

Chapter 2

***Leishmania infantum*-specific IgG, IgG1 and IgG2 antibody responses in healthy and ill dogs from endemic areas. Evolution in the course of infection and after treatment**

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Abstract

The expression of IgG, IgG1 and IgG2 specific antibodies for *Leishmania infantum* was studied in five groups of dogs in Catalonia (Spain): I, 99 asymptomatic dogs (infected and uninfected) from a highly endemic area for leishmaniosis; II, 139 untreated dogs with clinically patent leishmaniosis; III, 11 naturally infected asymptomatic dogs monitored for up to five years since they were found seropositive to *Leishmania* antigen and without treatment; IV, 25 naturally infected dogs with clinically patent leishmaniosis and treated with either meglumine antimoniate and allopurinol or allopurinol alone; and V, six experimentally infected dogs, treated with meglumine antimoniate and controlled for five years. The levels (ELISA units) of IgG, IgG1 and IgG2 in asymptomatic dogs (group I) were very variable (24 ± 33 , 32 ± 31 and 26 ± 31 , respectively), and, as expected, lower than in ill dogs (group II) (168 ± 34 , 84 ± 71 and 172 ± 31 , respectively). In both groups, the correlation between IgG and IgG2 levels ($r=0.95$, $P<0.001$ in group I and $r=0.63$, $P<0.001$ in group II) was higher than between IgG and IgG1 levels ($r=0.01$, $P>0.05$ in group I and $r=0.31$, $P<0.001$ in group II). In group III, IgG and IgG2 expression increased during infection, while IgG1 expression remained the same. In dogs of group IV, IgG levels after one year of treatment decreased more in responsive (mean values, 163 ± 42 before treatment (b.t.) and 100 ± 36 after treatment (a.t.)) than in unresponsive dogs (158 ± 29 b.t. and 124 ± 51 a.t.), especially for IgG1 (94 ± 89 b.t. and 20 ± 21 a.t. in responsive dogs and 35 ± 25 b.t. and 22 ± 13 a.t. in unresponsive dogs) rather than for IgG2 (156 ± 16 b.t. and 114 ± 45 a.t. in responsive and 151 ± 11 b.t. and 125 ± 36 a.t. in unresponsive dogs). Similar results were observed in the evolution of experimentally infected animals after consecutive and specific treatments. Overall results show the great variation in *Leishmania*-specific IgG1 expression in asymptomatic and symptomatic dogs, their lack of correlation with that of IgG2 and chemotherapy is more effective in dogs with initially high expression of IgG1.

Key words: *Leishmania infantum*, IgG, IgG1, IgG2, response to treatment, dog

Introduction

Canine leishmaniosis, caused by *Leishmania infantum*, is endemic in the Mediterranean basin and its seroprevalence ranges from 10% to 37% (Amela *et al.*, 1995; Bettini & Gradoni, 1986; Fisa *et al.*, 1999). The clinical features of such infection are variable. Some dogs develop active disease, whereas others do not or the disease resolves spontaneously. The immune response to *L. infantum* in the dog is humoral and cellular, which results in a wide array of responses: from resistant dogs with predominant cellular response to susceptible dogs with exaggerated humoral response (Abranches *et al.*, 1991; Cabral *et al.*, 1998; Carrera *et al.*, 1996; Pinelli *et al.*, 1994).

The serological follow-up of natural and experimental *Leishmania* infections in dogs, with or without treatment, has been reported elsewhere. Treatment of ill dogs induces temporary clinical improvement, often accompanied by a decrease in the specific antibody levels (Lanotte *et al.*, 1979; Mancianti *et al.*, 1988; Riera *et al.*, 1999). However, in other cases clinical improvement has not been associated with decrease in the titre of specific antibodies (Ferrer *et al.*, 1995).

