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Development and Clinical Trial of a Novel DNA Vaccine as Immunotherapy during Canine Leishmaniasis

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Abstract

Background: Visceral Leishmaniasis (VL) is a zoonotic disease caused by *Leishmaniainfantum* in the Mediterranean area. Dogs are the main reservoir of *Leishmaniainf antum* parasites. Disease management represents a serious problem, since anti-*Leishmania* drugs have limited efficacy in both symptomatic and asymptomatic dogs, which are infective to phlebotomine vectors. In many tropical and sub-tropical countries the development of a safe and easily-available vaccine has high priority. DNA vaccines represent one of the most recent innovations in the field of immunization.

Findings: This study aimed to evaluate the effect of a DNA vaccine based on two *Leishmania* antigens (Cpb1, PO) in leishmaniotic dogs. Twelve leishmaniotic dogs from a Leishmaniasis-endemic area (Naples, Italy) received three consecutive injections of DNA vaccine at 15-days intervals. Another group of five leishmaniotic dogs received the same amount of pVAX-1 without the coding sequences of *Leishmania* antigens. *Leishmania* DNA load, INFγ, IL-4 mRNA expression levels and clinical parameters were tested before and after the therapy, every 3 months for a period of 12 months.

Analysis of the data in the vaccinated dogs showed: i) a decrease *Leishmania* DNA load in lymph node samples, ii) an increase of INFγ and IL-4 mRNA expression levels in PBMC samples. All vaccinated dogs also showed an improvement in the clinical symptoms.

Conclusion: Our results show that the vaccine developed in this study may represent a useful tool in the treatment of leishmaniotic dogs. However, since the duration of positive effects is limited in time, further trials are needed to evaluate the effectiveness of immunotherapy alone or in association with conventional therapy.

Keywords: DNA vaccine; Real-time PCR; *Leishmania infantum*; Cytokine; Dog

Introduction

Visceral Leishmaniasis (VL) or *kala-azar* is a zoonotic disease caused by *Leishmaniainfantum*, in the Mediterranean area, and by *Leishmania chagasi* in Latin America and China [1]. It is endemic in less developed countries; however, incidences are increasing in non-endemic areas due to changing patterns of international travel and to population migration [2]. VL currently affects 12 million individuals and it is an opportunistic infection in immunocompromised patients [3].

Dogs are the main reservoir of *Leishmaniainfantum* parasites, and they play a central role in the transmission to humans through phlebotomine sandflies [4,5]. A large amount of evidence has demonstrated that the prevalence and incidence of canine Leishmaniasis (CanL) has, until now, been underestimated [6,7]. In addition to dogs, wild canids, such as jackals (Canis aureus) and red fox (Vulpes vulpes), are potential feral reservoirs for *Leishmania* infantum [8].

After infection, some dogs can control the parasite and don't develop the disease in the short term, sometimes remaining subclinical for years or for their entire lifetime, whereas others may present progressive disease [9]. The presence of latent infection in dogs is typical and contributes to maintaining the long-term presence of the parasite in endemic regions. Disease management represents a serious problem since both symptomatic and asymptomatic dogs are infective to phlebotomine vectors [10], and the available antileishmanial drugs have limited efficacy in dogs [11,12].

The data currently available for canine VL show that the clinical appearance and evolution of Leishmaniasis is a consequence of complex interactions between the parasite and host immune response. The outcome of infection depends on the ability of host macrophages to effectively destroy the parasite. This activity is determined by the balance between a heterogeneous set of cytokines [13]. Previous studies on the immune responses in experimentally infected dogs, have revealed that T lymphocytes and the cytokines play a crucial role in determining whether infection evolves toward a protective immunity status or a progressive and manifested disease [14].

Two different T helper (Th) cell subsets, termed Th1 and Th2, which differ from each other in the profile of secreted cytokines, have been described [15]. In the murine model for Leishmaniasis, the Th1 response confers protection, whereas the Th2 response renders the host susceptible to infection [16-18]. Studies on the immune response in dogs experimentally infected with *Leishmania infantum* have shown that the protective immunity is associated with the production of IL-2, IFN-γ and TNF-α by peripheral blood mononuclear cells (PBMC)

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in addition to the induction of parasite-specific cytotoxic T cells [9]. Furthermore, the activation of macrophages by IFN-γ has been shown to result in nitric oxide production which mediates the killing of intracellular parasites [19, 20]. On the other hand development of the disease is associated with the production of other interleukins such as IL 10, IL 12, IL 18 etc.

