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A *Leishmania infantum* multi-component antigenic protein mixed with live BCG confers protection to dogs experimentally infected with *L. infantum*

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Abstract

The capacity of a quimeric protein, formed by the genetic fusion of five antigenic determinants from four *Leishmania* proteins, formulated with BCG, to protect dogs against *Leishmania infantum* infection is described. The data showed that after i.v. administration of 500,000 parasites of the *L. infantum* M/CAN/ES/96/BCN150 strain, zymodeme MON-1, the animals became infected as suggested by the humoral response against the parasite antigens. All control unvaccinated dogs had parasites in the lymph nodes at day 150 post-infection. One of these unvaccinated infected dog was parasite negative at day 634 behaving, thus, as resistant. In contrast, only 50% of the immunized dogs had parasites in the lymph nodes at day 150 post-infection. Four of these dogs became parasite negative by day 634 post-infection. The control animals developed at various times during the follow-up period clinical symptoms associated with Leishmaniasis. The control diseased dogs developed also in the liver and spleen some of the abnormal histological features associated with natural visceral Leishmaniasis. The immunized dogs, however, were not only normal at the clinical but also at the anatomo-pathological level. A positive delayed type hypersensitivity (DTH) response was observed in nine of the immunized protected dogs. The data indicated that Q + BCG confers 90% protection against infection and at least 90% protection at the clinical level.

Keywords: *Leishmania infantum*; Visceral leishmaniosis; Vaccine; BCG; Antigens

1. Introduction

Parasitic diseases represent a serious health problem world-wide with concomitant negative effects on the economy of the populations affected by them. Leishmaniasis caused by *Leishmania* protozoan, is no exception. About 22 different species of *Leishmania* are responsible for a wide spectrum of the human and animal forms of Leishmaniasis that occur mostly in the tropics and subtropics being the active form of visceral Leishmaniasis often a progressive and fatal disease when not treated. In parts of Asia, the

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Mediterranean region and South America there are over 2 million new cases of Leishmaniasis each year with 367 million people at risk (Control of Leishmaniasis: WHO expert committee, 1990). Moreover, the *Leishmania*/human immunodeficiency virus co-infection is beginning to represent a serious human threat (Alvar et al., 1997). 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 measures that could limit the spread of both infections (Wolday et al., 1999).

In Europe, the visceral form of Leishmaniasis is caused by *L. infantum* and is prevalent in various Mediterranean countries in which domestic dogs constitute an important reservoir of the infection. The symptoms developed in both humans and dogs with visceral Leishmaniasis are rather similar. In mice, the *L. major* infection can either end in a rapid resolution of the infection or lead to a disseminated, chronic and fatal disease. It seems that the outcome is largely dependent on the mouse strain used as host. Resistance seems to be mediated by a Th l type response while the disease is mediated by a Th 2 type response (Reed and Scott, 1993). In dogs resistance or susceptibility to the disease is probably also associated with a dichotomous immune response. In these animals, resistance is associated with enhanced specific lymphoproliferative responses and positive delayed type hypersensitivity (DTH) (Solano-Gallego et al., 2000) together with little or no specific circulating antibodies. Susceptibility, on the other hand, courses with high anti-*Leishmania* antibody titters and depressed lymphoproliferative ability (Cabral et al., 1992; Pinelli et al., 1994). It has been assessed that in dogs an IgG2a response is associated to asymptomatic infections (Nieto et al., 1999). However, as indicated by these authors and by Stacey (Stacey and Blackwell, 1999) it might be that it is the balance between IgG1/IG2a the critical factor that determines susceptibility or resistance.

Several candidate vaccine molecules have been identified through their abilities in a murine model to provide protection. In addition to membrane associated proteins, such as gp46, dp72, gp63, LPS and Kmp-11, other proteins as P-2/A-2, P-4 and P-8, expressed preferentially in the amastigote stage, have been tested (Coutinho et al., 1996). Significant protection against *L. pifanoi* infection was found in mice immunized with the antigens P-4 and P-8 while minimal protection was observed after immunization with the P-2/A protein. The *L. pifanoi* derived P-8 antigen was also found to provide cross-protection against *L. amazonensis* or *L. major* infection and to induce efficient protection in mice of different H-2 haplotypes (Soong et al., 1995; Haberer et al., 1998). Furthermore, parasites were eliminated completely in P-8 immunized BALB/c and CBA mice. In addition to these proteins, significant protection has been induced by vaccination with DNA encoding the LACK protein (Gurunathan et al., 1997).

