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Early suppression of lymphoproliferative response in dogs with natural infection by *Leishmania infantum*

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Abstract

Dogs are the domestic reservoirs of zoonotic visceral leishmaniasis caused by *Leishmania infantum*. Early detection of canine infections evolving to clinically patent disease may be important to leishmaniasis control. In this study we firstly investigated the peripheral blood mononuclear cell (PBMC) response to leishmanial antigens and to polyclonal activators concanavalin A, phytohemagglutinin and pokeweed mitogen, of mixed-breed dogs with natural *L. infantum* infection, either in presymptomatic or in patent disease condition, compared to healthy animals. *Leishmania* antigens did not induce a clear proliferative response in any of the animals examined. Furthermore, mitogen-induced lymphocyte proliferation was found strongly reduced not only in symptomatic, but also in presymptomatic dogs suggesting that the cell-mediated immunity is suppressed in progressive canine leishmaniasis. To test this finding, naive Beagle dogs were exposed to natural *L. infantum* infection in a highly endemic area of southern Italy. Two to 10 months after exposure all dogs were found to be infected by *Leishmania*, and on month 2 of exposure they all showed a significant reduction in PBMC activation by mitogens. Our results indicate that suppression of the lymphoproliferative response is a common occurrence in dogs already at the beginning of an established leishmanial infection.

Keywords: Dog; Leishmaniasis; *Leishmania infantum*; Lymphocyte proliferation

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1. Introduction

Dogs are the domestic reservoirs of *Leishmania infantum*, the agent of zoonotic visceral leishmaniasis in the Mediterranean Basin (World Health Organization, 1991). These animals may develop a severe syndrome characterized by chronic evolution of viscerocutaneous signs which result from *Leishmania* multiplication in macrophages of spleen, liver, bone marrow, lymph nodes and skin. After an incubation period of 1 month to several years (Keenan et al., 1984), symptoms appear with weakness, weight loss, diffuse dermatitis, splenomegaly, lymph node enlargement, anemia and renal failure. At this stage, the disease is refractory to conventional drugs successfully used in human therapy. Cross-sectional studies carried out in natural disease foci have shown that only about 50% of the infected dogs display viscerocutaneous involvement, while the remaining animals have no specific signs (Lanotte et al., 1979; Pozio et al., 1981). These asymptomatic dogs have antileishmanial serum antibodies and can be positive to parasite isolation. As suggested by longitudinal studies, asymptomatic cases may represent resistant dogs which can control or even eliminate the parasite, or susceptible presymptomatic dogs which tend to evolve toward patency within a variable period of time (Pozio et al., 1981). Discrimination between these subgroups of animals could be epidemiologically important, since symptoms tend to be associated with higher infectivity to phlebotomine vectors (Gradoni et al., 1987).

The aim of this study was to evaluate leishmanial antigen- and mitogen-induced lymphoproliferative response in naturally infected asymptomatic preclinical dogs, and to confirm our results in a cohort of naive Beagle dogs exposed to naturally transmitted *L. infantum*. We employed lymphocyte proliferation assay (LPA) using crude *Leishmania* soluble (LS) antigen as well as two recombinant leishmanial antigens for the specific response to *L. infantum*, and the mitogens concanavalin A (Con A), phytohemagglutinin (PHA) and pokeweed mitogen (PWM) for T and B cell polyclonal activation.

2. Materials and methods

2.1. Leishmania serology and parasitology

Serum samples were examined by immunofluorescence antibody test using *L. infantum* promastigotes as antigen and a serum dilution of 1 : 40 as threshold titre (Pozio et al., 1981). Sera were also examined by an ELISA test employing the recombinant leishmanial antigen K39 and recently developed as diagnostic kit for canine leishmaniasis (Gradoni et al., 1997). Sera showing absorbance value $\geq$0.4 were considered positive. Lymph node and/or bone marrow aspirate samples were partly smeared on slides and stained with Giemsa's stain for micoscopical examination, and partly seeded into Evans' modified Tobie's medium (biphasic) and 'sloppy' Evans medium (semi-solid) (Evans, 1987).

