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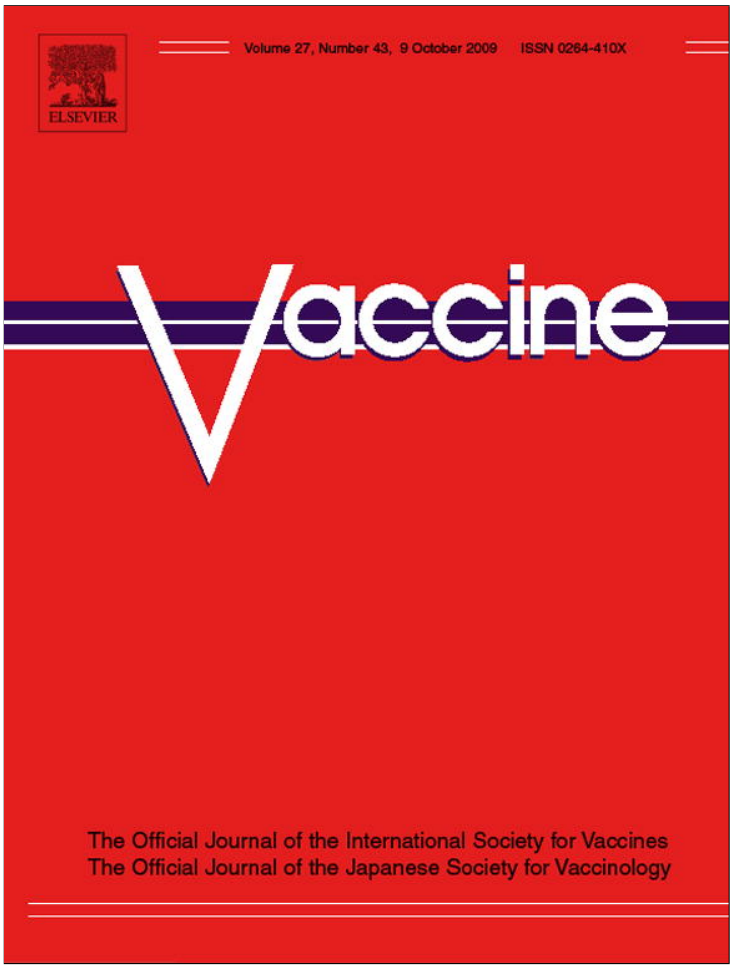

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The Chimerical Multi-Component Q protein from *Leishmania* in the absence of adjuvant protects dogs against an experimental *Leishmania infantum* infection

J. Carcelén[a,*], V. Iniesta[a], J. Fernández-Cotrina[a], F. Serrano[a], J.C. Parejo[b], I. Corraliza[c], A. Gallardo-Soler[c], F. Marañón[d], M. Soto[e], C. Alonso[e], C. Gómez-Nieto[a]

[a] *LeishmanCeres Laboratory (GLP Compliance certificated), Unidad de Parasitología y Enfermedades Parasitarias, Facultad de Veterinaria, Universidad de Extremadura, Avda. de la Universidad s/n, 10071 Cáceres, Spain*
[b] *Unidad de Genética, Facultad de Veterinaria, Universidad de Extremadura, Avda. de la Universidad s/n, 10071 Cáceres, Spain*
[c] *Departamento de Bioquímica y Biología Molecular y Genética, Universidad de Extremadura, Avda. de la Universidad s/n, 10071 Cáceres, Spain*
[d] *Laboratorios LETI S.L., Sol 5. Tres Cantos, 20750 Madrid, Spain*
[e] *Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain*

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ABSTRACT

The protective potential against *Leishmania* infection of the *Leishmania* chimerical Q protein administered as a single (Q) or double dose (Q+Q) without adjuvant was analyzed in a double-blind placebo controlled experiment in dogs. During vaccination the protein induced an intense early anti-Q response but no reactivity against total *Leishmania infantum* proteins was detected. Several end-points were taken into consideration. In the vaccinated animals the amount and intensity of clinical symptoms was lower than in the control group. Pathological signs of disease were observed in liver, kidney and spleen of all dogs from the control group in contrast to the normal appearance of the organs of the vaccinated animals. Vaccination was able to induce parasite clearance in most dogs. Only 1/7 dog was parasite DNA positive in skin in the Q group in contrast to 6/7 dogs in control and 4/7 in Q+Q. Significant anti-SLA clearance was observed in the vaccinated animals at the end of the study. Differences between control and vaccinated animals were also observed at the biochemical level, DTH and nitrite oxide production.

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1. Introduction

The disease caused by the *Leishmania* sp infection has a worldwide distribution being endemic in at least 88 countries. In these countries leishmaniasis is a serious public health problem since 350 million people are at risk. In humans the clinical form of the disease can range from cutaneous and mucocutaneous lesions to visceral active forms, depending upon the *Leishmania* species infecting the host. The visceral form of leishmaniasis (VL) affects an estimated population of 500,000 people each year having negative effects on the economy and social costs of the populations affected by them [1]. In the last decades of the 20th century *Leishmania*/Human Immunodeficiency Virus (HIV) co-infection has been a serious human threat. After the introduction of highly active antiretroviral therapy (HAART) leishmaniasis can be better controlled in HIV-infected individuals having decreased the incidence of visceral leishmaniasis among AIDS patients [2]. The evidence that co-infection with *Leishmania* in HIV-infected subjects can affect the course of either one or both diseases highlights the importance of developing control tools that could limit the spread of both infections [3].

In the Mediterranean basin the visceral form of leishmaniasis, caused by *Leishmania infantum* infection represents a severe endemic disease. Domestic dogs constitute the main reservoir of the disease, playing a key role in the transmission to humans [4]. Since VL is considered to be fatal if untreated canine leishmaniasis seems to be a disease of both veterinary and public health importance. Thus, in a zoonotic visceral leishmaniasis foci a reduction in *Leishmania* transmission would result in an effective preventative measure against the disease in humans. The control of the visceral leishmaniasis clinical form depends on action against the phlebotomines and the animal reservoirs. However, since the chemotherapy available at present against leishmaniasis is far from satisfactory, having a high cost and low efficacy with relapses occurring in most treated dogs [1], vaccination may be the best option to develop an effective strategy for controlling the parasite infection [5,6].

There is general agreement that the establishment of a protective anti-*Leishmania* immune response requires the presentation of appropriate antigens by antigen-presenting cells, the induction and expansion of CD4+ Th1 lymphocytes and the activation of macrophages for efficient killing of parasites. In this context,

* Corresponding author. Tel.: +34 927257131; fax: +34 927257110.
E-mail addresses: jesualdo@unex.es, leishman@unex.es (J. Carcelén).

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most of the recent research dealing with the development of anti-*Leishmania* vaccines has been addressed to the identification of *Leishmania* molecules and carrier systems, able to elicit favourable cytokine phenotypes in *in vivo* models [7,8]. In theory, a cocktail vaccine composed of several *Leishmania* antigens should confer higher protection than a vaccine based on a single antigen. Nevertheless, exceptions to this rule exist since a few single recombinant antigens have been shown to induce protection against canine leishmaniasis. In fact, some of the reported molecularly defined vaccine candidates have shown promising results both in murine and canine models [9].

