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Analysis of the humoral immune response against total and recombinant antigens of *Leishmania infantum*: correlation with disease progression in canine experimental leishmaniasis

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Abstract

Leishmaniasis by *Leishmania infantum* in the Mediterranean Basin constitutes an important problem in both human and veterinary medicine. Based in both the importance of canids as reservoirs for the human disease and the fact that the canine disease may be an excellent model for the human condition, the present work has been conducted to analyze clinical and immune mechanisms associated with canine experimental leishmaniasis. Six-month-old mixed-breed dogs were intravenously infected with *L. infantum* promastigotes and the infection course was monitored along a 343 days-period. On day 75 post-infection (p.i.), amastigotes were observed in the lymph nodes of all dogs. The analysis of the humoral response against total *L. infantum* antigens by both ELISA and Western blotting evidenced a correlation between the levels of IgG isotypes (IgG1 and IgG2) and disease progression. It was observed that in those animals showing either a regressive or an oligosymptomatic form of the disease, the anti-*Leishmania* IgG1 antibodies were undetectable whereas those animals developing active disease showed high levels of anti-*Leishmania* IgG1 antibodies. Additionally, the time-course of antibody production against *L. infantum* recombinant antigens in the experimentally infected dogs has been analyzed. The present data suggest that reactivity against the heat-shock protein 70 (HSP70) may be used as diagnostic marker of early steps of infection, and that the appearance of anti-histone antibodies is associated with progression of infection to disease status.

Keywords: *Leishmania infantum*; Dogs; Experimental infection; Clinical features; IgG1; IgG2

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1. Introduction

Parasites of the genus *Leishmania* are intracellular protozoa that infect macrophages of mammals including humans. These protozoa are distributed in tropical and sub-tropical areas and it is estimated that a third of the World's population live in endemic areas. In the Mediterranean area, visceral leishmaniasis (VL) caused by *Leishmania infantum* is an endemic disease that affects both humans and dogs. *L. infantum* parasites are transmitted to hosts by sand fly vectors of the genus *Phlebotomus.* It is assumed that the domestic dog is the main reservoir for the human disease. The incidence of canine VL in the Mediterranean countries varies between 10–37% (Bettini and Gradoni, 1986; Ozbel et al., 1995). Therefore, the prevalence of canine VL represents a serious risk of infection for the human beings, particularly for immunocompromised patients (Badaro et al., 1986). Furthermore, it has been suggested that *Leishmania* parasites can play a cofactor role triggering the pathogenesis of the HIV infection (Tremblay et al., 1996). Given the fact that canine and human leishmaniasis share similar disease symptoms, including fever, hypergammaglobulinaemia, hepatosplenomegaly and anaemia, dogs represent an appropriate animal model for the human disease. Additionally, the study of canine VL is justified by itself considering the prevalence of the disease among the dog populations. From the analysis of clinical and pathological symptoms accompanying canine VL disease, two main groups of responses to infection have been evidenced in both naturally and experimentally infected dogs (Abranches et al., 1991; Pinelli et al., 1994; Carrera et al., 1996). Most of the infected animals are susceptible and develop active disease, while a small percentage is resistant to the infection since they do not develop the disease or it resolves spontaneously. The most common manifestation of canine disease is a viscero-cutaneous leishmaniasis (VCL), showing renal, hepatic, ocular, cutaneous and even cerebral alterations (Nieto et al., 1992; García-Alonso et al., 1996a, b).

In order to develop specific assays for the serodiagnosis of canine leishmaniasis a search for parasite antigens recognized by sera from VCL dogs has been performed in our laboratory. As a result of this work, several *L. infantum* antigens were isolated and characterized among which it is noteworthy to mention the acidic ribosomal proteins P2a and P2b (Soto et al., 1995a), the ribosomal protein P0 (Soto et al., 1995b), the histones H2A (Soto et al., 1995c) and H3 (Soto et al., 1996), the heat shock proteins HSP70 (Quijada et al., 1996) and HSP83 (Angel et al., 1996), and the KMP-11 (Berberich et al., 1997). The prevalence of specific antibodies against those recombinant antigens among the VL dogs has been also determined, indicating that these antigens represent valuable tools for serodiagnosis of canine leishmaniasis. However, since the prevalence values were determined by the use of sera from dogs with active disease, an important question to know is whether these antigens can be used for serodiagnosis of early infection. In order to direct solve this question, in this work, we have analyzed the time course of the immune humoral response against these cloned antigens in dogs experimentally infected with *L. infantum*. The results indicate that the humoral response against each one of the antigens shows different time-course of appearance and that specific features of the humoral response may be correlated with the VCL outcome. As a conclusion from this work, we have defined two main classes of antigens: (a) antigens that are recognized

early during infection, and (b) antigens that may be used as molecular markers of predictive value for the clinical evolution of the infection.

