	0.454	Asymptomatic	0
45	0.856	O, U	2				

Animals of the kala-azar endemic area were clinically evaluated at the moment of serum collection: (O) onychogryphosis; (L) loss of weight; (AP) apathy; (U) disseminated ulcers. The total IgG anti-*Leishmania donovani* antibodies were evaluated in dog sera, by the FML-ELISA assay using protein A peroxidase. The cut-off of the method was 0.435 (Abs 492 nm).

in the field. The IgG2 increase would represent then a marker of this cellular immune protective response.

Two subtypes of IgG antibodies are described in dog serum: IgG1 and IgG2. Dogs with visceral leishmaniasis, naturally infected with *L. infantum* produced both IgG1 and IgG2 antibodies, with IgG2 related to asymptomatic infections and IgG1 being associated with disease (symptomatic dogs, non or low-responsive to chemotherapy) [14]. Solano Gallegos et al. analyzed the *L. infantum*-specific IgG, IgG1 and IgG2 antibody response in healthy and ill dogs [22].

The authors observed that only IgG1 remained stable for a 5 years period, in a group of naturally infected asymptomatic dogs. Furthermore, IgG1 levels were specially decreased in the group of symptomatic dogs that were responsive to chemotherapy treatment while remain stable in the unresponsive group, suggesting a possible correlation between IgG1 increase, symptomatology and disease [22]. Recently, Nieto et al. described that IgG1 antibodies were undetectable in *L. infantum*-infected oligo-symptomatic dogs or in dogs showing a regressive disease [33]. On the other

hand high levels of anti-*Leishmania* IgG1 antibodies were seen in dog with the active disease [32,33].

In *L. chagasi* naturally infected dogs, the IgG1 was the predominant subtype, recognized by the recently described *L. donovani* nucleoside hydrolase recombinant antigen (*LdNH*), in ELISA assay [34]. The *LdNH* antigen of *L. donovani* corresponds to the proteic moiety of the GP36 glycoprotein [35] that was described as the specific human kala-azar marker of the FML complex antigen [36]. The increase in anti-*LdNH* IgG1 antibodies was predominant in 72% of the infected seropositive dogs and highly correlated to symptomatology ($P = 0.035$) [34]. Therefore, among the *L. chagasi* naturally infected dogs, a minor proportion remains with a predominant IgG2 response ([34] and this investigation). This might be due to the non-synchronic natural infection. The dogs bearing a recent infection, although positive in their anti-FML IgG response, would still maintain the higher IgG2 absorbance values, found in pre-immune animals. Otherwise, these dogs could belong to the group that shows natural genetic resistance to visceral leishmaniasis [14,33]. Noteworthy, dogs with visceral leishmaniasis are considered oligo-symptomatic if showing up to two symptoms and polysymptomatic when showing three or more symptoms of the disease. In this investigation, among the naturally infected animals, more oligo-symptomatic dogs were found in the group showing an IgG2 predominant response (77%) than in the IgG1 predominant group (48%). This difference was highly significant ($\chi^2 = 6.541$; $P < 0.025$), supporting the two previous hypothesis of natural resistance [14] or delay in the onset of disease.

Using QS21 as adjuvant of the outer surface proteins OsPA and OsPB of *Borrelia burgdorferi*, in a dog vaccine formulation against Lyme disease, Ma et al. observed a four-fold higher IgG1 and eight-fold higher IgG2 antibody responses in vaccinees than in control dogs [37]. Only the IgG2 subtype was significantly correlated with the protective anti-borrelial activity, as detected by a complement fixation in vitro assay [37]. Furthermore, a live attenuated *Salmonella typhimurium* vaccine was used to orally deliver the EgDf1 antigen of *Echinococcus granulosus* in dogs [38]. A pronounced increase in IgG2 specific antibodies and minor levels of IgG1 were seen in vaccinated dogs. Taken together, these results support the hypothesis that, in dogs, the IgG2 response might be associated to a TH1-like response whereas the IgG1 subtype would be associated to a TH2 response [38].

In this investigation, we confirmed the differential IgG1/IgG2 production profile in dogs with visceral leishmaniasis infection, with the IgG1 subtype associated to natural infection and the IgG2 subtype correlated to the highly protective FML-vaccine treatment [4–9]. These results might be the embasement for the development of new diagnostic tools for canine visceral leishmaniasis that will allow to distinguish sera sample of vaccinated or infected dogs. This information will be helpful in large scale screenings for epidemiological kala-azar control [3].

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