2.2. Dogs

In the first part of the study, three groups of mixed-breed, adult dogs of both sexes from the Naples area of Italy were used. The first group (controls) consisted of 10 healthy animals with no *Leishmania* on clinical, serological and parasitological examinations. The second group, hereafter designated as presymptomatic dogs, consisted of seven naturally infected animals diagnosed as having leishmaniasis by specific serology and parasitology, but not showing any clinical evidence of the disease when this study was performed. The dogs were housed thereafter in a kennel and followed up for several months after diagnosis. They all developed a viscerocutaneous disease 8 to 10 months later. The third group, designated as symptomatic dogs, consisted of 10 naturally infected animals in a chronic stage of viscerocutaneous leishmaniasis, characterized by organic alterations and by the most common clinical signs of the disease, such as systemic lymph adenopathy, diffuse furfuraceous dermatitis/depilation and skin ulcers. For both groups of infected dogs the duration of leishmaniasis was unknown.

In the second part of the study, five naive Beagles of both sexes (Stefano Morini, Reggio Emilia, Italy), 2 to 4 years old, were housed together in a kennel in a highly endemic area of canine leishmaniasis in southern Italy, during winter months. In this region, the *L. infantum* transmission season (coinciding with the presence of biting phlebotomine sandflies) lasts from late May to late September (Maroli et al., 1993). The dogs were under constant veterinary control to exclude concomitant infections/diseases. Specific serological and parasitological investigations for leishmaniasis were carried out periodically before, during and up to 6 months after natural exposure. By the end of the observation period all dogs showed evidence of an established *Leishmania* infection (positive serology and parasitology), with no evidence of other infections/diseases. Seroconversion and presence of *Leishmania* were shown in each animal at different times: specific antibodies were firstly detected on late July in three dogs, whereas first parasite demonstration was obtained on late October in two dogs. During the observation period, only one dog evolved to a patent clinical condition.

2.3. Leishmanial antigens

LS antigen was obtained by freezing and thawing a suspension of washed *L. infantum* promastigotes three times. The disrupted organisms were then centrifugated at $8000 \times g$ for 30 min and the protein concentration of the resulting supernatant was adjusted at 1 mg/ml. Recombinant leishmanial antigens rLeIF and rLdp23 were a gift from Dr. S. Reed, Corixa, Seattle, USA. They were shown to strongly stimulate cell-mediated immune response in murine and human hosts (Skeiky et al., 1995; Campos-Neto et al., 1995).

2.4. Lymphocyte proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation over Ficoll/Hypaque (density 1.077; Pharmacia, Uppsala, Sweden) for 30 min at $400 \times g$ at room temperature (Boyum, 1968). The mononuclear cell layer was

collected, washed three times in Dulbecco's phosphate buffered saline without calcium and magnesium (PBS), and centrifuged for 15 min at $400 \times g$. The pellet was resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μmg/ml gentamicin, 2 mM l-glutamine and 10^{-5} M 2-mercaptoethanol, hereafter designated complete medium. Mononuclear cell purity was 60–70%, as assessed by Giemsa stain, and cell viability, checked with the trypan blue exclusion test, was greater than 95%. The cell count was adjusted to 1.5×10^6 cells/ml.

LPA was performed using the [^{3}H] thymidine uptake assay as described by Oppenheim and Rosenstreich (1976) and Kristensen et al. (1982). Briefly, PBMC were cultured in triplicate into 96-well flat-bottomed microtitre plates (Falcon plastics, Becton Dickinson & Co., Oxnard, CA), in absence or in presence of antigens or mitogens. Optimal stimulant concentrations and culture times were determined in preliminary kinetic experiments. Each well contained 100 μl of cell suspension plus 100 μl of 20 μg/ml of each antigen, or 4 μg/ml Con A (Sigma, Saint Louis, MO, USA), 2.5 μg/ml PHA (Sigma), or 5 μg/ml PWM (Sigma) in complete medium. Cell cultures were incubated for 5 days (antigens) or 72 h (mitogens) in 90% relative humidity atmosphere, at 37°C and 5% CO_2. Cells were pulsed during the last 6 h of culture with 0.5 μCi of [^{3}H] thymidine (5 Ci/mmol, Amersham, Buchs, UK) and were harvested on glass-wool filters. The [^{3}H] thymidine uptake was measured in a β liquid scintillation counter (LKB-Wallac, Turku, Finland). Proliferative response was expressed as stimulation index (SI), representing the ratio of counts per minute (cpm) of the mitogen-stimulated cultures to the cpm of control cultures. A SI greater than 2.5 indicated a positive response (Cabral et al., 1998).