Recently, the generation of *Leishmania*-specific CD4+ T cell lines (Probst et al., 2001) as well as immunization with sequential fractions of a *Leishmania* cDNA expression library (Melby et al., 2000) has led to the identification of several antigens that afford partial protection against infection. Interestingly, the identified antigens are intracellular proteins among which are some of the histones. It has been also recently shown that histone H1 confers partial protection to BALB/c mice infected with *L. major* (Solioz et al., 1999). These data together with the finding that the acidic ribosomal Lip2a protein is able to induce proliferation of naive splenocytes and induce IFN-γ (Soto et al., 2000) led us to test the protective capacity of a quimeric multi-component antigenic protein, named Q, formed by the genetic fusion of five fragments from the acidic ribosomal proteins Lip2a, Lip2b, P0 and the histone H2A protein (Soto et al., 1998). The protein fragments forming the Q protein are highly immunogenic during *L. infantum* natural infections in dogs with visceral Leishmaniasis (Requena et al., 2000a). Live bacterial BCG was used as adjuvant since it has been reported that it activates macrophages inducing NO (Nozaki et al., 1997; Kreuger et al., 1998) and elicits long-lasting cellular and humoral immune responses (Warren et al., 1986; Warren and Chedid, 1988) in addition to mixed type 1 and 2 cytokines (Takayama et al., 1991). Several authors have described the use of BCG alone and a combination of BCG with several *L.* antigens and even leishmanin in protection assays (Weintraub and Weinbaum, 1977; Stefani et al., 1993; Pappas, 1983; Cabrera et al., 2000; Khalil et al., 2000; Misra et al., 2001). Our data showed that Q + BCG led to protection against infection in 90% of the immunized dogs as deduced

from the absence of parasites in popliteal lymph nodes and to clinical and pathological protection.

2. Materials and methods

2.1. Purification of antigens

Soluble *Leishmania* antigen (SLA) was prepared from cultured promastigotes using standard procedures (Scott et al., 1987). Shortly, cultured promastigotes were collected and disrupted by gentle sonication and successive steps (four times) of freezing in liquid nitrogen and thawing. Then, the supernatant containing the SLA antigens was collected after centrifugation at 13,000 rpm for 15 min. The Q protein was expressed in *E. coli* transformed with the appropriated PQE vector (Soto et al., 1998). The protein was purified as a histidine-tagged recombinant protein following standard procedures using agarose Ni–nitrilotriacetic acid (Ni–NTA) resin columns (Qiagen). The Q protein was passed through a polymixin B column to eliminate potential LPS contaminants. BCG was obtained from Calbiochem.

2.2. Animals, immunization and infection

A group of 6-week-old BALB/c mice ($n = 5$) were immunized i.p three times at a 2-week interval with a mixture of 2 μg of protein Q and 50,000 PFU of BCG, per dose. Another group of mice was immunized by the same route at the same intervals with 50,000 PFU of BCG, per dose. The antigen and BCG were in 50 μl of PBS. Also, 50 μl of PBS were administered i.p. to the control mice. Two weeks after the third immunization the mice were infected i.v. with 100,000 promastigotes of the *L. infantum* M/CAN/ES/96/BCN 150, zymodeme MON-1, strain in 50 μl of PBS (tail vein route). The parasites were prepared as indicated below. At day 30 post-infection, the mice were euthanized according to the requirements and guidelines established by the Animal Health Committee of the University. The relative parasite burden was estimated microscopically by the number of amastigotes in methanol fixed and Giemsa stained impression smears of the spleen. Several fields of the smears were observed. Also, the parasite burden was determined by an alternative method proposed by Stauber (Stauber, 1958). Briefly, a suspension of about 10 mg of the spleen was prepared by grinding the tissue in 1 ml of PBS. Three samples of 5 μl were spread on a slide. After staining with Giemsa the parasites were counted. This method gave an estimate of the number of parasites per mg of tissue. The results from different groups were compared by a two-tailed Student's *t*-test.

Twenty beagles were raised under parasite-free conditions. The beagles were raised and grown in our own Animal Facilities at the Veterinary Unit of the University of Extremadura (Cáceres, Spain) approved by the Ministry of Agriculture. The animals were maintained in quarantine for a period of 30 days before the initiation of the experiments according to the Guiding Principles for the Care and Use of Animals following the guidelines of the Ethical Committee. The dogs were 8–9 months old with an average weight of 16 ± 2 kg. Three i.p. immunizations with protein Q were performed in 10 dogs on days 0, 21 and 44. Each dose contained 4 μg of the Q protein/kg dog weight plus 10^6 BCG in 1 ml of PBS. The control animals ($n = 10$) were injected i.p. with an equivalent volume of PBS. Serum samples were taken at the times indicated in the figures and frozen at −80 °C. On day 108 (66 days after the third immunization), the animals were infected i.v. with 500,000 promastigotes of the *L. infantum* M/CAN/ES/96/BCN 150, zymodeme MON-1, strain. The strain (an autochthonous isolate) was originally derived from a dog having active visceral Leishmaniasis. Log-phase promastigotes were differentiated from amastigotes which had been isolated from the spleen of heavily infected hamsters (*Mesocricetus auratus*). A primary culture of differentiated promastigotes was used for infection. To preserve virulence, the parasites are continuously maintained by repeated passage in hamsters.