In the last decade, the polymerase chain reaction (PCR) has been successfully introduced and proven to be a sensitive and powerful tool to detect *Leishmania* in clinical samples as well as for parasite characterization [21-23]. Very recently, PCR has also been used to analyse the immune response profile either in humans or in infected dogs [24]. However, the multiple steps of post-PCR manipulation require time and pose the risk of DNA contamination. A recent approach to estimating the parasitic load of mouse livers experimentally infected with *Leishmania* infantum has been based on the use of a double real-time quantitative PCR test targeting the parasite DNA polymerase gene and the mouse brain-derived neutrophic factor gene [25,26]. Furthermore, a real-time PCR that simultaneously detects, quantitates and differentiates *Leishmania* organisms has been developed [27,28].

Control of Leishmaniasis currently includes the use of combined chemotherapeutic treatments and vector control by the use of insect repellent. Stamping out is a widely used practice in countries such as Brazil, however attempts to remove seropositive dogs has proven to be insufficient for eradicating visceral Leishmaniasis in dogs [29] .

It is generally accepted that an effective vaccine against canine Leishmaniasis is the best strategy for disease control. Among vaccine type, the DNA vaccine seems to be the most promising due to their ability to efficiently stimulate both humoral and cellular immune immunity in many infectious disease models [30].

A DNA vaccine typically consists of a foreign gene, encoding a protein antigen of interest, cloned into a bacterial plasmid under the control of an appropriate promoter that can be injected into the skin or muscle of the the host. After uptake of the plasmid, the protein is produced endogenously and intracellularly and the antigenic peptides can be presented on the cell surface in the context of both the MHC class I and MHC class II pathway [31].

In this study, a new DNA vaccine, which expresses both Cysteine proteinases b1 (CPb1) and acidic ribosomal protein PO (LiPO) of *Leishmania infantum* was constructed.

The immunotherapeutic potential of this vaccine was assessed in 12 naturally infected dogs.

The quantitative real-time PCR (qPCR) assay was used for measuring: a) *Leishmania* DNA load in lymph node aspirates; b) the expression levels of cytokines relative to either Th1 or Th2 patterns.

Both parasite DNA load and cytokine expression levels were also evaluated in a group of healthy uninfected dogs used as the control group.

Materials and Methods

Plasmid construction and purification

The coding sequences of two *Leishmania* antigens Cpb1 and PO were inserted into the pVAX-1 vector to obtain the pVAX/Cpb1/Po plasmid. pVax CPB1/PO encodesthe full-length sequences of the Cpb1 and PO genesplaced downstream of the cytomegalovirus (CMV) promoter and upstream of the bovine growth hormone (BGH) polyadenylation sequence in the pVax1™ vector (Invitrogen). The sequences of the Cpb1 and POgenes were derived from GenBank (accession number AJ628942.1 and X72714.1 respectively) and were synthesized by GenScript, USA Inc. in a concatameric formation with a linking sequence of repeats coding for a serine/glycine rich peptide flexible bridge rotating on a threonine hinge, as described previously (Robinson and Sauer, 1998). The pVax CPB1/PO plasmid was used for the invitro experiments and clinical vaccine trials described below. The plasmid pUC CPB1/PO encodes the Cpb1/PO fused genes cloned in the multiple cloning sites (MCS) of the pUC19 cloning vector and was used as negative control for *in vitro* experiments. Plasmid DNA for vaccine clinical trials was purified with the Endo Free Plasmid Giga Kit (Qiagen) using endotoxin-free material and eluted in endotoxin-free deionised water.