In the last few decades an increasing number of subunit vaccines, obtained by molecular biology recombinant methodologies, have been described. Some of them have shown to induce some degree of efficacy against experimental leishmaniasis. Recently, several purified proteins like the *leishmania* Fucose-Mannose Ligand (Leishmune®) [10,11], the purified excreted/secreted antigens (LiESAp) [12], the recombinant A2 protein [13], the cysteine proteinases type I (CPB) and type II (CPA) [14], and the multi-subunit recombinant *Leishmania* polyprotein MML, also known as Leish-111f [15–18], among others, have been tested as valuable tools for vaccine development. We have also recently shown that the intraperitoneal administration of a recombinant protein, named Q (PQ) formed by the genetic fusion of five intracellular antigenic fragments, from the *L. infantum* acidic ribosomal proteins Lip2a, Lip2b, P0 and histone H2A [19] when mixed with BCG, is able to protect dogs against a *L. infantum* infection [20]. We have also shown that the subcutaneous administration of the Q protein when mixed with BGC or CpG motifs is able to confer protection to mice against a *L. infantum* experimental infection [21].

Taking into account that the Q protein is a highly immunogenic antigen and that a single dose of the protein induces a strong and predominant IgG2 response in dogs, we have analyzed whether the administration of the protein alone might confer a similar level of protection as the one observed when the protein was administered in the presence of BCG in dogs. After administration of a single or two doses of the protein alone the efficacy of the vaccine was monitored for 330 days by analysis of several end-points. The data presented show that the Q protein when administered to dogs as a single subcutaneous injection in the absence of adjuvants promotes an efficient reduction in parasite load and a clinical protection against a *Leishmania* infection both at the anatomo-pathological and phenotypic levels.

2. Materials and methods

2.1. Animals

21 healthy beagle dogs, 9 males and 12 females, between 1 and 2 years old were purchased from ISOQUINEM S.L. (St. Feliu de Codres, Spain) and bred under vector-borne infection-free conditions. The animals were housed at the Animal Facility Service of the Veterinary Faculty of Cáceres (Uex) according to the Guiding Principles for the Care and Use of animals of the European and National laws. The features of the housing facility allowed controlling the environmental conditions through computerized systems that assured the best lodging and management of the animals for daily care. The study has been designed and conducted for compliance with Good Clinical Practices and Good Laboratory Principles (OCDE). The experiments were approved by the Ethical Committee of the University of Extremadura. All dogs were under constant veterinary supervision and received their routine vaccinations. All animals were also treated with antihelmintic and anti-ectoparasite drugs and maintained in quarantine for a period of 30 days, before the initiation of the experiments. Dogs were distributed into three groups (seven animals per group) taking into consideration sex, weight and age. Sex ratio (m/f) was 4/3.

2.2. Study design. Vaccine antigens and experimental infection

The expression and purification of the recombinant antigen used in this study have been described elsewhere [20]. Briefly, purification of the synthetic recombinant chimerical Q protein [19] expressed in the pQE31 vector was performed on Ni-nitrilotriacetic acid (NI-NTA) resin columns according to the method provided by the supplier (Quiagen®). The purified protein was dialyzed against 0.05 M Borax buffer (pH 9) and lyophilized. The vaccine doses were produced under GMP standards and were chemically and biologically defined as stable. The vaccine (containing 100 μg per dose of the lyophilized Q) and placebo vials were both supplied by Laboratories LETI S.L. reconstituted in 500 μl of sterile bi-distilled water and administered subcutaneously in the left flank of the animals. Group Q received a single Q dose on day 0. Group Q+Q received two Q doses on days 0 and 21, respectively. Placebo vials were administered to control dogs. The study was conducted under double-blind placebo conditions and neither the scientific staff nor the technician personnel were informed of the identity of the vials composition.

At day 60 after the initial day of vaccination (dpv) the animals were intravenously infected with 5×10^5 promastigotes of the *L. infantum* (M/CAN/ES/96/BCN 150, zymodeme MON-1) strain in a 1 ml of isotonic PBS. We have previously reported [21,22], and further corroborated by laboratory data, that this challenge condition induces infection in all animals. The same strain was employed as antigen in all immunological follow-up evaluations. The strain (an authoctonous isolate) was originally derived from a dog having active visceral leishmaniasis. Infection was carried out with log-phase grown promastigotes differentiated from amastigotes that had been isolated from the spleen of heavily infected hamsters (*Mesocricetus auratus*). A primary culture was used. Routine biopathological, immunological and parasitological evaluation of the animals was carried out during the entire period of the experiment (330 days). Serum samples were obtained from each dog for laboratory analyses, as described below. Clinical veterinarians performed the clinical examinations and obtained the analytical data in a double-blind placebo controlled fashion. All biopathological, parasitological and immunological analyses were performed according to standard procedures at the LeishmanCeres Laboratory (GLP Compliance certificated), Parasitology Unit, Faculty of Veterinary Sciences at Cáceres, Spain.

2.3. Vaccine safety evaluation and assessment of susceptibility to infection

Dogs were monitored during the vaccination period (60 days) to evaluate local and/or general reactions upon vaccination. Complete clinical examinations and general health evaluation, including individual rectal temperature and body weight measurements, were carried out weekly. After challenge a physical examination of each dog was carried out monthly. Dogs were clinically classified, according to presence or absence of infection signs [23–25]: asymptomatic (A) without any signs of the disease; oligosymptomatic (O) having 2–3 clinical symptoms such us lymphadenopathy and/or localized alopecia and/or weight loss; symptomatic (S) having characteristic clinical symptoms of visceral leishmaniasis, such us cutaneous alterations, onychogryphosis, keratoconjunctivitis, apathy and cachexia.

During each one of the clinical examinations blood samples were collected for haematological evaluations, biochemical profiles (urea, creatinine and alanine-amino transferase) and serum protein electrophoresis. Urine samples were taken by using bladder catheters. Whole blood cell counting was performed using

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an automatic blood cell counter (ABCvet®). Serum levels of urea, creatinine and alanine-amino transferase were determined (Reflotron®, Roche Diagnostic Ltd, UK) to evaluate renal and liver functions. The determination of serum protein content and electrophoresis was performed using an automatic system, model SAS-3 (Helena®, Beaumont UK). Gels were acid blue stained and washed with bi-distilled water. Finally, electrophoresis readings were analyzed using the Platinum software (Helena®, Beaumont, UK).

Urine analysis for blood, urobilinogen, bilirubin, protein, nitrite, ketones, ascorbic acid, glucose and pH-value was carried out using test strips (Medi-Test Combi 9®, Macherey-Nagel).

2.4. Anatomo-pathological studies

After necropsy specimens from liver, kidney and spleen were taken and fixed in 4% phosphate-buffered formalin, embedded in low-fusion paraffin, cut in 4-μm-thick sections, and stained with hematoxylin-eosin (Isokit®, Bio-Optica, Italy). Slide-mounted sections were examined by light microscopy (Nikon®).

2.5. Parasitological evaluation

The assessment of parasitological infection was performed by the aseptic collection of spleen and lymph node samples from euthanized dogs, followed by culturing in Schneider's medium containing 10% fetal calf serum (FCS) and 100 μg/ml of penicillin–streptomycin (Sigma–Aldrich®, Spain). The presence of parasites was determined by direct examination of the microtitre wells in an inverted microscope. The samples were cultured in sextuplicate for 2 weeks. When parasites could be observed in a single well of the sextuplicate the sample was considered positive. In our hands tissue culture is as sensitive as PCR. Also aspirates of lymph nodes were taken at day 150 post-infection (150 dpi). Spleen and lymph node parasite burden was assessed in impression smears obtained after necropsy of animals. The smears were stained by Giemsa and examined under optical microscopy. For the spleen and lymph nodes the relative parasite burden was estimated as the number of parasites per 1000 nucleated cells.