2.3. ELISA assays

For the ELISA assays standard ELISA plates (Costar) were coated overnight at 4 °C with 100 μl of a solution of the Q antigen at a concentration of 2 μg/ml of SLA at a concentration of 8 μg/ml diluted in PBS. After coating, the plates were washed three times with PBS containing 0.5% Tween 20 and further incubated for 30 min in blocking solution (5% nonfat dried milk powder in PBS–0.5% Tween 20) at 37 °C with shaking. Afterwards, the wells were

washed three times with PBS–0.5% Tween 20. After the washing process, the wells were incubated with the sera at a 1/200 dilution for 30 min at 37 °C, with shaking. Then, the wells were washed as described. As secondary antibody, a horseradish peroxidase labeled goat anti-dog IgG (Nordic Immunology, Tilburg, The Netherlands) was used. After incubation for 30 min at 37 °C the plates were washed and developed with ortho-phenylenediamine (Sigma) as substrate. The reaction was stopped after 15 min by addition to each well of 50 μl of H_2SO_4 3N. The absorbance was measured at 490 nm.

2.4. Biopsies of popliteal lymph nodes and culture of parasites

Aspirates of popliteal lymph nodes (after local anesthetics) were taken on days 150, 450 and 634 post-infection to detect the presence of parasites. The aspirates were cultured in Schneider's medium containing 10% FCS for 15 days. Each point was done in sextuplicate. The presence of parasites was determined by direct examination of the microtiter cells in an inverted microscope. When parasites could be observed in a single well of the sextuplicate the sample from which the aspirate was taken was considered parasite positive. The parasite burden of the spleen of euthanized dogs was assessed by counting the number of amastigotes in methanol fixed and thiadine–eosin (DADE n.v.) stained impression smears. The number of amastigotes per 1000 nucleated cells was determined microscopically.

2.5. Delayed type hypersensitivity (DTH)

The leishmanin reagent was an inactivated suspension of 5×10^6 *L. infantum* promastigotes per ml of a saline solution containing 0.5% of phenol. About 300 μl of that solution were injected intradermically to the dogs under the skin of the groin. Skin reactions were recorded after 72 and 96 h. The size of the induration in mm (width of the skin pinch at the site of injection in mm) was measured with the aid of a digital caliber. The values indicated represent the mean of each one of the measurements (made three times). Since the skin pinch at 0 h was very uniform in all dogs (2.3–2.5 mm) an induration larger than 3.3 mm was considered positive.

2.6. Clinical analysis and anatomo-pathological studies

Tissue samples from the liver were collected in 10% formalin and processed by routine paraffin embedding. Sections of 2 μm thickness were cut and stained with hematoxylin–eosin and reticuline (Argentaffin reticular fibers, Microm Laborgeräte, S.L.). The sections were observed and photographed in a Nikon microscope. Laparoscopy was done at the non-invasive surgical unit of the Veterinary Hospital of the University following the protocols derived in the unit (Usón et al., 1996).

3. Results

Several authors have indicated that in nature and after an experimental infection with *L. infantum* a large number of dogs are susceptible to infection and develop an active form of Leishmaniasis while a percentage behave as clinically resistant (Lanotte et al., 1979; Berrahal et al., 1996). The dogs are classified as resistant either because they do not develop clinical manifestations associated to visceral Leishmaniasis or because they resolve the infection spontaneously. In order to set clinical, humoral and parasitological parameters that would allow to perform protection experiments we published data showing that 3 months after infection all animals develop antibodies against a soluble *Leishmania* antigen fraction and against particular purified antigens of the parasites and that they had parasites in the popliteal lymph nodes. After a follow-up of 13 months, two dogs developed evident signs of clinical Leishmaniasis, one dog behaved as asymptomatic, having parasites in the lymph nodes and that one dog cleared the parasites at the end of the experimental period behaving, thus, as resistant (Nieto et al., 1999). We have reproduced this experimental infections and confirmed that the i.v. administration of 500,000 parasites of the M/CAN/ES/96/BCN 150, zymodeme MON-1 strain induces infection in all animals and disease in about 75% of them.

3.1. Immunoprotection of BALB/c mice against L. infantum infection

Before testing the efficacy of the Q + BCG mixture in dogs we performed a pilot study comparing the

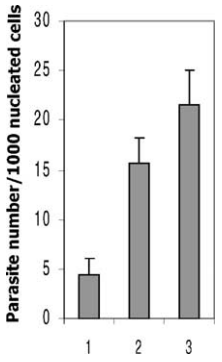

Fig. 1. Number of *L. infantum* parasites per 1000 nucleated cells in impression smears of the spleen of unvaccinated, BCG and Q + BCG immunized mice. (1) Q + BCG immunized mice; (2) BCG immunized mice; (3) unvaccinated control mice.

parasite burden in the spleen of Q + BCG and BCG immunized BALB/c mice relative to the controls to which PBS was administered. At day 30 post-infection, the sera of all mice were positive against SLA (data not shown). Fig. 1 shows the relative parasite burden observed in the spleen of the Q + BCG, BCG and the unvaccinated controls. There was an 80% difference between the amount of parasites in the spleen of the Q + BCG immunized mice and the controls ($P < 0.0004$). The BCG also conferred some protection since about a 28% reduction in parasite burden was detected in the BCG immunized mice relative to the control mice ($P < 0.03$). A 72% difference in parasite burden was observed between the Q + BCG and BCG immunized mice ($P < 0.0005$). A similar result was obtained when the absolute parasite load per mg of tissue was estimated. The Q + BCG immunized mice had as a mean 850 (±90) parasites per mg of tissue while the number of parasites per mg in the controls was 4480 (±500). The BCG immunized mice had as a mean 3160 (±250) parasites per mg of tissue. Based on these data we then designed an experiment to test the efficacy of a Q + BCG mixture to protect dogs, natural hosts, from *L. infantum* infection.