Expression of Cpb1/PO genes in transfected cells

The human embryonic kidney cell line HEK 293 was transfected with pVax CPB1/PO and with the negative controls pUC/Cpb1/PO, using the CalPhos Mammalian Transfection Kit (Clontech), according to the manufacturer's instructions. Transfected cells were harvested 48 h later and washed with phosphate-buffered saline three times. Total RNA was extracted with Trizol reagent (Invitrogen) and treated with DNaseI (Invitrogen). Complementary DNA (cDNA) was obtained from RNA with the SuperScriptTM First-Strand Synthesis System (Invitrogen). CPB1/PO cDNA was amplified by PCR. The amplified products were analyzed with a 1% agarose gel. Robustness of plasmids expressing CPB1/PO was evaluated by replicating the expression experiments three times.

Also, production of Cpb1/PO protein was detected by immunoblotting (B) using an antiserum leishmaniotic dog Vaccine clinical trial.

This study included 12 dogs, hospitalized at the Department of Clinical Veterinary Sciences of the University of Naples, naturally infected by *Leishmaniainfantum*. These dogs were living in the endemic area of Campania Region in South Italy and included different breeds, ranged in age from 1 to 10 years. All dogs were diagnosed as having CanL on the basis of clinical manifestations, immunofluorescence antibodies IFAT, and qPCR tests.

Criteria for inclusion in this study were: Clinical findings compatible with Leishmaniasis (weight loss, peripheral lymphadenopathy, skin lesions, ocular lesions, epistaxis, lameness, ulcers, diarrhea and moderate glomerulopathy), alterations of ematobiochemical parameters compatible with Leishmaniasis (thrombocytopenia, anaemia, leukopenia; total proteins and beta globulins higher than normal value), presence of anti-L*eishmania* antibody with titres above 1:80, and a *Leishmania* DNA load in lymph node aspirate ranging from 100 parasites/ml.

Animals were scored for clinical and laboratory parameters on a scale from 0 to 9 and the values were added up to give a clinical score [32].

Vaccination protocol

At the time of diagnosis, 12 dogs which were treated contemporaneously with three intramuscular doses and three subcutaneous doses of 500 μg of plasmid, Pvax Po\CPB1 plasmid on days 0, 15, 30.

Another group of five leishmaniotic dogs received the same amount of pVAX-1 without the coding sequences of *Leishmania* antigens (control group). These dogs were kept without treatment until there has

been a worsening of clinical symptoms, then, 6 months after the start of the study, they were subjected to specific treatment.

The efficacy of the therapy was evaluated at 0, 15 and 30 days from the beginning of the therapy, and 3, 6 and 12 months from the beginning of the therapy.

All dogs were given a clinical score on the basis of the presence and severity of clinical manifestations, the alterations of laboratory parameters, and the IFAT titre. In addition the *Leishmania* DNA load in the lymph node aspirates and mRNA expression levels of INF γ and IL4 in the blood were also measured at these times.

The Ethics Committee gave a positive opinion for treatment for compassionate use of dogs studied.

Sampling

Whole blood samples were collected from the dogs in EDTA-containing tubes by cephalic venipuncture. Lymph node aspirates were obtained by using a thin biopsy needle. Samples were stored at –80°C.

DNA extraction from tissue samples and from parasite cultures and RNA isolation and production of cDNAs were carried out as previously described [33].

Selection of primers and probes

All TaqMan primers and probes were selected as previously reported [33]. All primers and FAM probes were provided from Applied Biosystems.

Real-time PCRanalysis

Real-time PCR was performed as previously described [34,35].

Statistical analysis

Statistical analysis was carried out with GraphPad InStat software 3.0 application. The significance of differences between the two groups was evaluated by using the Anova tests. All conclusions were based on significance levels of $p < 0.05$. Boxplot of results were generated by using the same software.