Various studies have described the levels of specific *Leishmania* IgG subclasses (IgG1 and IgG2) in ill, asymptomatic and treated dogs, sometimes with conflicting results (Bourdoiseau *et al.*, 1997; Deplazes *et al.*, 1995). The varying immunoresponse of infected animals and the small number of animals studied may account for this controversy. This study aimed to determine the level of *Leishmania*-specific total IgG (IgG), IgG1 and IgG2 antibody responses in the sera of a wide range of canine populations: symptomatic and asymptomatic dogs from endemic areas and naturally and experimentally infected, as well as the evolution of the infection in animals that were either treated with anti-*Leishmania* specific drugs or not. The results of this study may clarify the significance and prognostic value of the *Leishmania*-specific IgG subclasses in canine leishmaniosis.

Material and methods

Animals and sera

Five groups of dogs were established.

Group I. Ninety-nine asymptomatic dogs, infected or not, living in an endemic area (the Priorat, a rural region in the north-east of Spain). Examination for external signs of disease and peripheral blood collection were carried out during the antirabies vaccination campaign performed during spring and early summer, coincidental with the start of the phlebotomine season.

Group II. One hundred thirty-nine naturally infected symptomatic dogs without previous specific chemotherapy. They were examined at the Veterinary Teaching Hospital, Facultat de Veterinària (UAB), and routinely checked for parasitological, serological and general biochemistry analyses (total protein concentration, protein serum electrophoresis, urea nitrogen and creatinine values). Dogs were from 7 months to 11 years old with a mean \pm standard deviation age of 5 ± 3.6 years.

Group III. Eleven asymptomatic dogs from the Priorat area that seroconverted to *Leishmania* antigen. They were monitored for *Leishmania*-specific antibodies and external clinical signs of disease at annual intervals for up to six years since they had been found seropositive.

Group IV. Twenty-five naturally infected symptomatic dogs treated at the Veterinary Teaching Hospital, Facultat de Veterinària (UAB). One blood sample was collected before chemotherapy and at least two sera samples were taken during a follow-up of at least one year, after specific chemotherapy. Either meglumine antimoniate (20 mg Sb⁵/kg every 24h x 30 days) and allopurinol (10 mg every 12h x 12 months) (16 dogs) or allopurinol alone (10 mg every 12h x 12 months) (9 dogs) were administered, according to the clinician criteria. All dogs were routinely checked for signs of disease and for general biochemistry analyses (total protein concentration, protein serum electrophoresis, urea nitrogen and creatinine values).

Group V. Six beagle dogs experimentally infected by intravenous inoculation of 5x10⁷ promastigotes in 0.5 mL of saline solution of *L. infantum* (MCAN/ES/92/BCN-83/MON-1). They were kept at the Animalarium in the Facultat de Veterinària (UAB). All were subjected to treatment with meglumine antimoniate (20 mg Sb⁵/kg every 12h x 20 days); four received a second treatment with liposome-encapsulated meglumine antimoniate (9.8 mg Sb⁵/kg every 24h x 20 days) and one was treated again with liposome-encapsulated meglumine antimoniate and allopurinol (10 mg every 12h x 8 months). They were monitored for five years, during which clinical, haematological and parasitological controls were performed under the auspices of the UAB animal care committee.

The sera were stored at -40°C until analyzed.

ELISA

The ELISA was performed as described elsewhere (Riera *et al.*, 1999). Anti-dog IgG1, IgG2 and IgG conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, TX, USA) were used as second antibodies. The working dilutions for each conjugated antibody were determined according to the titration performed for the lot (1:2000 for anti-dog IgG1, 1:2000 and 1:5000 for anti IgG2 and 1:15000 and 1:20000 for anti-IgG). The reaction was quantified as units (U) related to a positive serum used as calibrator and arbitrarily set at 100 U. The cut-off was established at 20U for IgG, 22U for IgG1 and 11U for IgG2 (mean + 4 standard deviations of 32 dogs from non-endemic areas).

Delayed type of hypersensitivity (DTH)

DTH was performed by injection of leishmanin reagent as described elsewhere (Solano-Gallego *et al.*, 2000).