Lymph nodes and eyelid skin samples were analyzed by the *Leishmania* TaqMan assay after necropsy. Briefly, samples were washed by incubation in 1 ml. of lysis buffer (10 mM Tris–Cl pH 8.0, 0.1 M EDTA pH 8.0, 20 μg/ml pancreatic RNAase and 5% SDS) [26] and100 μg of proteinase K at 56 °C overnight. Afterwards, genomic DNA from 200 μl of sample mix was obtained with the UltraClean™ BloodSpin™ Kit (Mo Bio Laboratories, Inc.). This spin filter method kit yields PCR ready DNA. 5 μl of DNA was used for the PCR. TaqMan-MGB probe and PCR primers were designed, as previously reported [27] to target conserved DNA regions of the kinetoplast minicircle DNA from *L. infantum*. Primers Leish-1 (5′-AACTTTTCTGGTCCTCCGGGTAG-3′) and Leish-2 (5′-ACCCCCAGTTTCCCGCC-3′) were run under universal conditions in the TaqMan assay. The TaqMan-MGB probe (FAM-5′-AAAAATGGGTGCCAGAAAT-3′-non-fluorescent quencher-MGB) was designed to target a conserved region of the kinetoplast (Applied Biosystems, Foster City, CA). The eukaryotic 18S RNA Pre-Developed TaqMan Assay Reagents (Applied Biosystems, Foster City, CA) were used as internal reference of canine genomic DNA. *Leishmania* primers and probe were added at 900 and 200 nM, respectively. Duplicates were amplified for each sample, both with the *Leishmania* and the 18S RNA assays, in a 25 μl final volume reaction mixture with the TaqMan Universal PCR Master Mix with UNG Amperase (Applied Biosystems, Foster City, CA). The thermal cycling profile was 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min [27]. Each amplification run contained positive and negative controls. Samples from a dog under parasite-free conditions were used as calibrators by the comparative Ct method (2-ΔΔCt) allowing determining the presence/absence and levels of parasite DNA in any PCR sample, indepently of the amount of DNA added or the presence of inhibitors.

2.6. Immunological evaluation

2.6.1. ELISA and IFAT analyses

Peripheral blood samples from each dog were collected at the beginning of the quarantine period, before vaccination (pre-bleed, day 0) and after immunization at days 15, 45 and 60 (0 dpi-challenge), and every month after challenge. ELISA was performed as described previously [20]. Briefly, high-binding ELISA plates (96 well, Costar®) were coated for 30 min at 37 °C with 100 μl of sterile PBS containing 2 μg/ml of Q protein or 8 μg/ml of SLA [Soluble *Leishmania* Antigen, obtained from late-log-phase cultures of *L. infantum* promastigotes (M/CAN/ES/96/BCN 150, zymodeme MON-1)]. Sera from immune and control dogs were diluted 1/200 and tested in triplicate. 1/2000 dilution horseradish peroxidase-conjugated sheep anti-dog IgG2 (Q and SLA assays) and 1/500 dilution goat anti-dog IgG1 (SLA assay) from Bethyl Laboratories Montgomery, TX, USA, were used as secondary antibodies. Sera of known reactivity against the antigens obtained from parasite-free and from *Leishmania* naturally infected dogs were included as negative and positive controls, respectively, and analyzed in triplicate in each plate. Plates were developed with OPD substrate (with H_2O_2 in citrate buffer) and the optical density (OD) was read at 492 nm. The cut-off value was set as the mean of absorbance values in negative controls plus 3 SD. IFAT was performed as described [20] using *L. infantum* promastigotes (M/CAN/ES/96/BCN 150, zymodeme MON-1). Sera were assayed in serial two-fold dilutions from 1/20 to ≥1/640 in phosphate-buffered saline (PBS) to determine total IgG levels (IFAT). The immunofluorescent assay was developed using a rabbit fluorescein-labelled anti-dog IgG conjugate (Nordic Immunological Laboratories; Tillburg, the Netherlands) diluted 1/160. The IFAT was considered positive at a dilution of the sera higher than 1/80.

2.6.2. Western blotting

Total proteins from *L. infantum* (M/CAN/ES/96/BCN 150, zymodeme MON-1) promastigotes were separated by SDS-PAGE and analyzed by Western blotting. Nitrocellulose membranes were blocked for 30 min with Tris-Buffered Saline (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 immune and control sera at the 1/40 dilution in the same blocking solution. After three washes with TBS-0.05% Tween-20, membranes were incubated with horseradish peroxidase-conjugated sheep anti-dog IgG2 (1/500) (Bethyl Laboratories Montgomery, TX, USA) for 45 min at 37 °C in TBS-0.05% Tween-20. After washing, the specific binding of antibodies was colour-revealed by the use of 4-chloro-1-naphthol as peroxidase substrate (Sigma–Aldrich®, Spain).

2.7. Delayed type hypersensitivity (DTH)

Dogs were tested for DTH 3 days before necropsy. Leishmanin reagent was an inactivated suspension of *L. infantum* (M/CAN/ES/96/BCN 150, zymodeme MON-1) promastigotes resuspended at the appropriate concentration in PBS-0.5% phenol (5×10^6/ml). The reagent solution (0.2 ml) was injected intradermally in the right shaved groin. The contralateral area received 0.2 ml of the diluent. Skin reactions were recorded after 48 and 72 h. Indurated areas were measured three times using a digital calliper

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and compared with the values of the diluent control. The largest diameter was recorded and averaged. Since the control skin reactions were very uniform in all dogs (2.6 ± 1.01 mm) an induration larger than 3.61 mm was considered positive.

2.8. Measurement of nitrite concentration

Nitrite concentration, as an indicator of nitrite oxide production (NO), was measured in the supernatant of cell cultures from homogenized canine lymph nodes. Cell cultures (10^6/ml) were incubated for 48 h. The cell supernatants were assayed by the Griess reaction as described [28]. Nitrite concentration was calculated using a $NaNO_2$ standard curve of known concentrations. Data are expressed as μM $NO_2^-/10^5$ cells/48 h.

2.9. Statistical analysis

The data were analyzed for statistical significance by analysis of variance (ANOVA) and Tukey's HSD post hoc Test at $\alpha = 0.05$ for differences among groups, and Pearson's linear correlation method for testing the association between variables.

3. Results

3.1. Vaccine safety

During the immunization period (60 days) dogs were monitored and weekly checked for the appearance of external clinical manifestations or disease symptoms. No local or general adverse reactions were observed. Fever was no detected in any of the dogs during the course of the study. All dogs remained at a constant body weight. Neither alopecia nor skin lesions at the injection site were detected. A mild enlargement of the left preescapular lymph node was observed during the first week of immunization that retrieved afterwards. The total white blood cell count remained within normal limits throughout the vaccination period (Table 1). Normal levels of lymphocytes, monocytes, granulocytes, eosinophils and platelets in the blood of vaccinated animals and controls were observed (data not shown). A slight increment in erythrocyte number and haemoglobin (within the physiological ranges detected in dogs) was observed 60 days after vaccination (before challenge, 0 dpi). There was not differences between the vaccinated groups and the controls. The levels of creatinine, urea, alanine-amino transferase in sera and urine (Table 1) were within physiological ranges in all groups. Thus, renal and liver damages due to vaccination could be excluded. The evaluation of biochemical parameters related to total protein and globulin levels showed that there were not significant differences among groups.