3.2. Immunization of dogs with the Q protein

Although the antibody response against the Q protein in some of the immunized dogs was low a positive response was observed in most of the animals at day 21 (Fig. 2b). The response was variable and may indicate that even within the same strain of animals high and low early responders may be found. The response against the protein increased after the second immunization becoming more uniform at day 44. Even at day 44, the response of two of the Q immunized dogs remained negative. The response increased in all animal after the three dose of Q. The response was positive in all animals at day 0 post-infection. As an average, the reactivity against the Q protein in all animals at day 634 was lower than at day 186. In four

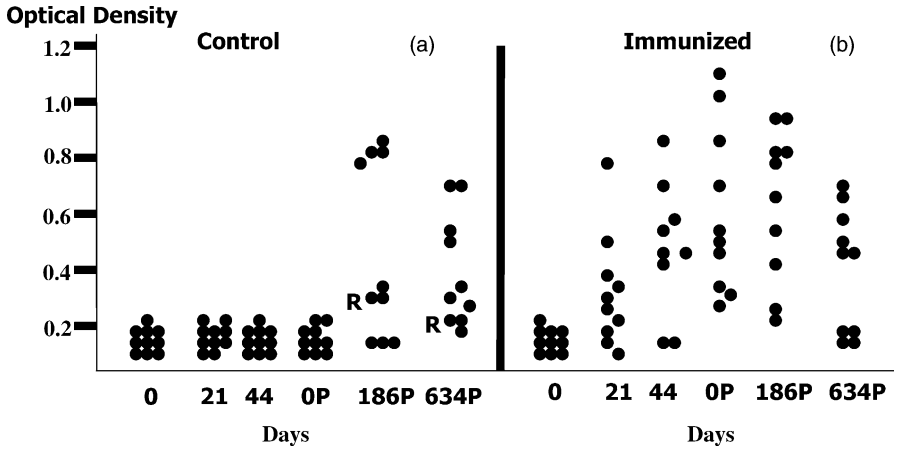

Fig. 2. Anti-Q IgG response in the control (a) and the Q + BCG immunized dogs (b). The dogs were immunized on day 0, 15 and 44. On day 108 (0 post-infection), the control and the immunized dogs were infected with 500,000 promastigotes of *L. infantum*. The sera of the animals were analyzed on the days indicated in the figure. The dark circles (●) indicate the value of the O.D. of each serum. Here, P means post-infection and R means the resistant control dog.

dogs, the anti-Q reactivity was similar to that found in the preimmune sera. It was also observed that in control dogs (Fig. 2a) the response against Q was negative but that at day 186 post-infection there was a clear positive reactivity against Q in the sera of eight animals. These dogs remained Q positive until the end of the experiment. Since the i.v. administration of an amount of SLA from 500,000 parasites does not trigger any significant reactivity against the Q protein the anti-Q reactivity observed in the control dogs must have been caused by active infection as it has been also observed in the sera of dogs with patent natural visceral Leishmaniasis.

3.3. Antibody response against SLA in unvaccinated and immunized dogs

That after administration of the parasites both the control and the immunized dogs became actively infected was indirectly shown by the presence of anti SLA antibodies in the sera of all the animals. Fig. 3a shows that none of the sera of the unvaccinated dogs recognized the SLA antigens before infection from days 0 to 108 but that all of them became SLA positive at day 186 (78 days post-infection). The sera of all dogs remained positive up to the end of the experiment. Variability in response against SLA was observed. Fig. 3b shows that the sera of the immunized dogs were also negative before infection but that they became positive at day 186 post-infection. In these animals, there was also a variable response against SLA. That the response against SLA was due to infection by replicating parasites and not to the amount of parasite antigen administered was shown by the fact that the i.v. administration of a total parasite antigen extract equivalent to 500,000 parasites, without adjuvant or in the presence of BCG, does not induce antibodies against SLA (laboratory data). It should be noticed also that the administration of the Q + BCG mix does not induce a positive response against SLA. The value of the O.D. observed in the controls versus that of the immunized dogs differs significantly at day 634 post-infection ($P = 0.02$). The same value of $P = 0.01$ was observed when the IFI titers were compared between controls and the immunized dogs at day 634 post-infection (data not shown).