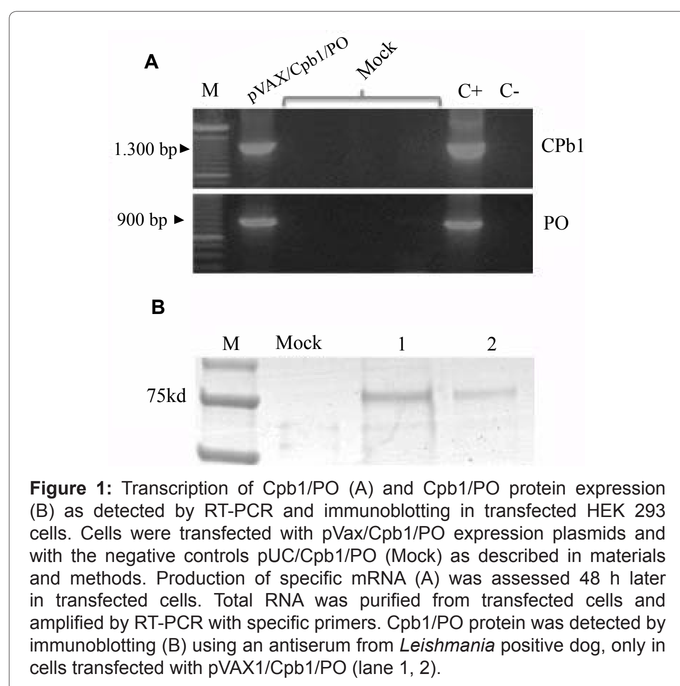

Figure 1: Transcription of Cpb1/PO (A) and Cpb1/PO protein expression (B) as detected by RT-PCR and immunoblotting in transfected HEK 293 cells. Cells were transfected with pVax/Cpb1/PO expression plasmids and with the negative controls pUC/Cpb1/PO (Mock) as described in materials and methods. Production of specific mRNA (A) was assessed 48 h later in transfected cells. Total RNA was purified from transfected cells and amplified by RT-PCR with specific primers. Cpb1/PO protein was detected by immunoblotting (B) using an antiserum from *Leishmania* positive dog, only in cells transfected with pVAX1/Cpb1/PO (lane 1, 2).

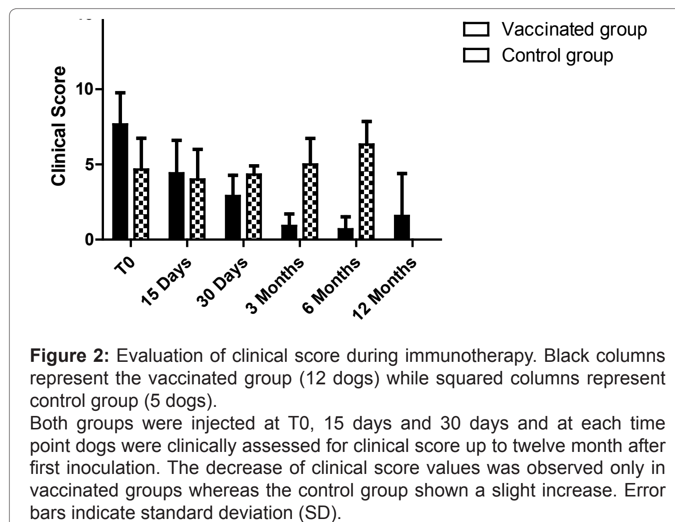

Figure 2: Evaluation of clinical score during immunotherapy. Black columns represent the vaccinated group (12 dogs) while squared columns represent control group (5 dogs).
Both groups were injected at T0, 15 days and 30 days and at each time point dogs were clinically assessed for clinical score up to twelve month after first inoculation. The decrease of clinical score values was observed only in vaccinated groups whereas the control group shown a slight increase. Error bars indicate standard deviation (SD).

Results

Plasmid Pvax/Cpb1/PO was designed in order to investigate the immunotherapeutic potential of DNA vaccines against canine Leishmaniasis. To provide an enhanced immunological response, the proteins were linked together with a sequence encoding a glycine bridge. DNA sequencing confirmed the correct synthesis of Cpb1/PO in Pvax1. To ensured that Cpb1/PO fusion proteins could indeed be transcribed in mammalian cells, the presence of specific mRNA was established by RT-PCR from total RNA isolated from HEK 293 cells transfected with Pvax/Cpb1/PO plasmid. As shown in Figure 1, an amplicon of approximately 1330 bp and 900 bp corresponding to Cpb1 and PO respectively was obtained from transfected cells (Figure 1A). Immunoblotting performed on HEK 293 cell lysates confirmed the presence of correct transcription of fusion protein Cpb1/PO (Figure 1B).