3.2. Humoral response against SLA and Q during the immunization period

Serum samples were obtained at days 0, 15, 30, 45 and 60 post-vaccination and assayed by ELISA using as antigen the recombinant antigen employed for vaccination (protein Q). Fig. 1 shows that the immunization of dogs with the recombinant antigen (groups Q and Q+Q) resulted in the production of high levels of anti-Q IgG2 antibodies. As expected from a highly immunogenic protein significant positive levels of anti-Q antibodies in the sera from immunized animals were observed as early as 15 days post-vaccination related to day 0 ($p < 0.0001$). In general, the kinetics of the IgG2 anti-Q response was similar in the Q and Q+Q groups. The response remained positive at day 60 post-vaccination although differences in the IgG2 reactivity level between the Q and the Q+Q groups were detected at day 60. The highest absorbance values were detected in the Q+Q group. An IgG2 response against Q was not observed in control animals. Fig. 2 indicates that in spite of having a high positive response against Q the sera of the Q vaccinated dogs were negative against SLA. These results were corroborated by IFAT (data not shown).

3.3. Protection against canine visceral leishmaniasis

3.3.1. Clinical parameters

After experimental infection control and vaccinated dogs were monthly checked for the appearance of external clinical manifestation of CVL. In agreement with previous data [24] three different forms of the disease were observed in the dogs from the control

Table 1
Haematological and biochemical evaluation in control, Q and Q+Q groups.

		Groups								
		Control			Q			Q+Q		
	Periods	Immunization		Infection	Immunization		Infection	Immunization		Infection
	Days	0 dpv	(60 dpv, 0 dpi)	330 dpi	0 dpv	(60 dpv, 0 dpi)	330 dpi	0 dpv	(60 dpv, 0 dpi)	330 dpi
Haematological and biochemical parameters	Erythrocytes (10^6/mm^3)	4.9 ± 0.3	5.9 ± 0.4	5.3 ± 1.1	4.7 ± 0.2	5.9 ± 0.5	5.5 ± 0.3	5.5 ± 1.8	5.9 ± 0.4	5.1 ± 0.9
	Hemoglobin (g/dl)	16.0 ± 1.0	16.6 ± 1.2	12.2 ± 2.9	15.7 ± 0.6	15.9 ± 0.9	13.0 ± 1.1	15.6 ± 0.8	15.5 ± 0.4	12.1 ± 2.4
	Leukocytes (10^3/mm^3)	9.2 ± 1.5	9.9 ± 1.8	5.2 ± 1.2*	9.7 ± 1.3	9.8 ± 0.7	6.7 ± 1.3	11.6 ± 1.9	11.0 ± 1.1	6.8 ± 2.4
	Total proteins (g/dl)	6.0 ± 0.4	5.8 ± 0.3	6.4 ± 0.9	6.1 ± 0.5	6.1 ± 0.2	6.1 ± 0.4	5.7 ± 0.4	6.2 ± 0.2	5.8 ± 0.3
	Globulins (g/l)[a]	5.1 ± 1		14.5 ± 9.6*	5.1 ± 0.9		7.8 ± 1.7	5.1 ± 1.2		9.4 ± 3.7
	Creatinine (mg/dl)	1.0 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
	Urea (mg/dl)	49.3 ± 7.2	34.3 ± 4.6	35.8 ± 2.0	49.2 ± 12.4	40.9 ± 3.9	39.6 ± 7.7	43.2 ± 7.2	35.7 ± 7.3	36.7 ± 3.6
	ALT (U/l)	41.2 ± 7.3	44.3 ± 11.6	30.7 ± 4.7	35.3 ± 6.2	39.6 ± 7.9	37.7 ± 14.4	43.0 ± 8.6	43.0 ± 7.2	31.8 ± 14.3
Urine analysis[b]	Protein (mg/dl)	0	0	6*	0	0	0	0	0	1

The table indicates the results of each group at pre-vaccination (0 dpv), pre-infection (60 dpv, 0 dpi) and at the end of the study (330 dpi). Haematological and biochemical results are shown as the average value ± standard deviations.
The "*" represents statistically significant differences between controls relative to groups Q and Q+Q.
dpv: days post-vaccination.
dpi: days post-infection.
[a] Globulin levels were analyzed at 0 dpi (pre-infection) and at 330 dpi (end of the study).
[b] Urine analyses: number of dogs with protein levels over 100 mg/dl.

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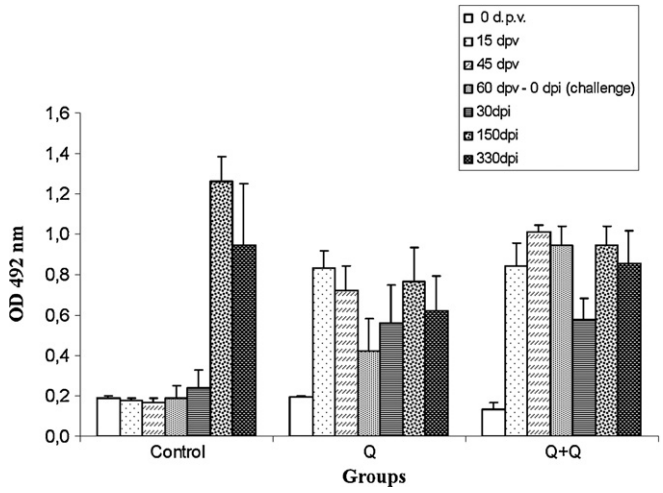

Fig. 1. Changes in the IgG2 anti-PQ antibody absorbance values by ELISA in serum samples of vaccinated (Q and Q+Q groups) and control dogs prior immunization (0 dpv), at different times post-vaccination [days 15, 45 and 60 (0 dpi-challenge)] and during the infection period until the end of the study (days 30, 150 and 330). Results are expressed as the mean average of absorbance in sera from each group ± standard deviation of triplicate experiments. Sera samples were used at a 1/200 dilution and OD readings were measured at 492 nm. Positive and negative control sera were included in each assay.