2. Materials and methods

2.1. Animals

A litter of five mixed-breed dogs, three males (n° 1, 2 and 4) and two females (n° 3 and 5) were raised under parasite-free conditions in the animal facility of the Veterinary Faculty of the University of Extremadura (Cáceres, Spain). Four of the animals (n° 1, 2, 3 and 4), 6 months old, were intravenously inoculated (on days 0 and 28) with 5×10^5 *L. infantum* promastigotes (autochthonous isolate M/CAN/ES/96/BCN 150, zymodeme MON-1) in 1 ml of PBS. Animal n° 5 was kept as the uninfected control and received 1 ml of PBS. The dogs were maintained according to the Guiding Principles for the Care and Use of animals. Every day the animals were clinically controlled by physical examination. Blood samples were taken on day 0, prior to infection, and every 15 days after the infection. The sera were frozen at −80°C until use. From day 75 p.i. (post-infection) aspirates of popliteal lymph node were performed monthly to detect the presence of amastigote forms by direct microscope examination and by culture in NNN medium. On day 343 the animals 1, 3 and 4 were treated with Glucantime® (Rhône Mérieux).

2.2. Recombinant antigens

The expression and purification of the recombinant antigens used in this study have been described elsewhere. Most of the recombinant antigens, HSP70 (Quijada et al., 1996), HSP83 (Angel et al., 1996), P2a and P2b (Soto et al., 1995a), P0 (Soto et al., 1995b), histones H2A (Soto et al., 1995c) and H3 (Soto et al., 1996) were expressed as fusion products with the maltose binding protein and purified by affinity chromatography on amylose columns (New England Biolabs). The recombinant protein KMP-11 (Berberich et al., 1997) was purified by affinity chromatography on Ni-NTA columns (Qiagen). The purified recombinant antigens were analyzed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then by Coomassie blue staining to assess the purity of the preparations. The protein content of each antigen preparation was determined by the Bradford assay (Bradford, 1976).

2.3. Enzyme-linked immunosorbent assays (ELISA)

The antigen used for the ELISA determinations was obtained from late-log phase cultures of *L. infantum* promastigotes (M/CAN/ES/88/CHUMI, LEM 2002). Briefly, parasites were harvested and washed several times in phosphate-buffered saline (PBS; pH 7.2) and the suspension was sonicated for 3 s with 3 s-interval during 15 min (Vibracell). The protein concentration was adjusted (Bradford, 1976) to 8 μg/ml. Aliquots of these proteins were stored at −80°C until used. The ELISA-plates (96 well; Inotech) were

coated with the antigen overnight at 4°C (100 μl per well of a solution of 8 μg/ml). After three washes with PBS-0.05% Tween 20 the plates were blocked with 5% skimmed milk for 30 min at 37°C. After three washing procedures, the plates were incubated for 30 min at 37°C with the sera at a dilution of 1/400 in PBS-Tween 20. The plates were again washed three times with PBS and incubated with 100 μl per well of goat anti-dog IgG1–Fc-peroxidase or sheep anti-dog IgG2–Fc-peroxidase (Bethyl Lab., INC, Montgomery, TX) for 30 min at 37°C. The IgG1–Fc and IgG2–Fc conjugates were diluted in PBS-Tween 20 at 1/500 and 1/3000, respectively. After the plates were washed, the enzymatic reaction was developed with *o*-phenyldiamine (Sigma Co.). The reaction was stopped 30 min later by addition of 50 μl of 3 N H_2SO_4 to each well. Absorbance values were read at 490 nm in an automatic micro-ELISA reader.

In ELISA assays with recombinant proteins, the plates were coated overnight at 4°C using 100 μl of the antigen diluted in PBS. The antigen concentration was 1 μg/ml for all the recombinant proteins. Incubation and blocking conditions were the same as indicated above. The conjugated anti-dog IgG was used at 1/8000 dilution.

2.4. Immunoblotting

Total proteins from *L. infantum* promastigotes were separated by SDS-PAGE and analyzed by Western blotting. Nitrocellulose membranes were blocked for 30 min with TBS (Tris 100 mM, NaCl 1.3 M, HCl 50 mM, EGTA 10 mM) containing 3% of skimmed milk and 0.05% Tween 20. Afterwards, the membranes were incubated for 1 h at 37°C with the sera at a dilution of 1/40 dilution in the same blocking solution. After three washes with TBS-0.05% Tween 20, membranes were incubated with either horseradish peroxidase labelled goat anti-dog IgG1–Fc or horseradish peroxidase labelled sheep anti-dog IgG2–Fc at dilutions of 1/50 and 1/300, respectively. After washing, the specific-binding of antibodies was color-revealed by the use of 4-chloronaphthol as peroxidase substrate (Sigma Co.).