3.4. Parasite content in popliteal lymph nodes

On day 150, 450 and 634 post-infection biopsies of lymph nodes were taken to evaluate the presence of parasites in that organ. Since the microscopic observation of stained biopsies may not be sensitive enough to detect small number of parasites, the biopsies were

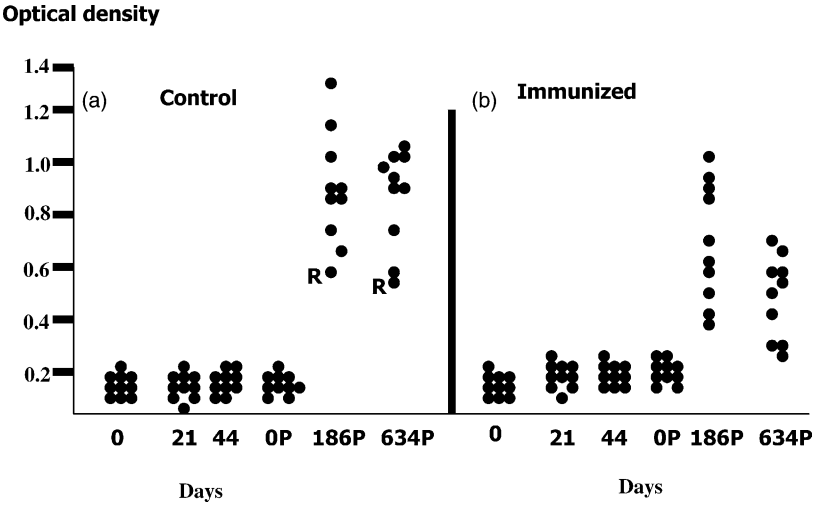

Fig. 3. Anti-SLA IgG response in the control (a) and the Q + BCG immunized dogs (b). The dogs were immunized on day 0, 15 and 44. On day 108 (0 post-infection), the control and the immunized dogs were infected with 500,000 promastigotes of *L. infantum*. The sera of the animals were analyzed on the days indicated in the figure. The dark circles (●) indicate the value of the O.D. of each serum. Here, P means post-infection and R means the resistant control dog.

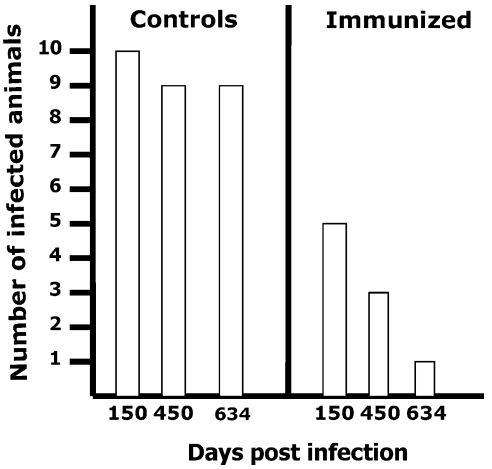

Fig. 4. Number of *L. infantum* parasite infected animals. The biopsies of the popliteal lymph nodes were cultured for 15 days as indicated in materials and methods. The numbers in the abscissa indicate the days post-infection in which the biopsies were obtained. The numbers in the ordinate indicate the number of dogs.

cultured for 15 days in Schneider's medium (Fig. 4). It was observed that on day 150 post-infection parasites could be detected in all of the control animals. On day 450, one of the control dogs became parasite negative and remained so until day 634. This result would be in agreement with reported data indicating that some dogs resist and resolve the infection. The reactivity of the sera of this dog against Q was low but it was significant against SLA. In contrast, on day 150 post-infection parasites could only be detected in 50% of the immunized dogs. Three of these animals became parasite negative at day 450. Another one of the immunized dogs which was parasite positive at day 450 became parasite negative at day 634. However, one dog remained parasite positive at that time. Thus, the data indicate that while at day 150 post-infection all of the control dogs were parasite positive due to infection, upon immunization with Q + BCG 50% of the animals were, at the level of the lymph nodes, able to resolve the infection 5 months post administration of the parasites. In summary, at day 634 only one of the immunized dogs was parasite positive while at that time nine of the control dogs were parasite positive. This would mean that Q + BCG had a protective efficacy of 90%. To have an estimate of the statistical significance of this value we asked the question of what would be the probability that in the immunized population of dogs nine of them could have been, by chance, naturally resistant. Using our own data and the data reported by other (Killick-Kendrick et al., 1994; Ciaramella et al., 1997) we assumed that between 20 and 10% of dogs may naturally resist infection after an experimental administration of *L. infantum* virulent parasites. The formula used to calculate the probability was the n-term of the binomial expansion:

$$P = \binom{n}{r} p^r q^{n-r}$$

in which p is the probability of being naturally sensitive and q the probability of being naturally resistant in the population and r the number of dogs sensitive in the immunized sample of size n (10 dogs). The probability that nine dogs in our size sample of the ten could have been naturally resistant is 10^{-5} to 10^{-8} depending on whether q was taken as 20 or 10%, respectively. Thus, we think that the protective efficacy value given above has a high degree of statistical significance.