Statistical analysis

Statistical analysis, Student's *t*-test (paired and independent) and coefficient *r* of Pearson were performed by using the SPSS programme.

Results

Leishmania-specific IgG, IgG1 and IgG2 in asymptomatic and symptomatic dogs (Groups I and II) (Fig. 1 and Table 1)

The level of *Leishmania*-specific IgG in asymptomatic dogs from the Priorat endemic area (group I) was very variable and ranged from 1 to 242 U. Only 62 animals were considered seronegative for total IgG (<20U) according to the cut-off established. The level of the IgG subclasses also was very variable and ranged from 1 to 219U for IgG1 and from 2 to 229U for IgG2. The IgG2 rate was correlated with that of total IgG ($r=0.948$, $P<0.001$) but the IgG1 rate was neither correlated with IgG2 nor with IgG ($r=0.086$, $P=0.395$; $r=0.008$, $P=0.934$ respectively). In these dogs, when the levels of IgG and IgG2 were low, that of IgG1 was variable, but when the levels of IgG and IgG2 were high, that of IgG1 was always low.

In ill dogs (group II), the levels of IgG, IgG1 and IgG2 were higher than for group I ($P<0.001$), as expected. High correlation between IgG and IgG2 levels ($r=0.631$, $P<0.001$) and a weaker correlation between IgG and IgG1 ($r=0.310$, $P<0.001$) were found, whereas no correlation was observed between IgG1 and IgG2 levels ($r=0.063$, $P=0.460$).

The clinical and biochemical features of 139 ill dogs are recorded in table 2.

Table 1. Anti-*Leishmania* specific immunoglobulins (mean and standard deviation) in different groups of dogs

<i>Groups of dogs</i>	<i>Total IgG</i>	<i>IgG1</i>	<i>IgG2</i>
I. (Asymptomatic) n=99	24±33	32±31	26±31
II. (Symptomatic) n=139	168±34	84±71	172±31
IV. (Pre-treatment) n=25	164±35	76±77	156±14
IV. (Post-treatment) n=25	111±41	22±18	121±40
IV A (Pre-treat. Responsive) n=16	163±42	94±89	156±16
IV A (Post-treat. Responsive) n=16	100±36	20±21	114±45
IV B (Pre-treat. Unresponsive) n=9	158±29	35±25	151±11
IV B (Post-treat. Unresponsive) n=9	124±51	22±13	125±36

ELISA values quantified as units (U) related to a positive serum used as calibrator that was considered to have 100 U.

Table 2. Abnormal clinical and biochemical features in 139 dogs with patent leishmanial disease

PHYSICAL	% OF DOGS	BIOCHEMISTRY	% OF DOGS
Skin involvement	79.7	Ratio albumina/globulines less than 0.59	63.2
Lymphadenomegaly	49	Hypergammaglobulinemia	57.8
Weight loss	35.7	Hypoalbuminemia	60.1
Somnolence (apathy)	33	Hypergammaglobulinemia and hypoalbuminemia	52
Abnormal locomotion	16	Azotemia	24.2
Diarrhea	13	Hypercreatinemia	18.7
Vomiting	10	High total serum protein	49.2
Ocular lesions	10		
Pale mucoses	10		
Hyperthermia	10		
Abnormal nails	8		
Epistaxis	8		

Longitudinal analysis of Leishmania-specific IgG, IgG1 and IgG2 in animals that seroconverted (Group III) (Fig. 2)

Eleven dogs from the Priorat region seroconverted to the *Leishmania* antigen during the serological control of leishmaniosis performed in this area every year. They were followed up until five years after seroconversion and showed no external clinical signs of the disease. They were considered infected after detection of seroconversion. The mean values of the antibody response showed that the level of IgG and IgG2 increased during infection. However, the level of IgG1 remained roughly the same.