3. Results

3.1. Clinical evolution of experimentally infected dogs

The clinical evolution of the four experimentally infected dogs was studied throughout a 343 days-period. 75 days post-infection (d.p.i.), amastigotes were observed in popliteal lymph nodes of all infected animals. Dogs 1, 3 and 4 remained parasitologically positive during the study, whereas dog 2 became negative at day 202 p.i. A moderate lymphoadenomegaly was the first symptom observed in all the dogs at day 94 p.i. At this time dogs 1 and 2 developed a subclinic or oligosymptomatic form of the disease (more evident in dog 1) showing exfoliative dermatitis on the nose and ear tips. Dog 1 showed onychogryposis from day 150 p.i. and a more intense and generalized dermopathy from day 186 p.i. Dog 2 showed a slight dermatitis with alopecia from day 108 p.i. which disappeared at 202 d.p.i. Dogs 3 and 4 developed a typical VCL symptomatology with progressive weight loss, alopecia, exfoliative and ulcerative dermatitis in tail, ears and

Table 1
Clinical features from dogs experimentally infected with *L. infantum*

Animal	Disease form	Symptomatology (d.p.i.)
1	Latent	Adenopathy (94) Dermatitis (123) Onychogryposis (150)
2	Latent Regressive	Adenopathy (94) Dermatitis (108) Regression (202)
3	Patent (VCL)	Weight loss (94) Adenopathy (94) Dermatitis (123) Onychogryposis (136) Ocular alterations (189)
4	Patent (VCL)	Weight loss (94) Adenopathy (94) Dermatitis (123) Onychogryposis (136) Ulcerations (205)

VCL – viscero-cutaneous leishmaniasis.
d.p.i. – days post-infection.

extremities. From day 136 p.i. onychogryposis became to be evident until 312 d.p.i. Keratoconjunctivitis was also observed 6 months p.i. in dogs 3 and 4. Thus, from a clinical point of view, three different evolutive forms of leishmaniasis were observed: a subclinic self-cured form (dog 2), a latent or oligosymptomatic form (dog 1), and a patent or symptomatic evolutive form (dogs 3 and 4). Thus, according to the clinical-pathological symptoms, despite the small number of animals used in the present experimental canine leishmaniasis study, the different outcomes of *L. infantum*-infection were similar to those observed during natural infection (Lanotte et al., 1979) (Table 1).

3.2. Kinetics of anti-Leishmania IgG1 and IgG2 antibodies in dogs experimentally infected with L. infantum

Fig. 1 shows the time course of the relative IgG1 and IgG2 reactivity against total *L. infantum* antigens in the sera from each one of the infected dogs. Serum samples were obtained at the indicated days and assayed by ELISA using *L. infantum* total proteins as coating antigen. Positive levels of anti-*Leishmania* antibodies in the sera from the infected animals were observed on day 40 p.i. except for serum from dog 3 that was positive on day 78 p.i. The reactivity values and evolution of IgG1/IgG2 isotypes were different depending on the animal considered. Thus, dog 1 showed a positive IgG2 reactivity against *Leishmania* antigens through the entire period of infection. However, only serum sample on day 40 p.i. from dog 1 showed a significant reactivity of anti-*Leishmania* IgG1 antibodies, the reactivity of the rest of serum samples remained close to background level (reactivity of serum from control dog n° 5). Serum samples from dog 2