2.5. Data analysis

Differences in proliferative responses were analyzed for significance using the Student's *t* test.

3. Results

3.1. LPA in mixed-breed dogs

The crude LS antigen did not induce any specific response in either group of infected dogs, whereas the recombinant antigens elicited only a moderate proliferative response in presymptomatic animals, with a mean SI of 2.6–2.8 (Fig. 1).

The highest proliferative response to mitogens was obtained with Con A, whereas PHA induced the lowest one. The mean SI values observed for the three groups after mitogen stimulation are shown in Fig. 2. Nine out of 10 dogs from the control group showed high SI values ranging 25.9–90.1 using Con A, 4.6–42.1 using PHA and 16.3–39.3 using PWM. These parameters were much lower for one dog (7.3, 1.4 and 2.4 respectively). Among the presymptomatic animals the SI values were also different and, on average, significantly lower than those of normal dogs (−62%, $P < 0.05$). Five out of seven animals showed very low SI ranges (2.5−5.1 with Con A, 0.6–4.5 with PHA and 1.5–5.8 with PWM), whereas in two dogs the response to mitogens was within the range recorded

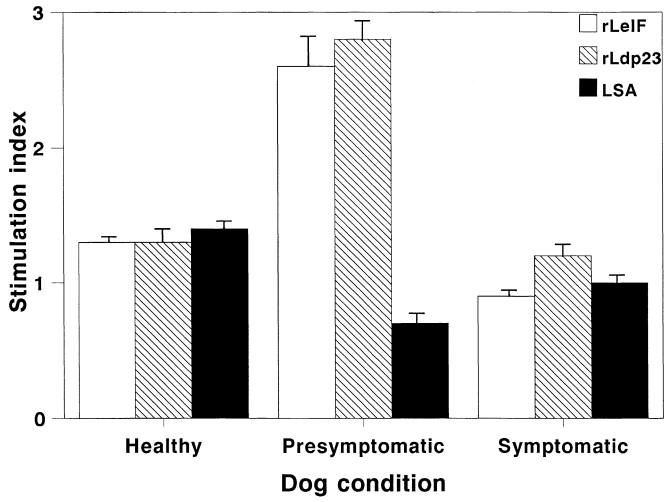

Fig. 1. Lymphocyte proliferative response of PBMC from healthy (10), presymptomatic (7) and symptomatic (10) dogs with leishmaniasis, after incubation with recombinant (rLeIF and rLdp23) and crude leishmanial soluble (LSA) antigens. Data are presented as mean stimulation index + SEM.

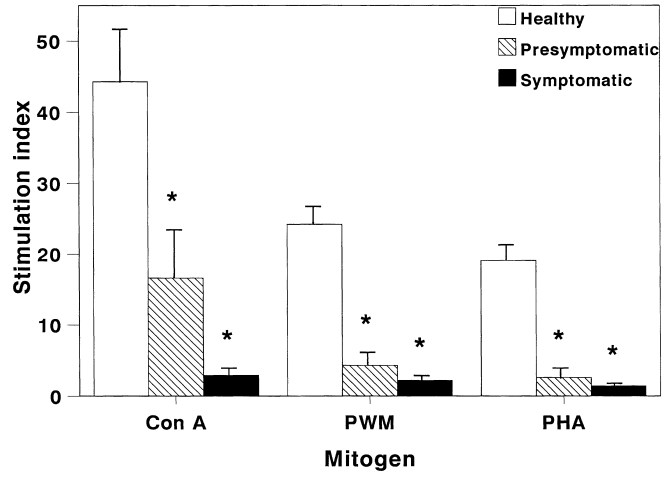

Fig. 2. Lymphocyte proliferative response of PBMC from healthy (10), presymptomatic (7) and symptomatic (10) dogs with leishmaniasis, after incubation with Con A, PWM and PHA. Data are presented as mean stimulation index + SEM. Asterisks indicate significant differences between values for infected animals and healthy controls ($P < 0.05$).

for responsive healthy animals. All the symptomatic dogs displayed a homogeneous proliferative response which was very low compared to controls (−93%), but not significantly different from presymptomatic animals. The SI value range was 1.3–4.2 (Con A), 0.6–2.9 (PHA) and 0.7–4.2 (PWM).

