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Immunological comparison of DNA vaccination using two delivery systems against canine leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) is a fatal disease caused by the intracellular protozoan parasite *Leishmania infantum*. Dogs are the primary reservoirs of this parasite, and vaccination of dogs could be an effective method to reduce its transfer to humans. In order to develop a vaccine against VL (apart from the choice of immunogenic candidate antigens), it is necessary to use an appropriate delivery system to promote a proper antigen-specific immune response. In this study, we compared two vaccine delivery systems, namely electroporation and cationic solid–lipid nanoparticle (cSLN) formulation, to administer a DNA vaccine containing the *Leishmania donovani* A2 antigen, and *L. infantum* cysteine proteinases of type I (CPA) and II (CPB) without its unusual C-terminal extension. The protective potencies of these two vaccine delivery systems were evaluated against *L. infantum* challenge in outbred dogs. Our results show that the administration of pcDNA-*A2-CPA-CPB*$^{-CTE}$ *GFP* vaccine as a prime-boost by either electroporation or cSLN formulation protects the dogs against *L. infantum* infection. Partial protection in vaccinated dogs is associated with significantly ($p < 0.05$) higher levels of IgG2, IFN-γ, and TNF-α and with low levels of IgG1 and IL-10 as compared to the control group. Protection was also correlated with a low parasite burden and a strong delayed-type hypersensitivity (DTH) response. This study demonstrates that both electroporation and cSLN formulation can be used as efficient vaccine delivery systems against visceral leishmaniasis.

1. Introduction

Leishmaniasis has been classified as one of the most neglected diseases (McDowell and Rafati, 2014). The three major clinical forms of this disease in humans are cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, and visceral leishmaniasis (VL). There are nearly 20 commonly recognized species of *Leishmania* that are known to cause CL or VL in humans (Desjeux, 2004). The *Leishmania donovani* complex (*L. donovani*, *Leishmania infantum*, and *Leishmania chagasi*) is responsible for more severe clinical manifestations than CL (Maurıcio et al., 2000), and leads to death, if left untreated. The numbers of leishmaniasis cases are increasing worldwide. Approximately 57,000 human deaths result annually from VL (Reithinger and Davies, 2002). VL is emerging as an important opportunistic infection among people with HIV-1 infection (Desjeux and Alvar, 2003; Alvar et al., 2008).

A variety of vaccination strategies against *Leishmania* have been attempted so far in mice, dogs, and humans (Coler and Reed, 2005). Of them, DNA-based vaccines have practical advantages, especially because they are generally less costly to produce than peptide or protein vaccines, can be produced on a large scale with high purity, and are stable under a wide variety of environmental conditions (Gurunathan et al., 2000).

The *L. donovani* A2 antigen, a gene family expressed specifically in the amastigote stage (Charest and Matlashewski, 1994) and associated with visceralization (Zhang and Matlashewski, 2001), has been tested previously as a candidate vaccine against *Leishmania*. Immunization with A2 antigen as a recombinant protein or a DNA

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vaccine significantly protected BALB/c mice, and more recently dogs, against *L. donovani* and *Leishmania amazonensis* infections (Ghosh et al., 2001; Coelho et al., 2003; Zanin et al., 2007; Fernandes et al., 2008; Resende et al., 2008). The saponin-adjuvanted recombinant A2 antigen, called Leish-Tec®, was licensed for prophylaxis against canine leishmaniasis and has been used in Brazil for partial protection in the high-dose *L. infantum* beagle dog model (Fernandes et al., 2008). In addition, recombinant *Leishmania tarentolae* expressing A2 antigen proved to be an effective live vaccine against *L. infantum* infection in mice (Mizbani et al., 2009). Among the other *L. infantum*antigen candidates, cysteine proteinases of type I (CPB) and II (CPA) have been examined in experimental vaccinations in both mouse and dog models (Rafati et al., 2005, 2006). Our previous study in dogs showed that DNA vaccination using *cpa* and *cpb* genes followed by administration of recombinant CPA and CPB proteins could induce partial protection (Rafati et al., 2005).

In general, DNA delivery methods can be classified into viral- and nonviral-based systems (Escors and Breckpot, 2010). Nonviral-based systems are subdivided into physical and chemical types. Electroporation (EP) is a physical delivery system involving the application of short electric pulses to the vaccination site, resulting in the formation of transient pores in the plasma membrane of the adjacent cells (Trollet et al., 2006; Cukjati et al., 2007). This allows macromolecules such as nucleic acids to enter the cytoplasm (Becker and Kuznetsov, 2007). The cationic solid–lipid nanoparticle (cSLN) formulation as a chemical delivery system has more advantages than other carrier systems, such as high stability in body fluids and tissues, ability to release drugs for sustained periods, biodegradability, ease of manufacture (Joshi and Müller, 2009), and low cost (Wissing et al., 2004; Shidhaye et al., 2008). Our previous study showed that the use of cSLN formulation as a delivery system could protect CPA, CPB and CPB-^{CTE} genes from extracellular enzymatic degradation, and also displayed remarkable low cytotoxicity (Doroud et al., 2010). Moreover, this delivery system together with pcDNA encoding CPA and CPB could partially protect BALB/c mice against cutaneous *Leishmania* infection (Doroud et al., 2011b). Our recent study in mice showed that both electroporation and cSLN formulation were able to deliver the DNA vaccine containing the *L. donovani* A2 antigen with *L. infantum* cysteine proteinases to promote a strong T helper (Th1) immune response and protect against *L. infantum* infection (Saljoughian et al., 2013).