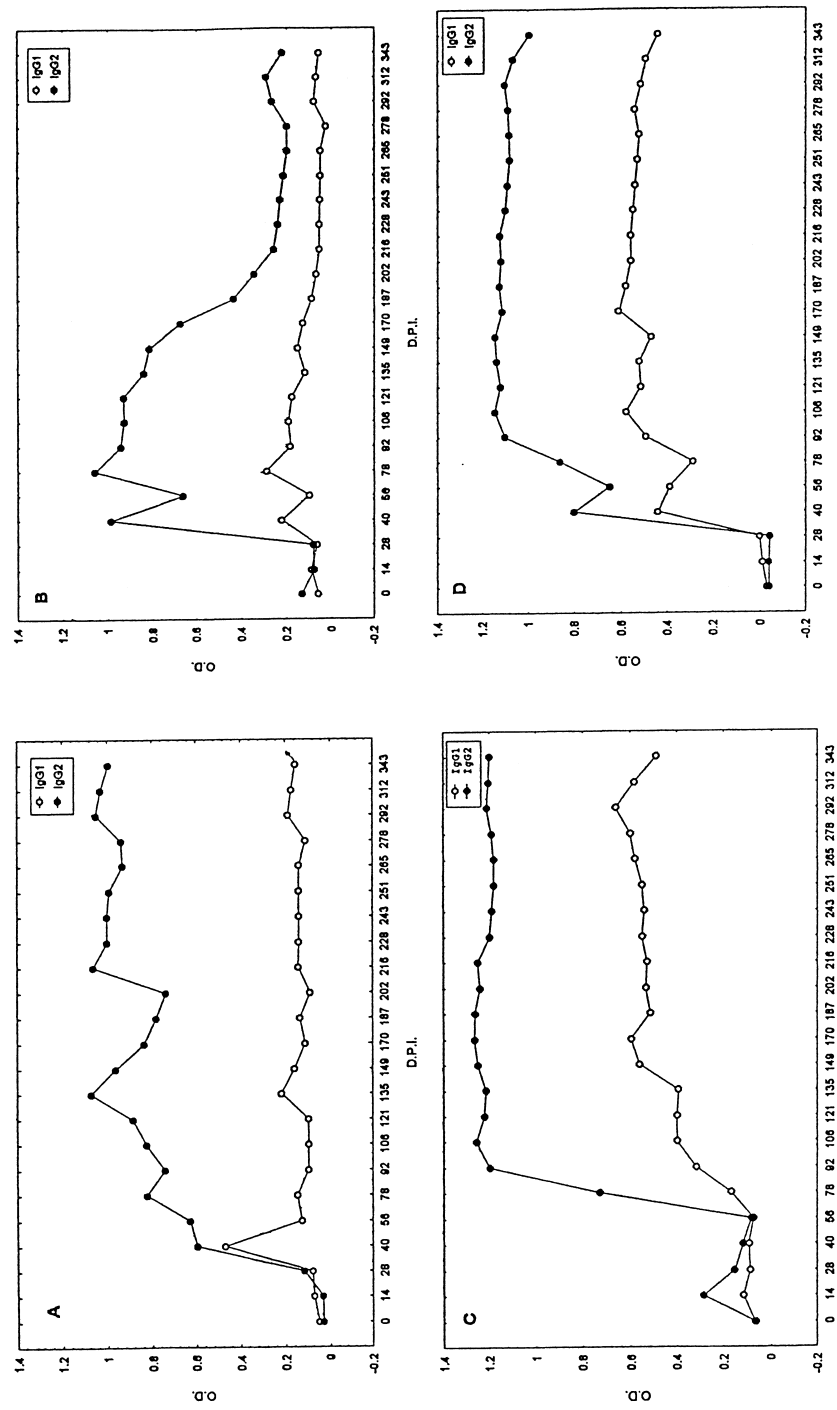

Fig. 1. Kinetics of specific anti-*L. infantum* IgG1 (solid circles) and IgG2 (open circles) antibodies in experimental canine leishmaniasis. (A) Dog 1, latent oligosymptomatic form. (B) Dog 2, latent regressive form. (C) and (D), dogs 3 and 4, patent evolutive forms (viscerocutaneous leishmaniasis).

showed significant IgG2 anti-*Leishmania* reactivity from day 40 to 170 p.i., from day 170 to 216 the reactivity values of serum decreased, and from day 228 to the end of experience the reactivity values of serum samples were negative. In addition, in this dog the IgG1 anti-*Leishmania* reactivity of all serum samples were close to those of control serum (dog n° 5). The humoral response elicited by *L. infantum* infection of dogs 3 and 4 showed a remarkable homology. Thus, significant serum levels of anti-*Leishmania* antibodies of both IgG1 and IgG2 isotypes were observed from day 78 p.i. for dog 3 and from day 40 p.i. for dog 4 to the end of the experience. In our conditions, the IgG2 reactivity was about two-fold higher than the IgG1 reactivity. However, it should be noted that both IgG1 and IgG2 specific reactivity showed similar kinetics of induction.

3.3. Western blotting analysis

Western blotting was used to identify the parasite antigens recognized by serum samples from the experimentally *L. infantum*-infected animals. Also, this technique was used to analyze weather or not specific polypeptides were recognized by antibodies from either IgG1 or IgG2 isotype. Fig. 2 shows the reactivity of the sera from the experimentally infected dogs against the *L. infantum* polypeptides as revealed by an IgG1 and IgG2 binding. The sera from days 78, 106 and 216 p.i. of all the animals were selected because they were considered as the most representative ones of the humoral immune response in the ELISA assays. As expected from ELISA data, the number of polypeptides recognized by the IgG2 antibodies was higher than the number of these polypeptides reacting with IgG1. The most intensively IgG2 labelled bands corresponded to proteins of apparent molecular masses of 84, 70, 55, 36–20 kDa. The sera from dogs 3 and 4 recognized a larger number of polypeptides. Interestingly, the low molecular weight