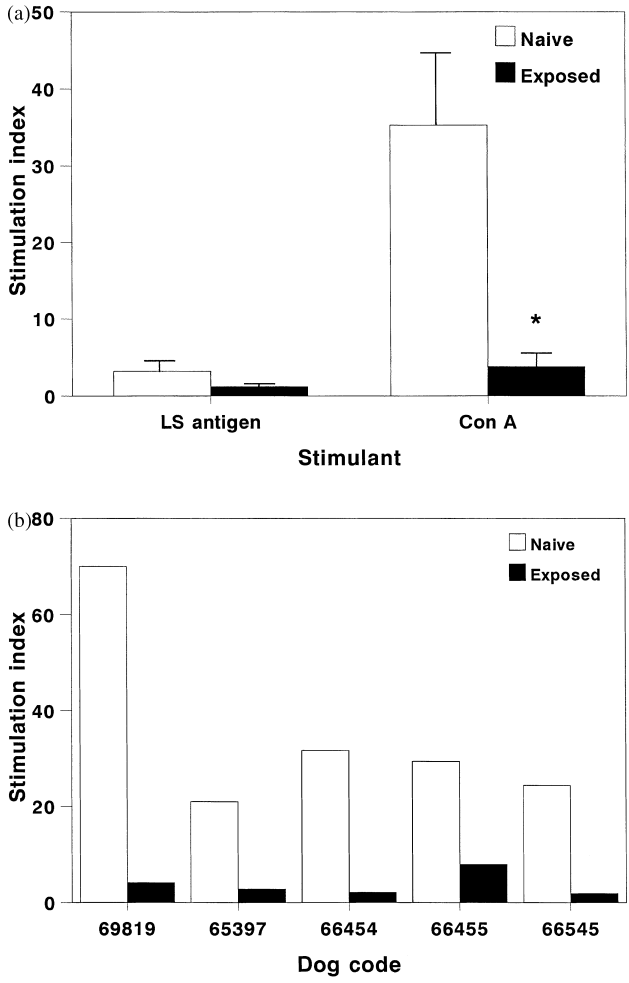

Fig. 3. Lymphocyte proliferative response of PBMC obtained from five Beagle dogs before ('naive') and after exposure to natural *L. infantum* transmission ('exposed'). (a) Mean stimulation index (+ SEM) after incubation with leishmanial soluble (LS) antigen and Con A. Asterisk indicates significant difference between values for naive and exposed dogs ($P < 0.05$). (b) Stimulation index for samples from each dog after incubation with Con A.

3.2. LPA in naive Beagle dogs

LPA was performed with LS antigen as specific stimulator and Con A as mitogen, on PBMC samples taken from Beagles before (March–April), and on month 2 of natural exposure to *Leishmania* (late July). No further PBMC samples were available in the follow-up period.

LS antigen stimulated no proliferative response in any dog before and during the transmission period (Fig. 3(a)). When PBMC from dogs before *Leishmania* exposure

were cultured in presence of Con A, the SI values were as high as those recorded in normal mixed-breed dogs (mean 35.3, range 21.0–70.0). These values were much lower in LPA experiments performed on samples taken after 2 months of exposure (−89%, $P < 0.05$), for a mean SI value of 3.8. This sharp decrease in proliferative response was observed in each dog, irrespectively of seroconversion time, *Leishmania* demonstration or appearance of symptoms. Two dogs resulted unresponsive (SI < 2.5) (Fig. 3(b)).

4. Discussion

Most investigations on the canine response to natural and experimental leishmaniasis have focused on the role of humoral and cell-mediated immunity. There is now evidence that clinically patent *Leishmania* infection is associated with cytokine-mediated impairement of T and B lymphocyte functions (Pinelli et al., 1994; Martinez-Moreno et al., 1995).