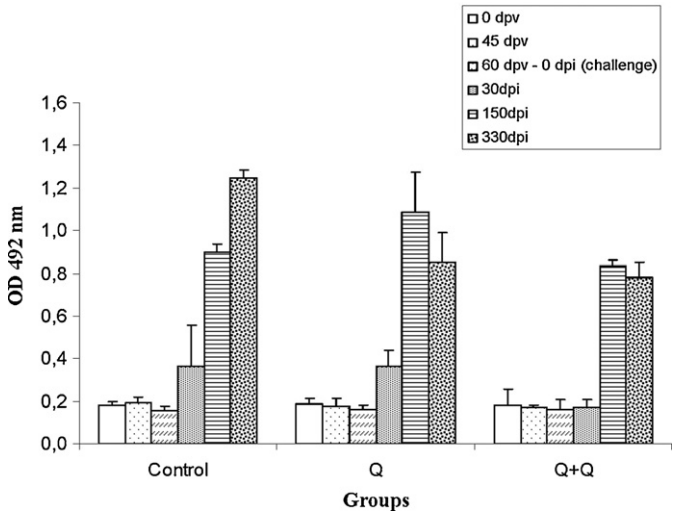

Fig. 2. Changes in the IgG2 anti-SLA antibody absorbance values by ELISA in serum samples of vaccinated (Q and Q+Q groups) and control dogs prior immunization (0 dpv), at different times post-vaccination [days 45 and 60 (0 dpi-challenge)] and during the infection period until the end of the study (days 30, 15 and 330). Results are expressed as the mean average of absorbance in sera from each group ± standard deviation of triplicate experiments. Sera samples were used at a 1/200 dilution and OD readings were measured at 492 nm. Positive and negative control sera were included in each assay.

group: a subclinic self-cured form, a patent form and a symptomatic evolutive form. Table 2 indicates that in the control group 3 animals developed patent clinical symptoms of leishmaniasis. In these dogs the external clinical symptoms were detected 5 months post-infection. A moderate lymphoadenomegaly was the first symptom observed in these dogs that developed into a progressive weight loss with alopecia, exfoliative and ulcerative dermatitis, onychogryposis, generalized lymphoadenitis and keratoconjunctivitis up until the end of the study. These 3 dogs accumulated 14 signs of clinical alterations. It was noted that also in this control group 3 other dogs showed an evolutive form of the disease. No symptoms of disease were observed in 1 dog. The 3 dogs showing the evolutive form of the disease accumulated 7 signs of clinical alterations.

In contrast, significant differences could be detected after clinical examinations of the dogs from the vaccinated groups ($p < 0.0001$). None of the dogs from the Q vaccinated group showed

Table 2
Clinical evaluation. Pathological analysis and DTH (skin test).

		Groups								
		Control			Q			Q+Q		
	Periods	Immunization		Infection	Immunization		Infection	Immunization		Infection
	Days	0 dpv	(60 dpv, 0 dpi)	330 dpi	0 dpv	(60 dpv, 0 dpi)	330 dpi	0 dpv	(60 dpv, 0 dpi)	330 dpi
Clinical symptoms (number of dogs)	Asymptomatic	7	7	1	7	7	4	7	7	2
	Oligosymptomatic	0	0	3	0	0	3	0	0	4
	Symptomatic	0	0	3	0	0	0	0	0	1
Histopathological evaluation	Kidney	(++) SG, (++) IN, (++) C, N			PS			SG (+), (+) C		
	Liver	(+++) GH, (+++) LG, (+++) DI, (+++) VD, (++) N			(+) SmG			(+) C, (+) SmG		
	Spleen	(++) HS, (++) FH, (++) CD			PS			(+) FH		
DTH test (number of dogs)	Positive	1			4			3		
	Negative	6			3			4		

This table summarizes the data at pre-vaccination (0 dpv) and the pre-infection (60 dpv, 0 dpi) period and at the end of the study (330 dpi).
Clinical symptoms. Results are expressed as number of dogs classified as asymptomatic (no signs of the disease), oligosymptomatic (with 2–3 clinical signs of visceral leishmaniosis) and symptomatic (with four or more signs of the disease).
Pathological symptoms. Results are expressed as a description of damages after histological evaluation of kidney, liver and spleen carried out after necropsy (330 dpi). SG (serose glomerulonephritis), IN (interstitial nephritis), C (congestion), GH (granulomatous hepatitis), LG (large granulomas), SmG (small granulomas), DI (diffuse infiltrate of lymphocytes, plasmocytes and macrophages with occasional amastigotes in the cytoplasm), VD (vacuolar degeneration), N (necrosis), HS (hyperplasic splenitis), FH (follicular hypertrophy), CD (cellular depletion), PS (physiological status). (+): low, (++): medium, (+++): intense.
DTH: data represent the number of dogs classified as positive or negative according to the skin reaction. Reactions ≥3.61 mm were considered positive. Induration size of each dog was recorded after administration of leishmanin 3 days before necropsy.
dpv: days post-vaccination.
dpi: days post-infection.

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signs of active leishmaniasis. In this group 4 dogs were clearly asymptomatic throughout the entire period of the study and 3 could be considered as oligosymptomatic due to hyperthermia and/or slight dermatitis together with moderate weight loss. All these dogs accumulated 7 signs of clinical alterations in contrast to the 21 detected in the control group. In the Q+Q group 4 dogs could be considered as oligosymptomatic showing hyperthermia and/or slight dermatitis together with moderate weight loss, accumulating 11 signs of clinical alterations. During the entire infection period of the infection 2 dogs were asymptomatic. A clinical manifestation of leishmaniasis was observed in 1 dog.

3.3.2. Biochemical and haemathological parameters

The analysis of the haematological parameters (Table 1) showed that the experimental infection induced in all groups a progressive decrease in the mean number of leukocytes. In the control group the differences observed in cell counts between pre-infection and those observed at the end of the study were statistically significant relative to the differences observed in the Q and Q+Q animals between these periods ($p = 0.0001$). In controls a slight decrease in erythrocytes and haemoglobin levels (although remaining within normal limits) were observed at day 330 post-challenge relative to pre-challenge. However, the differences between groups were not considered to be significant. The serum level of creatinine, urea and ALT was in a physiological range in all animals. There were not significant differences in total serum protein levels in the animals of all groups. However, differences in urine protein levels were observed in the animals from the infected control group when compared to the Q and Q+Q vaccinated animals ($p = 0.0231$). A significant increase ($p = 0.0001$) in globulin concentrations were observed in the animals having patent clinical symptoms of leishmaniasis (control infected group) when compared to Q and Q+Q vaccinated animals. Moreover, a significant positive correlation between increase in globulin levels and animals having clinical signs of disease was detected ($p < 0.0001$).

3.3.3. Anatomo-pathological analyses

Table 2 summarizes the pathological observations. The liver of all control dogs had a tumescent appearance with distended capsules and discoloured areas. Microscopically, a granulomatous hepatitis process, with a high number of large granulomas was detected in the liver of all control dogs. In this organ there was also an abundant diffuse portal infiltration of lymphocytes, plasmocytes and macrophages with occasional amastigotes in the cytoplasm. Some degree of hepatocyte destruction, vacuolar degeneration and necrosis together with disruption of the normal tissue architecture in the portal area and parenchyma was observed. The spleen of the control dogs showed prominent nodules on the surface with focal areas of necrosis. Microscopically an intense spleen hyperplasia with follicular hypertrophy and cellular depletion with lymphocytolysis was observed. These animals also presented signs of renal failure with serose glomerulonephritis and congestion, together with interstitial nephritis and necrosis of the tubular system.

In contrast, the liver of vaccinated dogs from Q and Q+Q groups showed a normal physiological appearance. Microscopically, no signs attributed to the disease was detected. Only minimal infiltrates, both in the portal spaces and in the hepatic parenchyma were observed. The tissue architecture of the spleen and kidney was typical of a physiologically normal splenic and renal status. In the kidney and spleen of the Q+Q vaccinated dogs only a low congestion process and a slight glomerulonephritis and follicular hypertrophy, respectively, could be detected.