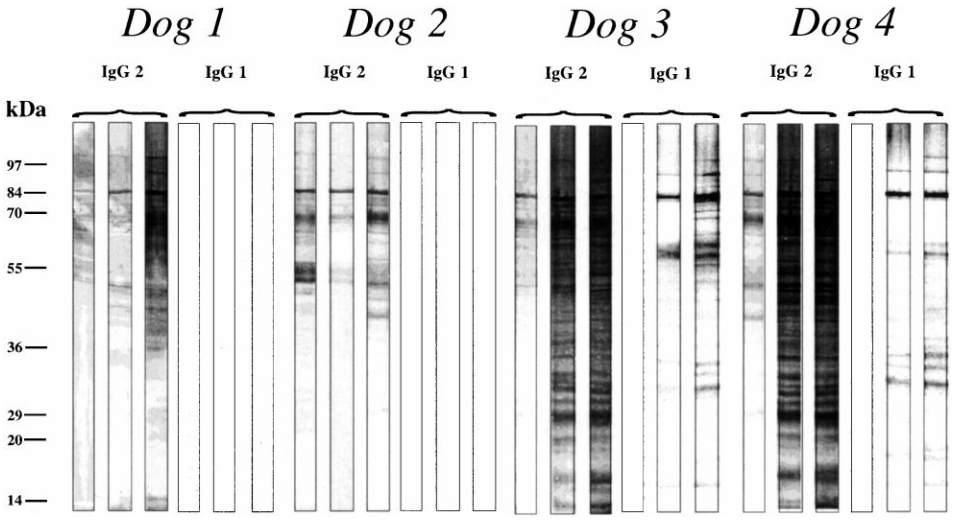

Fig. 2. Western blot analysis of *L. infantum* antigens recognized by sera (isotypes IgG1 and IgG2) from the experimentally infected animals (dogs 1, 2, 3 and 4), corresponding to days 78, 106 and 216 post-infection (p.i.).

polypeptides showed delayed reactivity since they were only labelled by the sera obtained from dogs 3 and 4 on days 106 and 216 p.i. In agreement with the ELISA determinations the dichotomous form of the disease and of the immune response between the infected animals was clear when the specific IgG1 response was observed. While the serum samples of dog 1 (with latent and oligosymptomatic infection) and dog 2 (with the regressive form) did not show any reactivity by IgG1 antibodies against *Leishmania* proteins, in sera from dogs 3 and 4 (with the patent VCL form) a strong IgG1 reactivity was observed mainly against polypeptides of high molecular weight. Finally, it was observed that a similar pattern of proteins was recognized by either IgG1 or IgG2 antibodies from dogs 3 and 4, indicating that the antigen-specificity of IgG isotypes was the same.

3.4. Humoral response against heat shock proteins HSP70 and HSP83

Fig. 3 shows the reactivity of sera against the recombinant *L. infantum* HSP70 during a follow-up period of 343 d.p.i. Anti-HSP70 antibodies were detected in the sera from all four dogs as early as 40 d.p.i. However, the time course of appearance of the anti-HSP70 reactivity was different depending on the evolution of the infection. Thus, the anti-HSP70 humoral response of dog 1 showing the asymptomatic infection form was variable along the study with maxima at days 78, 243 and 292. The sera from dog 2 in which the regressive form was observed showed the highest anti-HSP70, reactivity at 78 d.p.i. which decreased gradually thereafter to control levels (pre-infection sera) at day 228. In

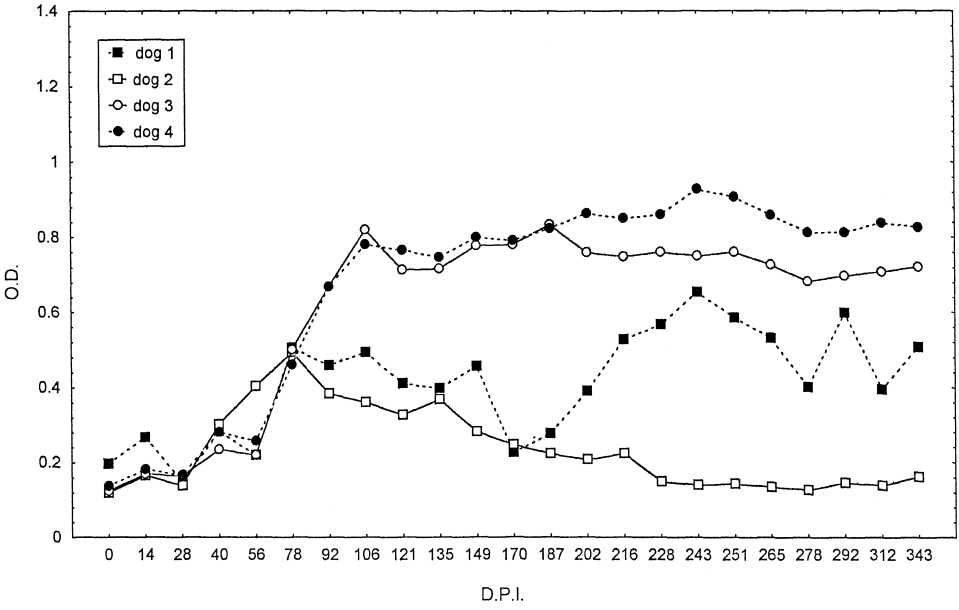

Fig. 3. Levels of anti-HSP70 antibodies in the *L. infantum*-infected dogs throughout the experimental period.