Before immunotherapy began, vaccinated dogs (Group1) had a mean clinical score of 7, while control dogs had a mean value of 5. Three months after the start of therapy, vaccinated dogs showed a progressive improvement in their clinical manifestations, as demonstrated by the decrease in their clinical scores (Figure 2). In addition the average clinical score was lower than before starting therapy. This decrease continued until 6 months after the beginning of therapy. At this point the average of clinical score of the vaccinated dogs started to progressively increase, and finally three dogs had a relapse.

The average of value of clinical score for the control group progressively increased from the beginning of the therapy to the end (Figure 2).

30 days after the start of the immunotherapy, vaccinated dogs showed a relevant decrease of *Leishmania* DNA that continues until 3 months after the beginning of therapy. From this time point the *Leishmania* DNA load started to progressively increase without reaching original load. On the contrary the control group shows a progressive increase of DNA *Leishmania* load (Figure 3).

The IFN-γ and IL-4 mRNA expression levels in group 1 and in the control group were monitored by real time PCR (Figure 4).

IFN-γ mRNA levels of vaccinated dogs show a significant increase at days15 and 30 post inoculation, whereas no difference

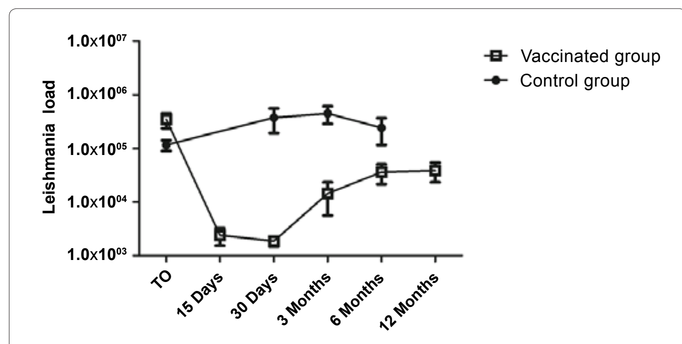

Figure 3: Evaluation of parasite load during immunotherapy. Squares represent vaccinated group (12 dogs) while circles indicates control group (5 dogs). Fine-needle biopsy were taken and *Leishmania* DNA copies was estimated by qPCR at each time point. The axis indicates the number of *Leishmania*/ml expressed in logarithmic values.
In contrast to control group the vaccinated group exhibits a reduction of parasite load since 15 days after first inoculation.

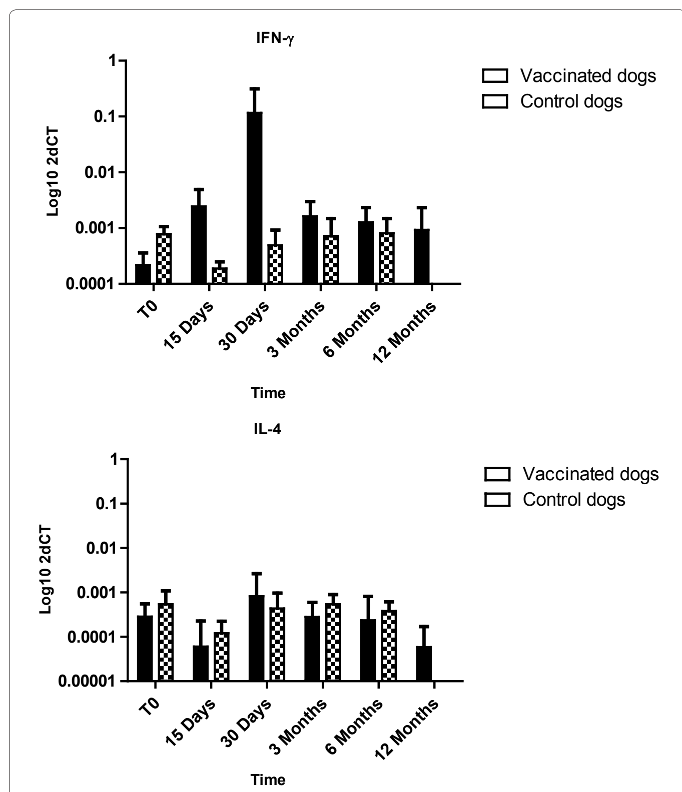

Figure 4: *Transcriptional analysis of PBMC cytokines mRNA expression levels during immunotherapy.*
Cytokine mRNA levels in PBMC shows no relevant differences between the vaccinated and control group with the exception of the 15 days and the 30 days time point of vaccinated group that shows a significant increase of IFN-γ. Error bars indicates standard deviation (SD).

in IL-4 mRNA levels where observed between the vaccinated and the control group.