3.3.4. Protection at parasite level

By analysis of aspirates of lymph nodes (data not shown) it was observed that on day 150 post-infection all dogs were parasite positive. Table 3 indicates that also on day 330 post-infection all control dogs were lymph node and spleen parasite positive. Even the asymptomatic dog was parasite positive as assessed by lymph node and spleen sample culture. In contrast, 2 animals from group Q and 1 animal from group Q+Q were lymph node parasite negative. In spleen, 3 animals from each vaccinated group, Q and Q+Q, were parasite negative. The analysis of the lymph node impression smears (Table 3) from the animals of the control group showed 5 parasite positive dogs in contrast to 1 in either of both Q and Q+Q groups. The analysis of spleen impression smears in control group showed that 6 dogs were parasite positive. The parasite negative dog corresponded to the asymptomatic dog indicated above. In contrast, all Q vaccinated dogs were parasite negative at the spleen level. In the Q+Q vaccinated group 2 animals were positive in spleen smears. All these observations were confirmed by a qPCR assay. Regarding to parasite burden no significant differences among parasite positive dogs were found. In these dogs the parasite load was estimated in a range which corresponds to 1–4 amastigotes/1000 nucleated cells. Interestingly, no significant differences were found in the dynamic range of parasite DNA expression in positive dogs by qPCR (data not shown). Parasite DNA (Table 3) was detected in 6 out of the 7 skin samples analyzed in the control group. In the Q group 1 of the 7 skin out samples were positive. Finally, in the Q+Q group 4 skin samples were positive. Similar levels of parasite DNA were detected in all parasite positive samples. Thus, the data showed that the Q and the Q+Q vaccination was able to induce parasite clearance or a significant reduction of parasite burden ($p < 0.0001$) in lymph nodes, spleen and skin on day 330 post-administration of parasites.

3.3.5. Immune response in dogs after experimental infection

3.3.5.1. ELISA assays. Figs. 2 and 3 show that all challenged dogs developed after infection a significant humoral response against SLA as an indication that they had been actually infected ($p < 0.0001$). The analysis of IgG subclasses revealed that after challenge an IgG2 predominant response was observed as early as 150 days post-challenge. The O.D. values against SLA and the evolution of the IgG2/IgG1 isotypes were different depending on the group considered. The IgG2 reactivity against SLA (Fig. 2) was significantly higher in controls than in the vaccinated animals particularly at day 330 post-infection ($p < 0.0001$). The mean IgG1 reactivity against

Table 3
Parasitological analysis.

	Culture						TS[a]						Real-time PCR[b]		
Groups	Control		Q		Q+Q		Control		Q		Q+Q		Control	Q	Q+Q
Sample	LN	S	LN	S	LN	S	LN	S	LN	S	LN	S		Skin	
Parasite negative	0	0	2	3	1	3	2	1	6	7	6	5	1	6	3
Parasite positive	7	7	5	4	6	4	5	6	1	0	1	2	6	1	4

Results are expressed as number of animals in each group that presented positive/negative parasite detection by culture and analysis of tissue smears (TS) in lymph node (LN) and spleen (S), and real-time PCR in skin, at day 330 dpi.

[a] In positive dogs no significant differences in parasite load were found. In these dogs parasite burden ranged from 1 to 4 amastigotes/1000 nucleated cells.

[b] In positive dogs no significant differences in the parasite DNA expression levels were found.

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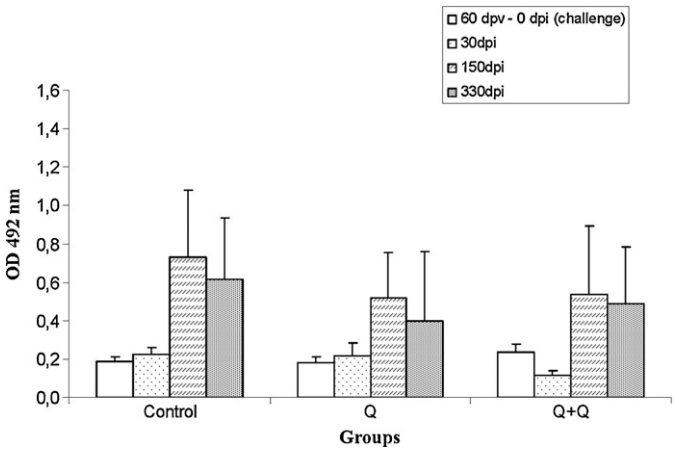

Fig. 3. Changes in the IgG1 anti-SLA antibody absorbance values by ELISA in serum samples of vaccinated (Q and Q+Q groups) and control dogs prior challenge (60 dpv–0 dpi) and during the infection period until the end of the study (days 30, 150 and 330). Results are expressed as the mean average of absorbance in sera from each group ± standard deviation of triplicate experiments. Sera samples were used at a 1/200 dilution and OD readings were measured at 492 nm. Positive and negative control sera were included in each assay.

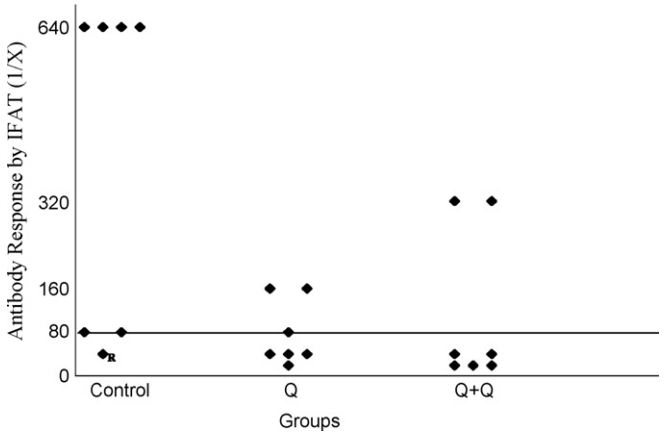

Fig. 4. IFAT analysis. IFAT was performed on slides coated with *Leishmania infantum* promastigotes (M/CAN/ES/96/BCN 150, zymodeme MON-1). Sera from vaccinated (groups Q and Q+Q) and control dogs and obtained at 330 dpi (end of the study) were assayed in serial two-fold dilution from 1/20 to 1/640. The threshold titre for positivity was set at 1/80. R: dog naturally resistant.

SLA (Fig. 3) was statistically different between controls and vaccinated animals ($p < 0.0001$) at day 150 post-infection. In the control group 3 dogs were IgG1-SLA negative. In the Q and Q+Q groups 6 and 5 dogs, respectively, were IgG1 SLA negative at day 330 post-challenge.

It was also observed that at day 150 post-infection there was a clearly positive reactivity against Q in the sera of all control infected animals (Fig. 1). The response was significantly higher than that observed in the vaccinated dogs ($p < 0.0001$), in spite of the fact that these vaccinated animals responded to Q during the vaccination period. These control dogs remained Q positive until the end of the experiment. The anti-Q reactivity was detected in this control group at the time of the increase in reactivity against SLA and the appearance of clinical signs of the disease. A significant positive correlation between these variables was detected ($p < 0.0001$). Even though a slightly increase in reactivity against the Q protein was observed in the Q vaccinated group at day 150 post-challenge relative to that observed in the pre-challenge day, in the Q+Q vaccinated dogs at day 150 the reactivity against the Q protein was lower than that detected before challenge.