3.5. Clinical manifestations

Following infection on day 108 the dogs were weekly checked for the appearance of external clinical manifestations. It was found that 5–6 months after infection some of the control dogs developed at different times a gradual onset of clinical signs of Leishmaniasis such as conjunctivitis, lymphadenitis and ulcerations. These external symptoms became obvious in nine of the dogs in the period between day 250 and 634 post-infection having, moreover, various symptoms as blepharitis, cachexia, ulcers, ganglion hypertrophy, anemia and alopecia. One of the unvaccinated control dogs did not show any of these external signs with the exception of intermittent periods of hyperthermia which appeared on day 174 post-infection and continued during the entire period of the follow-up period. This dog was the one indicated above as lymph node parasite negative on days 450 and 634 post-infection. A similar search was conducted on the immunized dogs. None of the dogs showed at any time signs attributable to Leishmaniasis with the exception of a local dermatitis in one of them (at day 300 post-infection). This dog, however, was parasite negative at day 450 post-infection. Thus, it may be concluded that the Q + BCG immunization

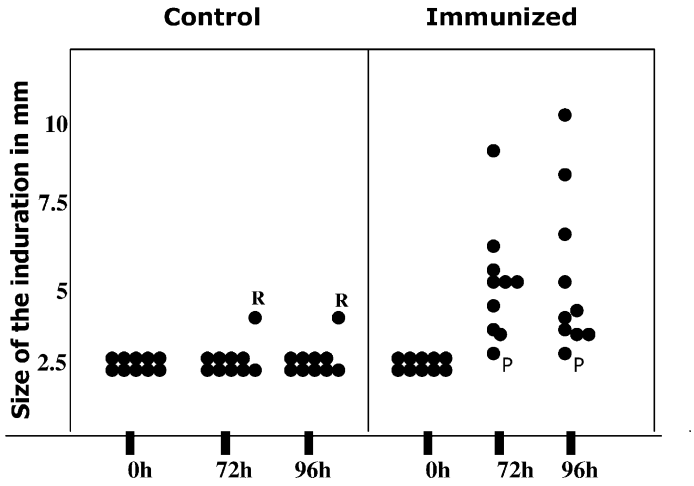

Fig. 5. Size of the induration in mm post administration of leishmanin. The dark circles (●) indicate the size of the induration observed in each dog. Skin reactions were recorded 72 and 96 h after administration of the leishmanin. The size of the induration was measured with the aid of a digital caliber. Here, R indicates unvaccinated dog parasite negative and P indicates immunized dog parasite positive.

induced clinical protection in all or at least nine of the vaccinated dogs.

3.6. Delayed type hypersensitivity assays

On day 634 post-infection, a delayed type hypersensitivity assay was performed. The size of the induration was determined 72 and 96 h after administration of leishmanin (Fig. 5). In none of the control dogs with the exception of the resistant one, a positive DTH response was observed. In contrast, in eight of the immunized dogs a positive DTH response was observed 72 h after administration of leishmanin. The size of the DTH response was variable among dogs. In some dogs the response at 72 h was more intense than at 96 h. The immunized dog which was DTH negative, either at 72 or 96 h after leishmanin administration, was the one observed as positive at the parasite level. In contrast, the control dog which was DTH positive was the one in which obvious clinical symptoms were not detected.

3.7. Anatomo-pathological analysis

Since in addition to clinical external manifestations the visceral form of Leishmaniasis courses with defined anatomo-pathological features, we performed a study of the alterations in morphology occurring in the liver and spleen of control and immunized dogs. Because all animals showed similar clinical signs six dogs from each group were chosen at random. From each group three dogs were sacrificed and the liver and spleen of three dogs were analyzed by laparoscopy. The non-sacrificed unvaccinated control diseased dogs were treated because of ethical requirements. It was observed that the liver of the control sacrificed dogs had a tumescent appearance with distended capsules and discolored areas (Fig. 6a). The appearance of the liver of the immunized animals was normal (Fig. 6b) similar to that observed in non-infected dogs. Microscopically, in the liver of the control dogs there was abundant diffuse portal infiltration of lymphocytes, plasmocytes and macrophages, hepatocyte destruction, vacuolar degeneration and necrosis (Fig. 6c) together with disruption of the normal tissue architecture (Fig. 6d) in the portal area and parenchyma. In the immunized dogs, necrosis was not observed with minimal infiltrates both in the portal spaces and in the hepatic parenchyma (Fig. 6e). The tissue architecture was normal (Fig. 6f). Macroscopically, the spleen of the sacrificed control dogs showed prominent nodules on the surface with focal areas of necrosis (Fig. 6g). In contrast, the appearance of the spleen of the immunized dogs was normal (Fig. 6h). Impression smears of the spleen of the sacrificed control and immunized dogs were examined to determine the parasite content.