Longitudinal analysis of Leishmania-specific IgG, IgG1 and IgG2 in treated dogs (Group IV) (Tables 1 and 3)

Leishmania-specific immunoglobulins were measured in 25 ill dogs that were monitored over one year after treatment. Both the antibody level and response kinetics varied between the groups studied. We classified the 25 dogs into two groups according to the clinical status of the animal one year after treatment, irrespective of the treatment received: Group IV.A, responsive animals, apparently healthy and with normal biochemistry values (10 were treated with Sb and allopurinol and six with allopurinol alone), and Group IV.B, unresponsive animals with signs of disease and/or abnormal biochemistry values (six treated with Sb and allopurinol and three with allopurinol alone). The mean values for total IgG, and IgG2 at diagnosis in groups IV.A and IV.B were not different (Student's *t* test, $P > 0.05$). However, the mean value for IgG1 at diagnosis in group IV.A (94 ± 89) was higher than in group IV.B (35 ± 25) (Student's *t* test, $P = 0.02$). When the 25 treated dogs were considered, the mean rates of total IgG, IgG1 and IgG2 significantly decreased after treatment (paired Student's *t* test, $P < 0.001$). In group IV.A, the mean levels of IgG, IgG1 and IgG2 also decreased notably after treatment (paired Student's *t* test, $P < 0.01$). The decrease was less obvious in dogs from group IV.B (paired Student's *t* test, $P = 0.048$ for IgG, $P = 0.053$ for IgG2 and $P = 0.112$ for IgG1). Individual results are shown in Table 3.

Table 3. *Leishmania* specific immunoglobulins levels and clinical outcome in 25 treated dogs

Levels of <i>Leishmania</i> specific immunoglobulins		Treatment outcome	
Before treatment	After treatment	Responsive n=16	Unresponsive n=9
High level of IgG, IgG1 and IgG2. n=12	High level of IgG and IgG2 remained. IgG1 level decreased	3	1
	High level of IgG, IgG1 and IgG2 remained	1	0
	Level of IgG, IgG1 and IgG2 decreased	6	1
High level of IgG and IgG2 and low level of IgG1 n=13	Level of IgG, IgG1 and IgG2 remained	2	5
	Level of IgG, IgG1 and IgG2 decreased.	4	2

Longitudinal analysis of IgG, IgG1 and IgG2 in experimental canine leishmaniosis after infection and treatment (Group V) (Fig. 3)

Figure 3 represents the evolution of the total IgG, IgG1 and IgG2 of six dogs experimentally infected with *L. infantum*. After inoculation, once the animals showed a pathological protein serum electrophoresis, they were treated with a free form of meglumine antimoniate.