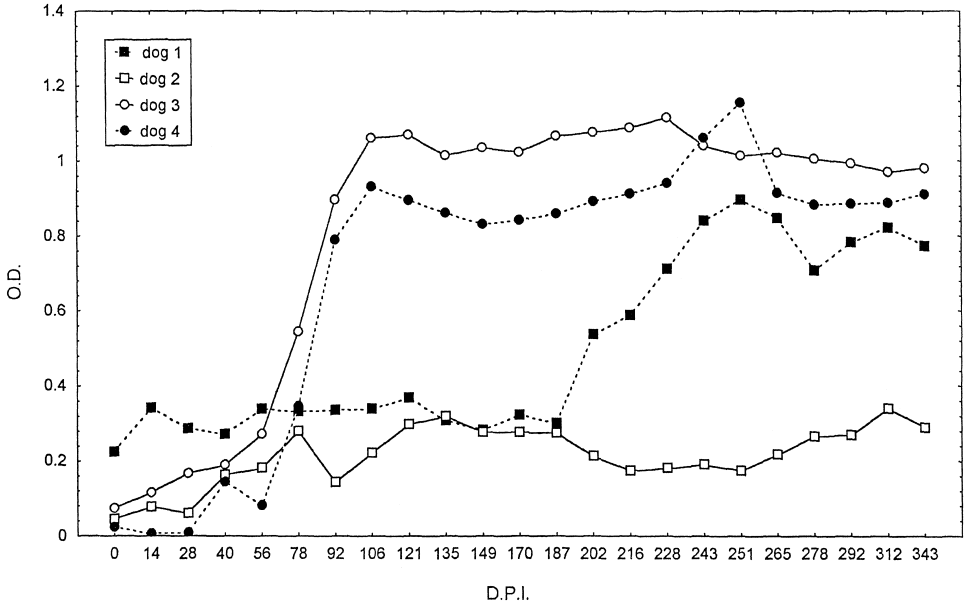

Fig. 4. Levels of anti-HSP83 antibodies in the *L. infantum*-infected dogs throughout the experimental period.

contrast, the sera from dogs 3 and 4 showed the highest anti-HSP70 reactivity at 106 d.p.i. that was maintained up to the end of the experience.

The evolution of the humoral response against the HSP83 is illustrated in Fig. 4. The reactivity of the serum samples against the HSP83 protein followed a similar pattern to that observed against the HSP70 protein, although with slight differences in the level of reactivity and the kinetics of appearance. The serum samples from dog 1 showed the highest positive reactivity against the protein at 202 d.p.i. as an indication of a delayed induction relative to the response against the HSP70. The anti-HSP83 reactivity of the serum samples from the dog 2, showing the regressive form, were very low along the entire experimental period. The time-course of appearance and the intensity of the anti-HSP83 responses in the sera from dogs 3 and 4 was similar in both cases and also similar to the response against the anti-HSP70 protein. In both animals, the higher reactivity values were observed at 106 d.p.i.

3.5. Humoral response against histones H2A and H3

Significant positive reactivities against the H2A and H3 histones were only detected in the serum samples from the dogs which progressed to the patent and symptomatic VCL form (dogs 3 and 4; Figs. 5 and 6). In sera from those dogs, positive reactivity values were clearly detected on day 92 p.i. The reactivity values showed by serum samples from dog n° 2 were not significantly different to that of control serum (serum obtained before infection). However, after 92 d.p.i., the serum samples of dog n° 1 showed a weak, but significant, anti-H2A reactivity, although clearly lower than that present in samples from

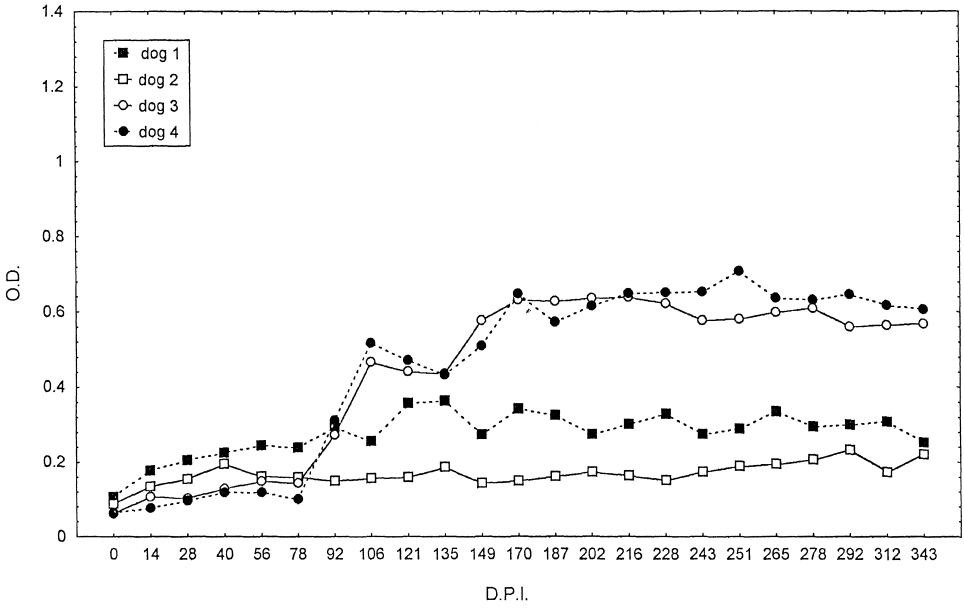

Fig. 5. Levels of anti-H2A antibodies in the *L. infantum*-infected dogs throughout the experimental period.