In this study, we evaluated the potency of the pcDNA-*A2-CPA-CPB-*^{CTE} *GFP* trifusion gene delivered by either a physical method (electroporation) or a chemical method (cSLN formulation) as a candidate vaccine against *L. infantum* infection in dogs, and also assessed its ability to induce protective immunity after a 20-month follow-up.

2. Materials and methods

2.1. Ethical consideration

All experiments in dogs, including maintenance, handling program, and blood and bone marrow (BM) sample collection, were approved by and found to be in accordance with the guidelines of the Animal Care and Ethics Committee at Pasteur Institute of Iran (Grant ID 564 dated 2011) and Veterinary Board of Tehran Medical School (700/4038 dated 2011) based on the specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medical Education (MOHME) of Iran (2005).

2.2. Plasmid construction and purification

pcDNA 3.1(-) (Invitrogen, Grand Island, NY, USA) vector containing *A2-CPA-CPB-*^{CTE} *GFP* genes was transformed into the DH5α *Escherichia coli* strain available from our previous study (Saljoughian et al., 2013). The A2-CPA-CPB trifusion expression was confirmed in COS-7 mammalian cells before the commencement of vaccination (data not shown). Large Luria-Bertani (LB) culture was prepared, the pcDNA-*A2-CPA-CPB*$^{-CTE}$*GFP* vaccine was purified by ion-exchange chromatography with QIAGEN EndoFree Mega Kit (Hilden, Germany), and each gene fragment was confirmed by polymerase chain reaction (PCR) (Saljoughian et al., 2013). The total concentration and purity of pcDNA were determined by a NanoDrop ND-1000 spectrophotometer.

2.3. Animal studies

This study included 30 outbred dogs (18 males and 12 females, aging 6 months to 4 years, and weighing 18 ± 4 kg), from nonendemic parts of Iran. The animals were housed individually in conventional kennels at the School of Veterinary Medicine, Tehran University, and fed with standard commercial diet (Nutripet, Iran). The animals were regularly maintained for 3–4 months in the animal facility. All the animals were treated for intestinal helminth infections and were immunized against distemper (DHP, produced by NOBIVAC, Intervet), canine parvovirus (CPV strain 154), canine adenovirus (CAV 2 strain Manhattan LPV3), and rabies (BHK, produced by Pasteur Institute of Iran) before study. All dogs responded negatively for the presence of *Leishmania* DNA by PCR (primers: RV1 and RV2 that targeted the region of kinetoplastid minicircle DNA of *L. infantum*) (Lachaud et al., 2002) of peripheral blood mononuclear cells (PBMCs) and serum anti-*Leishmania*-specific immunoglobulin G (IgG) antibody by enzyme-linked immunosorbent assay (ELISA). ELISA was performed with some modifications (serum 1/100 diluted in phosphate-buffered saline (PBS)/BSA 1%, 100 μL substrate system (KPL, ABTS), and the OD was measured at 450 nm) as described earlier (Rafati et al., 2005). The well-being of the animals was determined regularly by veterinarians, and all the invasive procedures were performed in accordance with the rules of ethical procedures in animal experimentation and biosafety.

2.4. Vaccine administration and parasite challenge

The dogs were divided into three groups of 10 (according to their weight, sex, and age), denoted as G1, G2, and G3. The first group (G1) was immunized subcutaneously (SC) with pcDNA-*A2-CPA-CPB*$^{-CTE}$*GFP* vaccine (200 μg/dog) via cSLN formulation, a chemical delivery previously described by Doroud et al. (2011a). The second group (G2) was immunized SC with the same amount of DNA via electroporation, with a field strength of 80 v/cm (constant), 10 pulses of 30 ms each using ECM 830 Electroporator (BTX, Holliston, MA, USA). The control group (G3) was immunized with PBS. Our previous studies in dog and mouse models showed that pcDNA did not significantly stimulate the immune response; hence, in this study, we did not consider the empty pcDNA as control group (Rafati et al., 2005; Saljoughian et al., 2013). All groups were immunized similarly for their booster immunization. All groups were tested by intravenous injection of 4×10^7 parasites/dog *L. infantum* (MCAN/ES/98/LLM-877) in stationary-phase promastigotes (kindly provided by the World Health Organization (WHO) collaborating center for leishmaniasis, Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain) 3 weeks after the booster vaccination.

2.5. Humoral response assay

Sera of the dogs were tested for the presence of IgG1 and IgG2a against either rA2, rCPs, or *Leishmania* (F/T) at six different periods (T0: before challenge at day 41; T2: 2 months after challenge at day 60; T6: 6 months after challenge at day 180; T11: 11 months