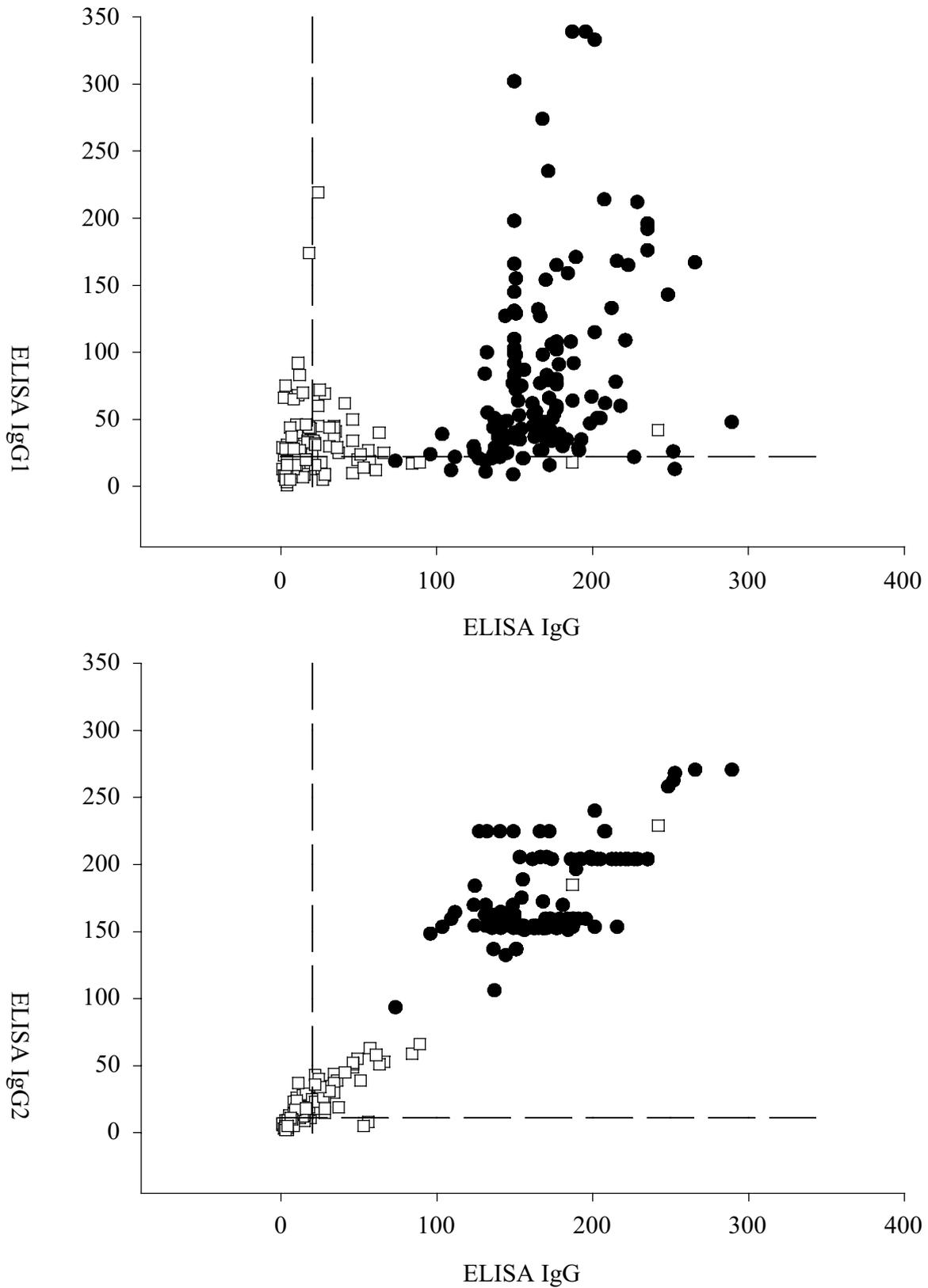
Dogs 2c29 and 6839 showed high levels of IgG and IgG2 but low levels of IgG1, once the disease appeared. Both animals died of renal failure, one immediately after treatment and the other one year later.

Dogs 6835 and 4103 showed high levels of IgG and IgG subclasses at the beginning of the disease. After repeated treatments and a lengthy and extensive follow-up, the levels of IgG and IgG2 decreased slowly. IgG and IgG2 levels remained positive throughout the study, while IgG1 decreased to background levels. These dogs did not have clinical relapses after the second treatment and the DTH to *Leishmania* antigen was positive at the end of the follow-up.

Dog 312854 first presented high levels of IgG and IgG subclasses, which did not decrease after two treatment regimes with free and encapsulated meglumine antimoniate. A third treatment with meglumine antimoniate and allopurinol resulted in a clinical and biochemical improvement. The level of IgG1 decreased but IgG and IgG2 remained high.

Dog 2c61 had high levels of IgG and IgG2 but low levels of IgG1 at the beginning of the disease. Its IgG profile remained the same until the end of the follow up. The disease evolved gradually and became chronic after the second treatment. Its DTH to *Leishmania* antigen and that of dog 312854 at the end of the study were negative.

Fig 1. Anti-*Leishmania* specific immunoglobulins in 99 asymptomatic (□) (Group I) and in 139 symptomatic (●) (Group II) dogs. Long dash lines, cut-off absorbances for each immunoglobulin.