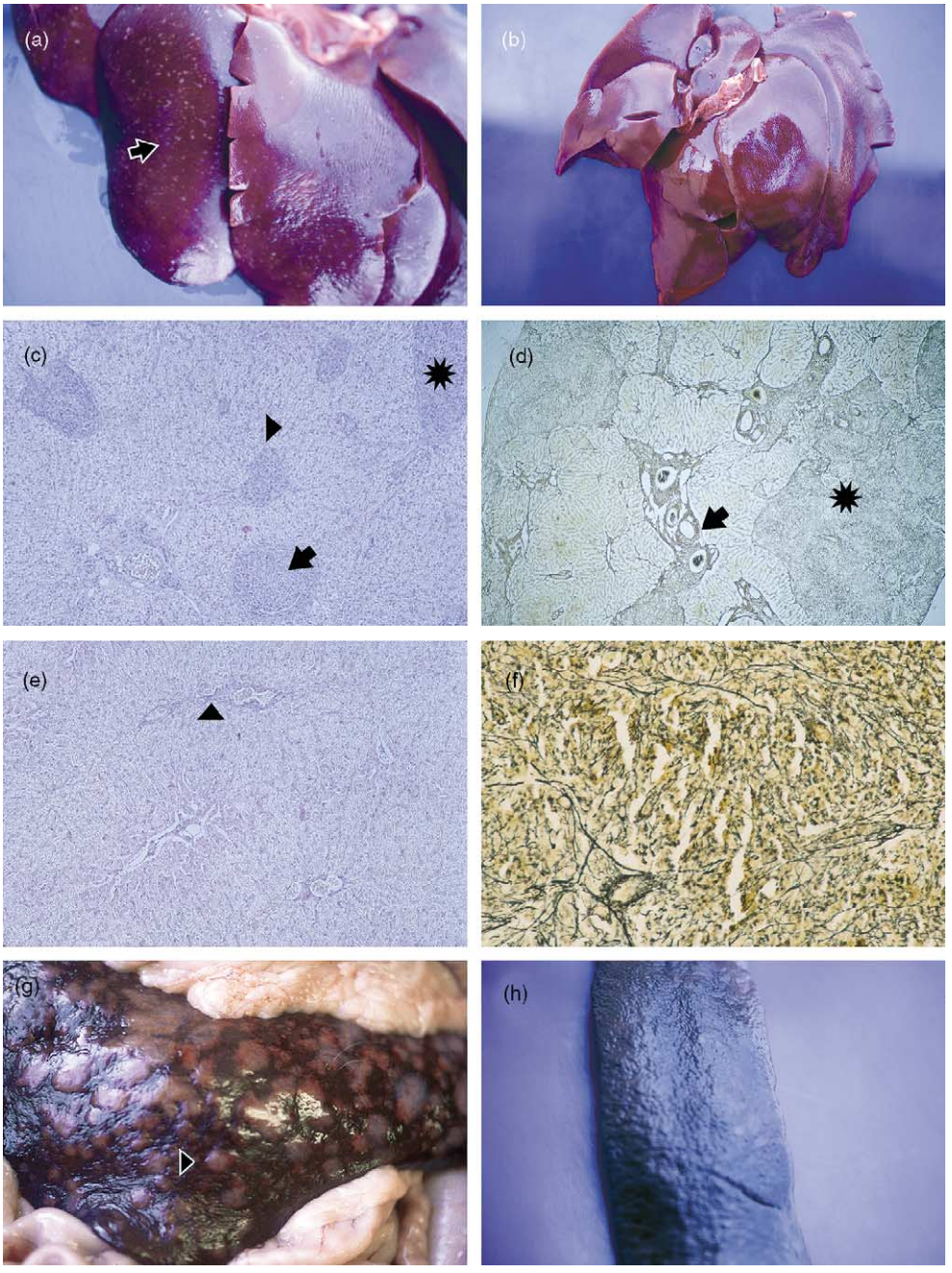

Fig. 6. Anatomical features of the liver and spleen of the control and the immunized dogs. (a) Macroscopic detail of the liver of a unvaccinated control dog with a tumescent appearance, distended capsules and whitish discolored areas. (b) Macroscopic view of the liver of a Q + BCG immunized dog with a normal appearance (c) Micrograph (10×) of the liver of a control dog showing diffuse infiltration of lymphocytes, plasmocytes and macrophages in parenchyma (➡) and in the portal areas (✷) with hepatocyte destruction and vacuolar degeneration and necrosis (▶) (hematoxilin–eosin staining). (d) Micrograph (4×) of the liver of a control dog showing disruption of the normal tissue architecture in the portal area (➡) and parenchyma (✷) (reticulin staining). (e) Micrograph (10×) of the liver of a Q + BCGimmunized dog showing that the infiltrates both in the portal areas (▶) and in the hepatic parenchyma are minimal. (f) Micrograph (10×) of the liver of a Q + BCG immunized dog showing that the tissue architecture is normal (reticulin staining). (g) Macroscopic detail of the spleen of a control dog showing large prominent nodules. (h) Macroscopic detail of the spleen of a immunized dog showing a normal appearance. The photographs were taken on day 634 post-infection. A representative photograph is shown.

Parasites were detected in the control diseased dogs (21, 39, and 25 parasites per 1000 nucleated cells) but not in the immunized animals.