Lymphocyte mitogenic stimulation by lectins provides an easy and simple mean to assess the host immunocompetence in a number of diseases. The mitogens employed in our study are classical polyclonal activators – Con A and PHA stimulate T cells, while PWM stimulates both T and B cells – irrespectively of their antigenic specificity and can be used to evaluate the functional status of lymphocyte populations. However, it is well known that these mitogens require more than one cell type, particularly the presence of macrophages and their cytokines, as well as cell–cell cooperation.

Our study has indicated significant immunological differences between healthy and ''apparently healthy'' (but presymptomatic) mixed-breed dogs with leishmaniasis, since cell proliferation to various mitogens was found significantly lower in the latter group. Because the natural history of the infection in these animals was unknown, it could not be excluded that the immunosuppression was the result of a prolonged latent infection, as in other parasitological disorders with chronic features (Cetron et al., 1993). However, our findings on *Leishmania*-exposed naive dogs strongly suggest that suppression of lymphoproliferative response may occur very early as a consequence of an established *Leishmania* infection. This may be substantiated by the following points: first, since they were living in the same environment the dogs were probably all infected during the first period of exposure, as indicated by early seroconversions; second, after 2 months of exposure the animals showed equally profound depression in cellular immune response; third, by the end of the observation period, a leishmanial infection was demonstrated in all dogs; and fourth, on the basis of clinical and laboratory monitoring it was excluded that immunological changes were related to other concomitant infections/diseases.

Few studies have been carried out on lymphoproliferative response to mitogens in dogs experimentally or naturally infected by *L. infantum*, with different results. In the study of Pinelli et al. (1994) on Spanish dogs, symptomatic animals showed a mitogenic proliferative response similar to uninfected control dogs. Although the lymphoproliferative assay employed by these authors was basically the same we used, the study population was different in part. Three out of six symptomatic dogs examined consisted of naive Beagles that had been experimentally infected with a single *Leishmania* dose and that slowly evolved to a patent clinical condition. In our study, naive Beagles have been

exposed to sandfly bites during 2 months, in a site where the force of leishmanial infection is extremely high (Maroli et al., 1993). It is much probable, as it appears from the 100% leishmaniasis incidence found in our dog cohort, that the animals received multiple infective bites, and hence higher antigen doses and/or repeated T-cell stimulation could have provoked rapid establishment of a marked Th2 profile (London et al., 1998), revealed by profound depression in lymphoproliferative response. The different results of ConA response in symptomatic, naturally infected mixed breed dogs of the Pinelli et al. study are more difficult to explain. The three Spanish dogs examined exhibited a mean SI value which was even higher than in healthy animals (269 versus 212, respectively), whereas in our symptomatic mixed breed dogs this was, on average, about one tenth of normal dogs. On the other hand, the Spanish dogs showed partial (1 animal) or full (2 animals) abrogation of IL-2 production. This observation is unclear and in contrast with the general notion that IL-2 is the main cytokine controlling the proliferative response in CD4 lymphocytes. It is also different from findings obtained in murine (Nickol and Bonventre, 1985; Evans et al., 1990) and human (Barral et al., 1986) hosts, which demonstrate that suppression of lymphocyte proliferation is a major immunopathological consequence of infection with a viscerotropic *Leishmania*. The low number of dogs used, the differences in breeds and in epizootiological background, including *Leishmania* strains involved, may account in part for such different results.

Martinez-Moreno et al. (1995) found 65% reduction in PBMC response to Con A stimulation in naturally infected dogs with clinical signs of leishmaniasis. In this case, the different lymphoproliferative assay employed could explain the different rate of response (−93%) observed in our symptomatic animals.

In conclusion, our results indicate that suppression of lymphoproliferative response is common in dogs already at the beginning of an established leishmanial infection. This study may provide a good model to investigate the progressive immune changes in the natural course of canine leishmaniasis, and may be helpful for correlating such changes with parameters relevant to disease control, such as infectivity to vectors or response to antileishmanial drugs and vaccines.

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