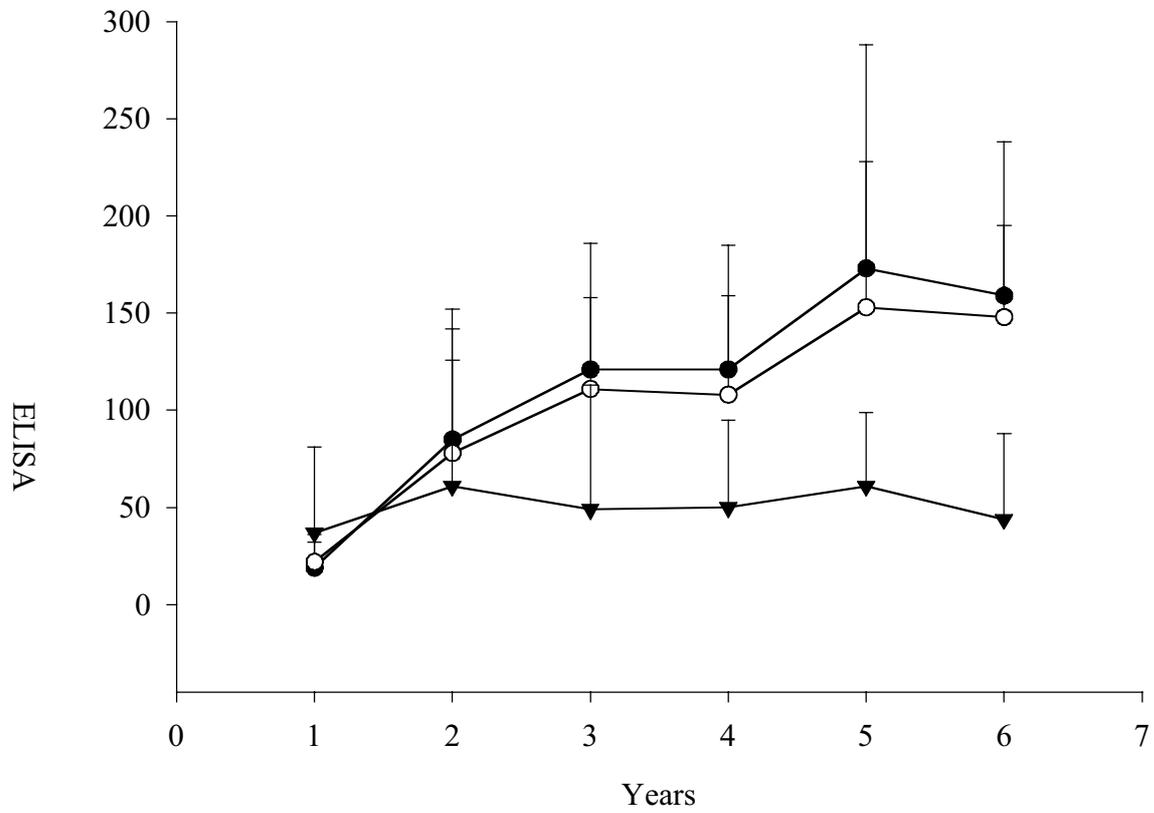


Fig 2. Anti-*Leishmania* specific immunoglobulins in 11 apparently healthy dogs over time after seroconversion. ELISA IgG (●), IgG1 (▼) and IgG2 (O). (Group III).