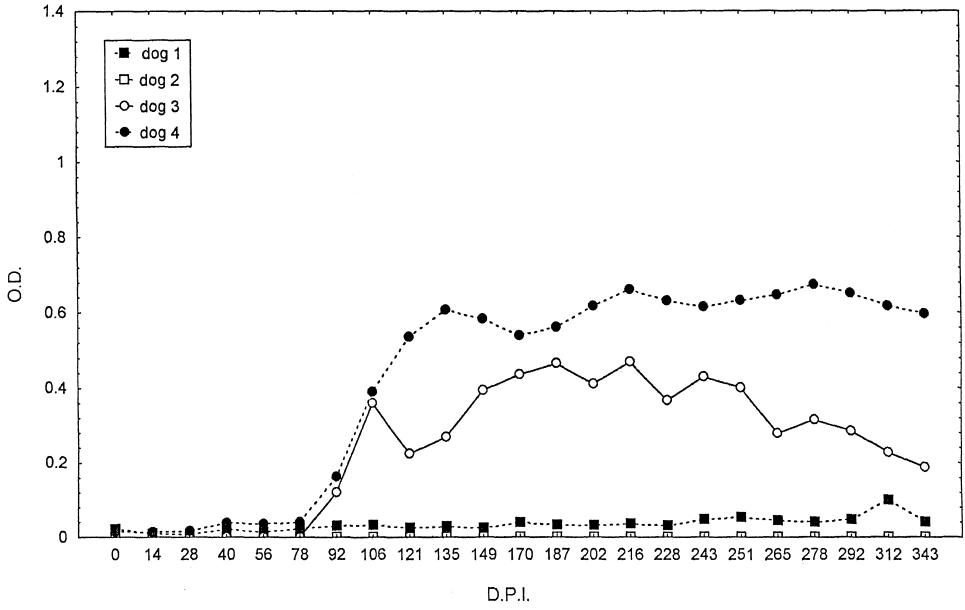

Fig. 6. Levels of anti-H3 antibodies in the *L. infantum*-infected dogs throughout the experimental period.

dogs n° 3 and n° 4. Anti-histone H3 antibodies were not observed in any of the serum samples from dogs 1 and 2.

3.6. Serum reactivity against the ribosomal proteins

During the 343 days of study, none of the serum samples from the experimentally infected dogs showed reactivity against the *Leishmania* ribosomal proteins P2a, P2b, and P0, indicating that these antigens were not able to trigger any humoral response in those animals (data not shown). These results were surprising since it is known that sera from dogs naturally infected with *L. infantum* contain high levels of antibodies against the acidic ribosomal proteins P2a and P2b (Soto et al., 1995a) and against the ribosomal protein P0 (Soto et al., 1995b).

3.7. Serum reactivity against the KMP-11 protein

As shown in Fig. 7 all the experimentally infected dogs developed a significant anti-KMP-11 antibody response. In dog 2, showing the regressive form of the infection, the highest anti-KMP-11 reactivity was on day 78 p.i. and it slowly decreased to reach background levels at day 265 p.i. In dog 1, showing an asymptomatic infection, moderated anti-KMP-11 reactivity values were detected from the onset on day 78 to the 202 d.p.i. Then, the anti-KMP-11 reactivity drastically increased to reach the highest level on day 243 p.i. remaining high to the end of the experiment. The anti-KMP-11 reactivity of serum samples from dog n° 3 was patent after 92 d.p.i., and at day 106 arose the high reactivity values that characterized the serum samples of this dog (Fig. 7). In dog n° 4, the anti-KMP-11 antibodies were observed from day 106 p.i. to the end of the experience (day 343). Although dogs 3 and 4 evolved to a patent VCL disease, the anti-KMP-11 reactivity values shown by the serum samples from dog 4 were lower than those shown by samples from dog 3 (Fig. 7).

4. Discussion

The aim of this study was to analyze the humoral response in dogs experimentally infected with *L. infantum* in order to determine the potential of antibodies to a set of recombinant antigens for diagnosis of active infection and prognosis of disease progression. Earlier studies have indicated that in a typical focus of canine leishmaniasis the patent cases represent 40–50% of the infected population, the pre-clinical cases represent 30–40%, and the resolving cases represent 10–30% (Bettini and Gradoni, 1986). It is remarkable that, although the data are not statistically significant, the animals experimentally infected in this experience have evolved differently, and representatives of the main outcomes to *L. infantum* infection have been observed. Thus, according to disease symptoms, the animal can be grouped in: (a) oligo- or asymptomatic dogs (numbers 1 and 2), and (b) dogs with patent VCL (numbers 3 and 4). Within the former group, while dog 1 remained parasitologically positive along the experience, dog 2 (from day 202 p.i.) was found parasite-free and, therefore, it can be considered as self-cured