3.3.5.2. IFAT. The IFAT data shown in Fig. 4 indicated that 6 control dogs were IFAT positive. Four of these dogs had high antibody titres (≥1/640). These animals were the ones affected by the evolving and patent leishmaniasis form, having the highest O.D. values against SLA. These IFAT titres were not observed in any of the Q and Q+Q vaccinated dogs.

3.3.5.3. Western blotting. Western blotting was used to identify the parasite antigens recognized by the sera of the control and Q vaccinated animals (Fig. 5). The sera from days 150 and 330 p.i. were selected. The most intensively IgG2 labelled bands corresponded to proteins of apparent molecular masses of 97, 84, 70, 55, 36, 29 and 14 kDa. As Fig. 5a shows at day 150 post-infection a strong IgG2 reactivity was observed in control infected dogs, mainly against high molecule weight protein bands. A similar pattern of reactivity was observed at day 330. The pattern of bands recognized by the sera of these control dogs is similar to that detected by the sera of naturally infected dogs that develop a typical VL disease (laboratory data). As expected, the intensity and the pattern of reactivity of the sera from dog 4th and 6th classified as oligosymptomatic and asymptomatic, respectively, were low at the end of the study. In contrast Fig. 5b shows that at day 150 post-infection the number of bands recognized by the Q vaccinated dogs is restricted to some proteins (mainly 36 kDa molecule weight). At day 330 post-infection the reactivity of the sera of the animals against *Leishmania* proteins was even lower and negative in some of them.

3.3.6. Delayed type hypersensitivity (DTH)

Table 2 summarized the number of dogs classified as positive or negative according to the skin reaction. It was observed that 4 of the immunized dogs from group Q and 3 from the Q+Q group developed a positive DTH response. These dogs were the asymptomatic ones described above. All control dogs were DTH negative with the exception of the asymptomatic one.

3.3.7. NO production

Nitrite oxide production in the lymph node cell cultures of all dogs from the Q and Q+Q groups was significantly higher (18.73 ± 7.90 and 15.11 ± 4.47 μM/10^5 cells/48 h, respectively) than that observed in controls (3.4 ± 1.04 μM/10^5 cells/48 h) ($p < 0.0001$).

4. Discussion

Since dogs are the main reservoir of visceral leishmaniasis (VL) there is an increased interest for the development of *Leishmania* parasite antigen based vaccines able to induce protective immunity. A large number of candidate vaccines [9] have been tested in dogs with different outcomes. Some of them have reached Phase III trials [29,30]. Recent data from our laboratory have shown that the recombinant chimerical Q protein from *Leishmania* is highly immunogenic [19] and that when it is administered in the presence of BCG and CpG motifs is able to confer protection to *L. infantum* experimentally infected dogs [20] and Balb/c mice [21], respectively. Since Th1 humoral and CD8+ cytotoxic T cells responses seem to be needed to confer protection [31] most vaccine formulations include the co-administration of parasite antigens with adjuvants to elicit a maintained and appropriate levels of antigen specific stimulation, to modulate the Th1/Th2 responses. In fact, there is general agreement that the correct balance in the Th1/Th2 cytokine dichotomy plays an important regulatory role in determining the outcome of *Leishmania* infection in dogs and humans [22,31–35].

Taking into account the observation that a single administration of Q in the absence of any adjuvant induces in dogs an IgG2

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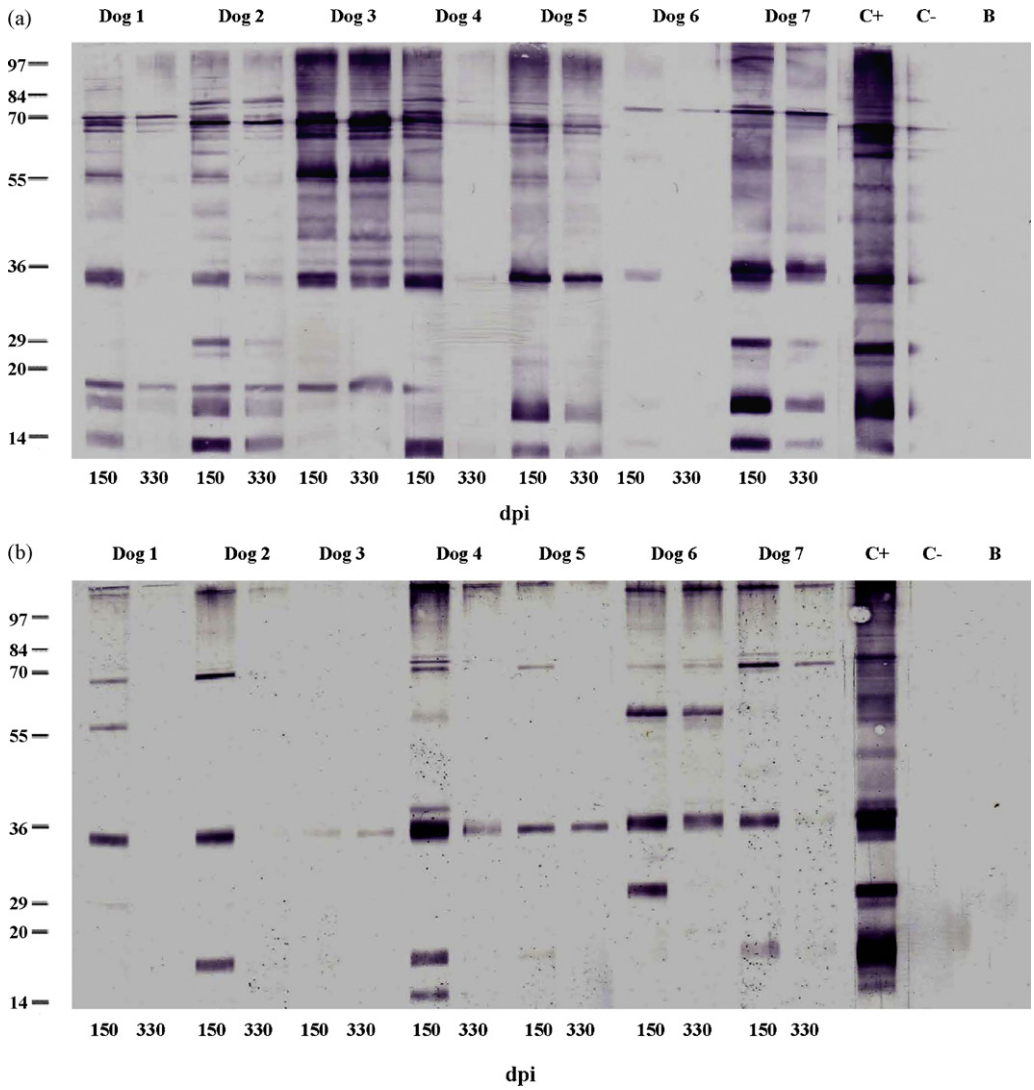

Fig. 5. (a) Western blot analysis of *L. infantum* antigens recognized by sera (isotype IgG2) from control dogs corresponding to days 150 and 330 post-infection (p.i.). C+: positive control, C−: negative control, B: blank. (b) Western blot analysis of *L. infantum* antigens recognized by sera (isotype IgG2) from vaccinated dogs (group Q) corresponding to days 150 and 330 post-infection (p.i.). C+: positive control, C−: negative control, B: blank.

humoral response (unpublished data), the protective efficacy conferred to dogs of a vaccine based on a single or a double dose of the recombinant Q protein was analyzed. The data presented in this report corroborate previous findings showing that the administration of 100 μg of the Q protein induced in all vaccinated dogs an early and intense long-lasting specific IgG2 antibody response. Other vaccine formulations already described are based on similar dosages of antigen [13,20,36] while in others larger amounts of antigen are needed to induce a positive and specific immune response in canines [10–12,29,30,37].