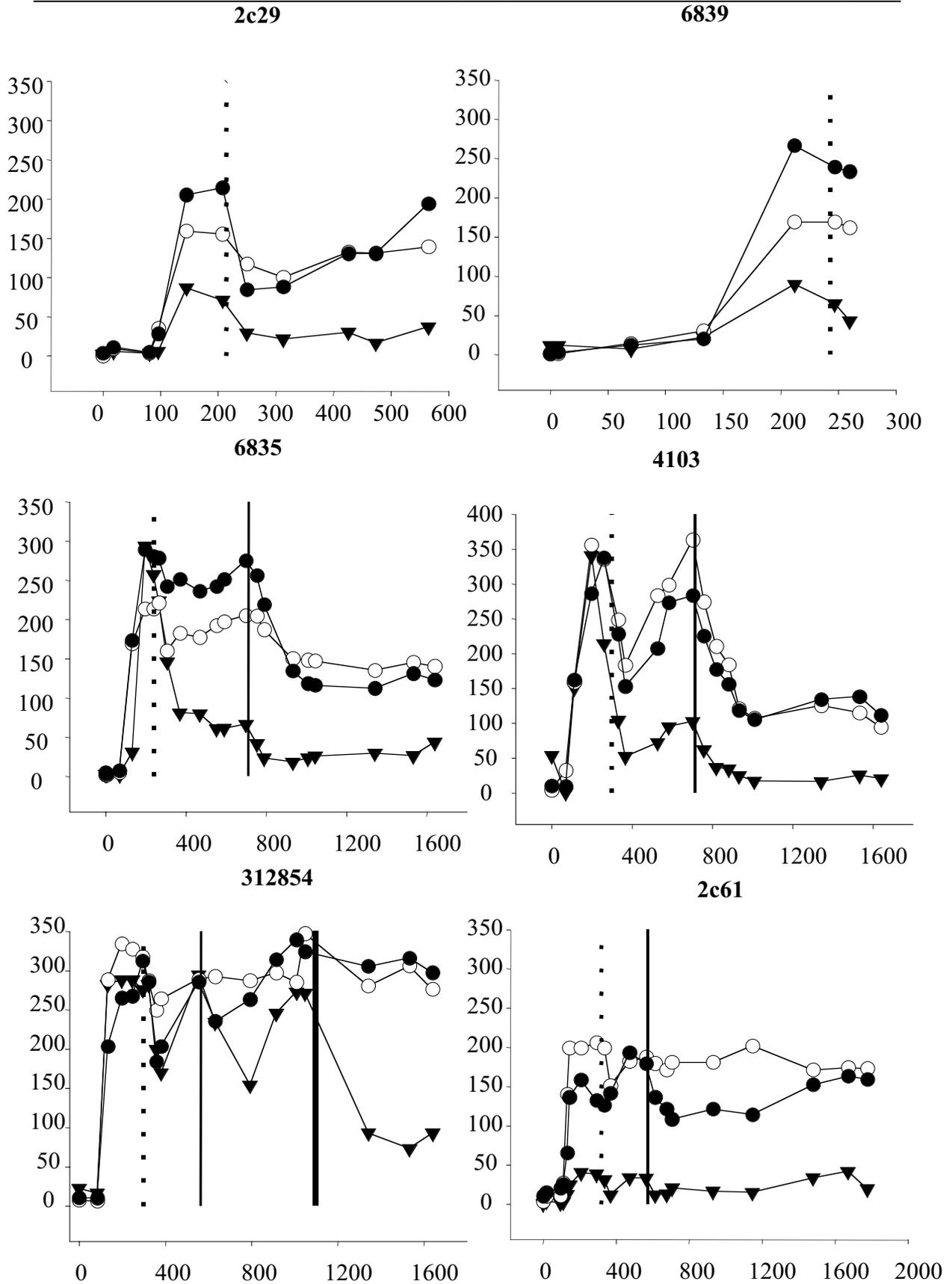


Fig 3. Anti-*Leishmania* specific immunoglobulins in six experimentally infected dogs after infection and treatment. ELISA IgG (●), IgG1 (▼) and IgG2 (○). Dotted lines, first treatment; heavy lines, second treatment; extra heavy line, third treatment. Y axis, ELISA; X axis, days after infection. (Group V).

Discussion

Many studies have aimed to associate the polarised T cell helper responses with *Leishmania* infections. Cytokines, T-cell subsets and immunoglobulin classes and subclasses have been examined in murine experimental models and in human leishmaniasis in order to understand the immunological mechanisms that control parasite persistence and multiplication.