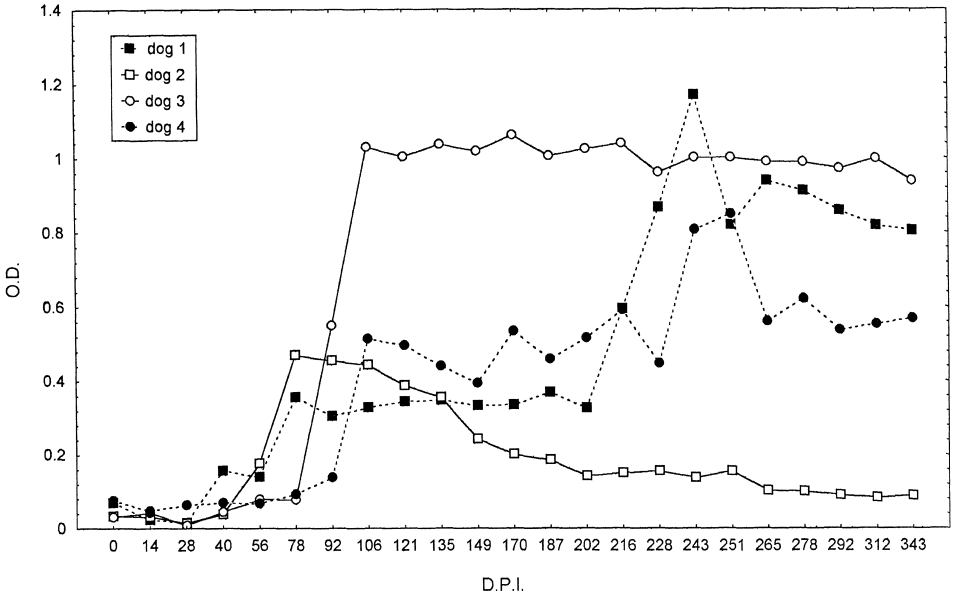

Fig. 7. Levels of anti-KMP-11 antibodies in the *L. infantum*-infected dogs throughout the experimental period.

(regressive infection). Animals showing regressive VL forms have been also described in canine experimental infections by Pinelli et al. (1995).

A conclusion drawn from this study, that is in accordance with previous ones (Gicheru et al., 1995), is that the solely presence of anti-*Leishmania* antibodies is not a conclusive sign of disease progression, because in all infected animals a strong humoral response against *L. infantum* antigens was induced. However, the analysis of IgG subsets in the infected dogs provides evidence of a direct correlation between induction of high levels of IgG1 anti-*Leishmania* antibodies and appearance of clinical symptoms of disease. Thus, while dogs 3 and 4, which evolved to a patent form of VCL, showed high values of both IgG1 and IgG2 reactivities, the latent and the regressive forms observed in dogs 1 and 2, respectively, associated with background levels of IgG1 reactivities. Therefore, regarding the dichotomous IgG1 response, it could be suggested an association between the presence of anti-*Leishmania* IgG1 antibodies and the chronic VCL. This conclusion agrees with the data from dogs naturally infected with *L. infantum* reported by Deplazes et al. (1995). These authors found that dogs infected with *L. infantum* produced both IgG1 and IgG2 antibodies with IgG2 being associated with asymptomatic infections and IgG1 being associated with disease. Given that IgG isotypes represent markers for the Th1/Th2 dichotomous immune response (Kawano et al., 1994), the presence of elevated levels of IgG1 reactivity in the dogs developing VCL (numbers 3 and 4) may be an indication of the production in these dogs of significative levels of Th2 derived cytokines. Although it is likely that the IgG isotype responses are merely downstream effects of cell-mediated immune responses, it cannot be excluded a direct effect of antibody subclasses in the pathology associated with leishmaniasis disease. For example, since it is known the IgG1

antibodies are important effectors of complement activation, it can be postulated that elevated levels of IgG1 antibodies may trigger complement activation which mediate inflammatory reactions leading to the pathological manifestations of the disease.

From the analysis of the time-course of appearance of specific antibodies against defined *L. infantum* antigens, several features must be pointed out. First, only the *Leishmania* antigens HSP70 and KMP-11 were recognized by the sera of all experimentally infected dogs. Second, it is noteworthy that the anti-HSP70 antibodies are observed as early as 40 d.p.i., indicating that this protein could represent a molecular tool for diagnosis of *L. infantum* infection in early steps. Third, the presence of high reactivity against histones H2A and H3 can be taken as an indication that anti-histone antibodies may be involved in the pathological process leading to disease progression. This suggestion is supported by the fact that only dogs that develop VCL disease showed significant reactivity against these histones. Fourth, no reactivity was detected against the ribosomal antigens (P2a, P2b and P0) in spite of the fact there is high response against these antigens in natural VCL forms. Two alternative hypothesis may explain this result: (a) the genetic background of the experimentally infected dogs conditions the humoral response against these antigens and (b) the humoral response against this class of antigens is elicited only at very late stages of the canine leishmaniasis disease. Although the high prevalence of antibodies against ribosomal proteins in dogs suffering of visceral leishmaniasis (Soto et al., 1995a, b) favors the second hypothesis more experimental data are needed to support it.

Acknowledgements

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