One of the first requirements needed to evaluate protection against CVL is the induction of an active infection in all animals, together with the reproduction of clinical and pathological features attributed to the disease. Our results showed that after infection all control dogs were SLA and parasite positive at day 150, confirming that the intravenous administration of 5×10^5 stationary phase promastigotes of the M/CAN/ES/96/BCN 150, zymodeme MON-1 strain successfully established active infection. It was also observed that in these dogs the representative outcomes of the disease [22,38–41] were detected. A similar pattern of disease has been reported after experimental infection using similar models of intravenous infection [12,22].

Given the complex nature observed in CVL development it was necessary to define the parameters that were going to be taking into consideration to estimate the level of protection The end-points selected were those considered to be representative of canine leishmaniasis such as the development of clinical manifestations (lymphadenopathy, cutaneous alterations, onychogryphosis, keratoconjunctivitis, apathy, weight loss and cachexia) together with physiological disorders (renal, liver and haemathological alterations), the analysis of parasites in tissue samples, the evaluation of the pathological consequences after infection, in organs closely related to the disease, and the analysis of the immune reaction against the Q protein and SLA lysate. It is likely that a balanced and effective cellular and humoral immune response, together with the control of parasite spreading and the maintenance of a consistent physiological status in organs determine the outcome of clinical VL. Thus, the protective efficacy was estimated by comparison of the disease features observed in controls dogs relative to those observed in vaccinated animals.

The examination of clinical external manifestations showed the existence of differences between Q vaccinated and control animals. Thus, while 4 dogs from the control group showed a patent symptomatic evolutive form of the disease, none of the Q and Q+Q

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vaccinated dogs developed a similar evolutive VL form. Moreover, while several symptoms associated to VL were observed in 6 control infected dogs, only 1 dog in the Q and 2 dogs in the Q+Q groups, showed some of these symptoms but with lower intensity. These findings are similar to that previously reported showing that most animals [20] were phenotypically protected after administration of tree doses of Q+BCG.

The biochemical and haemathological results were in agreement with the clinical data and the pathological observations. There was a decrease in the levels of leukocytes in all infected control dogs together with a significant increase in globulin concentration and renal alteration. In contrast, a physiological status was observed in the organs of the Q and Q+Q vaccinated animals.

The anatomo and histopathological analyses of spleen, liver and kidney showed, moreover, the existence of clear differences between control and vaccinated animals. In controls dogs an established pathology typical of canine leishmaniasis was observed in these organs, showing morphological alterations with an inflammation process, together with tissue degeneration and necrosis. In contrast, a physiological tissue architecture was observed in spleen, kidney and liver of the Q and Q+Q vaccinated groups.

Differences between control and vaccinated animals were also observed at the parasite level, since by culture in Schneider's medium parasites could be detected in lymph nodes and spleen of all control dogs. In contrast 1 dog from the Q+Q group and 2 of the dogs from the Q group were parasite negative at the lymph node level. In addition, 3 dogs from the Q and 2 from the Q+Q groups were negative in spleen cultures. It is noteworthy that at day 330 post-infection the asymptomatic dog from control and those from the vaccinated groups were parasite positive as detected by culturing of lymph nodes and spleen samples as an indication that parasite infection is not, per se, straightforwardly correlated with clinical VL.

Differences between control and vaccinated dogs regarding parasite burden was also detected in impression smears of spleen and lymph nodes. The data indicated that the vaccine was able to induce parasite clearance in some dogs and a significant parasite burden reduction in most vaccinated animals since at the lymph node level 5 dogs from the control group were parasite positive, while only 1 dog was positive in both Q and Q+Q groups. Moreover in spleen there were 6 positives in control group while none in the Q group and 2 positives in the Q+Q group. The protection conferred by Q vaccination was moreover confirmed by PCR analyses of eyelid skin samples. The absence of parasites in skin of the Q vaccinated dogs is of interest as it may potentially lead to reduce zoonotic transmission within endemic areas. The control of parasite burden in all vaccinated dogs was also suggested by humoral analyses against SLA and Q. Although all dogs developed positive anti-IgG2 reactivity against ELISA-SLA and IFAT, the control dogs showed significantly higher levels of anti-SLA IgG2 antibodies relative to vaccinated animals, particularly at day 330 post-infection. Also an IgG2 clearance was detected by Western blotting in the Q vaccinated dogs. Similar humoral response clearance was detected in Q+Q vaccinated group when analyzed. The sera of control dogs, with the exception of the asymptomatic one, were anti-Q positive at day 330 post-infection.

Since an anti-Q reactivity has been detected in the sera of symptomatic dogs and in Balb/c mice after infection, but not in the resistant C57BL/6, it has been suggested that a release of internal antigens and the induction of the anti-Q reactivity is closely related with a necrotic killing stage that promoted the massive destruction of parasites in infected tissues [42]. It seems that the necrotic stage is the most important control mechanism against the parasite, as it has been shown in human cutaneous leishmaniasis. After the necrotic stage and cure process of cutaneous lesions a life-long protection to reinfection is provided [43,44]. Most likely, then, the humoral seroconversion observed in the vaccinated animals as well as in the asymptomatic control dog could be interpreted in terms of control of parasite dissemination. Other interesting point was the observation that in spite of the high anti-Q reactivity observed in the Q and Q+Q groups after vaccination no reactivity against the total *L. infantum* proteins (ELISA-SLA) and IFAT was detected in the sera of these animals. The absence of response against SLA after Q vaccination is relevant because it allows distinguishing between vaccinated and infected animals in large-scale immunoprofilaxis programs.

Furthermore, the induction of DTH positive conversion in some of the protected dogs from the Q and Q+Q groups and the enhanced production of NO also indicates that the Q vaccine was able to elicit a cellular immune response and a leishmanicidal activity [28], by an NO-mediated macrophage effector mechanism, leading to the control of parasite replication. In fact, the asymptomatic dogs from Q and Q+Q vaccinated groups (4 and 3 animals, respectively) and the asymptomatic one from the control group were DTH positive

In view of these data we believe that most likely an early anti-Q immune response induced by vaccination could neutralize the exposure to the immune system of an amount of surface proteins and secretion parasite products that could trigger a disproportionate humoral response leading to the immunopathological consequences related to the disease. A disproportionate humoral response has been shown to occur in the late evolutive and pathological forms of the disease [43]. This hypothesis is also in agreement with previous reports [20,21] indicating that activated macrophages may present intracellular antigens during *Leishmania* infections [45] and that conserved internal proteins may have the capacity to induce T cell proliferation [45] and to afford protection [46,47].

In conclusion, our data indicate that vaccination with the Q protein administered as a single subcutaneous injection in the absence of adjuvants induces strong protection. The data shown suggest that a single dose of Q induces a higher degree protection than two doses. The protection seems to be correlated to a specific early and balanced humoral and cellular immune response that promotes an efficient killing of parasites even under a high virulent experimental infection. We believe that these data warrants a further evaluation of the immunoprophylactic effects of the Q vaccine in a pilot study under natural infection conditions.

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