Discussion and Conclusions

DNA vaccination has been demonstrated to induce both humoral and cellular immune responses. Th1 cells can activate macrophages to destroy intracellular microorganisms and also activate B cells to produce strongly opsonizing antibodies such as IgG2a and IgG2b [31].

In this study, a pVAX based plasmid expressing the Cpb1 and PO proteins of *Leishmaniainfantum* was designed and evaluated in dogs diagnosed with symptomatic Leishmaniasis. As shown in Figure 1, Cpb1/PO mRNA was observed in transfected HEK 293 cells, and the Cpb1/PO fusion protein was detected by Western blotting analysis.

Cpb1 and PO may represent an ideal candidate for the development of a DNA vaccine against Leishmaniainfection. Cysteine proteinases (CPs) are enzymes that belong to the papain superfamily, which are found in a number of organisms from prokaryotes to mammals. Previous data indicates that the Cysteine proteinases (CPA and CPB) inoculated as recombinant protein or as DNA vaccines is able to elicit a protective immune response against L. major in the murine model [36-38]. Analysis of the immune response showed that vaccinated animals developed a specific Th1 immune response, which was associated with an increase of IFN-gamma production. PO is an acidic ribosomal protein. Administration of the P0 antigen from *Leishmaniainfantum*, either as soluble recombinant or as a plasmid DNA formulation confers protective immunity to Leishmania major infection in BALB/c mice [39].

The protective immunity against *Leishmania* is believed to be due to the action of the subpopulation of lymphocytes T helper type 1 [40]. The T lymphocyte activation determines proliferation, differentiation and production of cytokines such as IL-12, IFN-γ and TNF-α [41]. In presence of TNF activated macrophages produce NO which is considered the main mechanisms for Leishmania elimination [42].

Results in this study demonstrate that vaccination with pVAX/Cpb1/PO determines a clinical improvement since 15 day after the first inoculation along with a decrease of DNA parasite load in the lymphnode. Improvement of clinical score and decrease of parasite burden progresses until 30 days post inoculation and stabilizes until 3 months time point.

The clinical improvement is probably due to a decrease of organ parasitism. The remarkable decrease in parasite burden is a consequence of activation of effective immunity response. The outcome of infection by *Leishmania* depends on the balance between host ability to induce immune mechanisms against *Leishmania* and the parasite strategies to interfere in the process of antigen presentation and thus modulate the immune response mediated by T lymphocytes [43]. Expansion in the number of CD4 + T lymphocytes subset has been correlated to a reduction in organs parasitism in dogs undergoing immunotherapy and in vaccinated animals [44,45].

Previous studies analyzing peripheral blood lymphocyte correlates protective immune response against *Leishmania* infantum with CD4+ Th1 cells expressing IFN-γ, IL-2 e TNF-α. Cytokine patterns (IL-4) of CD4+ Th2 subset has been correlated to symptomatic state of the disease [46].

In our study, at 15 days and 30 days time points there is a significant increase of IFN-Γ mRNA levels, coinciding with the lowest DNA parasite load and lowest clinical score in vaccinated dogs. IL-4 mRNA levels selected as Th2 response marker do not show significant variations along the clinical trial. Results suggest that pVAX/Cpb1/PO trigger an effective Th1 immune response. These effects are transient and more or less around the thirty months after the first inoculation we assist a progressive decline of the clinical status.

In conclusion, our results show that the vaccine developed in this study may represent a useful tool in the treatment of leishmaniotic dogs. However, further trials are needed to evaluate the effectiveness of immunotherapy alone or in association with conventional therapy.

Acknowledgement

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Results

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