Specific *Leishmania* IgG subclasses (IgG1 and IgG2) were first described in dogs with clinical patent disease and in treated dogs by Deplazes *et al.* (1995), who report that ill animals initially present high levels of both *L. infantum*-specific IgG1 and IgG2. After long treatment and clinical improvement, the level of IgG1 decreases and that of IgG2 remains constant. These authors suggest that titres of specific IgG1 and IgG2 are more reliable indicators of disease status than total IgG. They also hypothesise the association of IgG2 with an asymptomatic infection and that of IgG1 with the development of the disease. This expression of IgG subclasses in asymptomatic and symptomatic dogs, before and after treatment is not described by Bourdoiseau *et al.* (1997). Nieto *et al.* (1999) report a direct correlation between the appearance of clinical signs of canine leishmaniasis and high levels of both IgG1 and IgG2 in experimental infections.

Here, the study of several large cohorts of dogs highlights these discrepancies. The levels of total IgG and IgG2 were correlated in all the groups studied (asymptomatic, symptomatic and naturally and experimentally infected treated dogs) and, as expected, they were significantly higher in symptomatic than in asymptomatic dogs. However, the correlation between IgG1 and IgG was very variable, in agreement with a previous report (Solano-Gallego *et al.*, 2000), which has failed to correlate IgG subclasses in an asymptomatic canine population. Here, the asymptomatic canine population is composed by uninfected and infected dogs with differential immune responses to the parasite. If the cut off for IgG (>20U) is considered a tool for distinguishing infected and uninfected animals, non-infected animals (IgG<20U) have a variable IgG1 response, but asymptomatic infected animals (IgG>20U) have a low IgG1 response (Fig. 1). This low IgG1 response remains during the infection even when IgG and IgG2 responses significantly increase (Fig. 2).

In the naturally and experimentally infected symptomatic dogs, total IgG and IgG2 were high, as reported (Bourdoiseau *et al.*, 1997; Cavaliero *et al.*, 1999; Deplazes *et al.*, 1995), whereas IgG1 was extremely variable, ranging from background to high levels. Cavaliero *et al.* (1999) reported that IgG1 concentrations at the beginning of treatment were not as high as described by other authors (Deplazes *et al.*, 1995).

After treatment, anti-parasite IgG and IgG2 levels decrease very slowly and IgG1 expression drops quickly in responsive dogs (Deplazes *et al.*, 1995), whereas the decrease in unresponsive dogs is less marked or only temporary. In addition, IgG1 levels at diagnosis were significantly higher in responsive (group IV.A) than in unresponsive dogs (group IV.B), which also showed a large decrease in all immunoglobulins, similarly to experimentally infected dogs. Thus, low levels of IgG1 (Cavaliero *et al.*, 1999; Deplazes *et al.*, 1995) are valuable good prognosis indicators only if detected after treatment in animals with high expression of this immunoglobulin before chemotherapy.

The predominance of levels of specific IgG isotypes should be carefully associated with Th1- or Th2-like activity. The immunological mechanisms that regulate the susceptibility or resistance to visceral parasitism by *Leishmania* are still unclear. The polarised Th1-Th2 response reported in the infection by *L. major* in mice (Locksley & Reiner, 1995; Reed & Scott, 1993) is not so clear in human visceral leishmaniasis (Anam *et al.*, 1999; Mary *et al.*, 1999), in *L. infantum*-infected mice (Honoré *et al.*, 1998; Kaye *et al.*, 1991; Miralles *et al.*, 1994), or in *L. infantum*-infected dogs (Cabral *et al.*, 1998). A mixed Th1-Th2 response or other T-cell clones are probably involved. A CD4⁺ T cell population produces IL-5 and IFN- γ in asymptomatic human leishmaniasis (Mary *et al.*, 1999). Other accessory cells and signals, such as CD8⁺T cells, diverse antigen-presenting cells, variable interleukin interactions and chronicity may be key factors in the modulation of the IgG isotype response in dogs.

Data on the mechanisms that control residual intracellular *L. infantum* infection after chemotherapy and the function of cytokines in this regulation are scarce. However, this study suggests that control of infection after successful chemotherapy in dogs involves an antibody response.

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