4. Discussion

The pilot study performed on BALB/c mice infected with *L. infantum* promastigotes indicated that the animals became infected by the parasites and that the Q + BCG mix protected the animals against infection since it induced an 80% reduction in parasite load in the spleen of the animals. The study also showed that BCG alone induced some protection but that it was significantly lower. The course of the infection in dogs was in first instance monitored by analysis of the immune response against a soluble *Leishmania* antigen fraction. It was observed that at some time post-infection the sera of all dogs were positive against SLA. Active infection was further shown by culture of biopsies of lymph nodes. It was observed that at day 150 post-infection parasites could be detected in all of the control dogs. One of these dogs was parasite negative by day 450 post-infection and remained negative up to the end of the experiment. In contrast, in the immunized group of dogs only five dogs were parasite positive by day 150 post-infection. Two of these dogs became parasite negative by day 450 and still another dog became parasite negative by day 634 post-infection. Thus, if as indicated by several authors (Mathis and Peplazes, 1995; Campino et al., 2000) the biopsy culture for 15 days is as sensitive as PCR to detect the presence of parasites the data presented most likely indicate that the Q + BCG immunization induced parasite clearance, at least at the lymph node site, in nine out of the ten immunized animals or a protective efficacy of 90%. Since the probability that nine of the dogs could had been naturally resistant is rather low we believe that the 90% efficacy given has significant statistical value. Our data indicated that at the level of the spleen there was also at least a reduction of parasite burden since we could not detect parasites after analysis of 1000 nucleated cells in three of the sacrificed immunized dogs while parasites were detected in the unvaccinated control dogs.

Although it has been indicated (Lanotte et al., 1979; Berrahal et al., 1996) that signs of severe disease occurs in less than 50% of naturally infected dogs, it has been also shown that in endemic areas and after natural infection clinical signs of Leishmaniasis is progressive in almost all dogs (Ciaramella et al., 1997). In agreement with these data, we showed that after a clinical follow-up of 634 days 9 of the 10 control dogs developed clear signs of clinical Leishmaniasis while only one behaved as clinically resistant (the one which was parasite negative at day 634 post-infection). However, symptoms associated with Leishmaniasis, similar to those observed in the control dogs, were not detected in any of the immunized dogs with the exception of one in which there were intermittent local foci of dermatitis. It was observed that the protection detected at the clinical and the parasite level in the Q + BCG immunized dogs run parallel with the observations at the histological level since the gross morphology of the tissues (liver and spleen) of the dogs which were analyzed was normal. In contrast, large morphological alterations at the macro and microscope level were detected in the analyzed control dogs including necrosis and disruption of the normal architecture of the liver tissue.

Thus, the mice and dog data all together strongly indicated that the Q + BCG mix has a high protective value against *L. infantum* infection. We think that BCG is behaving as an adjuvant to the Q acting in a synergic way either by modulating the response to the Q or serving as a co-protective agent. In fact, it has been shown that BCG pretreatment of mice reduces the severity of the cutaneous disease due to *L. tropica* infection and induces a significant decrease in mortality without evidence of visceralization (Weintraub and Weinbaum, 1977). The data presented also showed that BCG induce some protection at the level of lowering the parasite burden in mice.

Our hypothesis to explain the protective potential of the Q + BCG mix is that the immunization may have induced Q parasite-specific and cross-reactive BCG mediated T cells that induce the activation of infected macrophages and, thus, the killing of intracellular or free parasites. In fact, it has been shown that there is cross-reactivity between BCG proteins and *L. donovani* parasites (Smarkovski and Larson, 1977). This cross-reactivity may account for the protection detected in mice after BCG administration. The hypothesis is in agreement with the report indicating that activated macrophages may present intracellular antigens during *Leishmania*

infections (Overath and Aebischer, 1999) and that conserved cytoplasmic proteins, in particular some histones, may have the capacity to induce T cell proliferation (Probst et al., 2001) and to afford protection (Solioz et al., 1999; Melby et al., 2000). In addition, the anti-Q response induced by immunization could neutralize the exposure to the immune system of an inappropriate amount of soluble antigens that would trigger a disproportionate humoral response. In the absence of antigen neutralization, uncontrolled immune–antigen complexes could mediate, moreover, pathological outcomes. It has been shown that some of the components of Q (LiP2a and LiP2b) accumulate in immune-complexes in certain tissues in dogs with active visceral Leishmaniasis (Garcia-Alonso et al., 1996) and that in experimentally infected hamsters there is a correlation between the amount of kidney immune-complexes and the severity of the disease (Requena et al., 2000b).

The induction of the leishmanin skin-test conversion observed in the Q + BCG animals indicates that the Q + BCG mixture was efficient in eliciting a cellular response. Interestingly, with the exception of one dog the highest DTH reaction was observed in the dogs which were parasite negative at day 150. However, clinical protection was achieved even in the dog in which a positive DTH reaction was not observed. Thus, since the number of DTH positive dogs correlates well with protection it is probable that leishmanin skin-test reactivity could be used as a subrogate marker of protection. It has been reported that there is a 98% correlation between positive DTH and cellular immune responses as shown by in vitro proliferative assays (Sassi et al., 1999; Solano-Gallego et al., 2000). Having seen that Q + BCG confers protection further experiments need to be designed to elucidate the actual role of BCG as a potential non-specific immune-modulator of the response triggered by the Q protein and to search for other suitable adjuvants which may circumvent the potential inconveniences of using BCG.

5. Conclusion

The data reported in this paper indicate that the quimeric recombinant Q protein formed by the genetic fusion of five antigenic determinants from four cytoplasmic proteins of the *L. infantum* parasite when mixed with live BCG induces protection in mice and dogs experimentally infected with the virulent *L. infantum* M/CAN/ES/96/BCN 150 strain, zymodeme MON-1. The protection was observed at the parasite, clinical and anatomo-pathological level in nine out of the ten dogs which were immunized with the mix.

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