Experimental Parasitology 128 (2011) 9–17

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Leishmania major: Protective capacity of DNA vaccine using amastin fused to HSV-1 VP22 and EGFP in BALB/c mice model

Azam Bolhassani [a,1], Elham Gholami [a,1], Farnaz Zahedifard [a], Neda Moradin [a], Parto Parsi [a,b], Fatemeh Doustdari [a], Negar Seyed [a], Barbara Papadopoulou [c], Sima Rafati [a,*]

[a] *Molecular Immunology and Vaccine Research Laboratory, Pasteur Institute of Iran, 69 Pasteur Ave., Tehran 13164, Iran*
[b] *Khatam University, Tehran, Iran*
[c] *Research Centre in Infectious Diseases, CHUL Research Centre and Department of Medical Biology, Faculty of Medicine, Laval University, 2705 Laurier Blvd., Quebec (QC), Canada G1V 4G2*

ARTICLE INFO

Article history:
Received 24 April 2010
Received in revised form 13 January 2011
Accepted 17 January 2011
Available online 26 January 2011

Keywords:
DNA vaccination
Leishmania
Amastin
Cutaneous leishmaniasis
Herpes simplex virus type1 VP22 (HSV-1 VP22)
Green fluorescent protein (GFP)

ABSTRACT

An intercellular spreading strategy using herpes simplex virus type 1 (HSV-1) VP22 protein is employed to enhance DNA vaccine potency of *Leishmania major* amastin antigen in BALB/c mice model. We evaluated the immunogenicity and protective efficacy of plasmid DNA vaccines encoding amastin-enhanced green fluorescent protein (EGFP) and VP22-amastin-EGFP. Optimal cell-mediated immune responses were observed in BALB/c mice immunized with VP22-amastin-EGFP as assessed by cytokine gene expression analysis using real time RT-PCR. Vaccination with the VP22-amastin-EGFP fusion construct elicited significantly higher IFN-gamma response upon antigen stimulation of splenocytes from immunized mice compared to amastin as a sole antigen. Mice immunized by VP22-amastin-EGFP showed partial protection following infectious challenge with *L. major*, as measured by parasite load in spleens. These results suggest that the development of DNA vaccines encoding VP22 fused to a target *Leishmania* antigen would be a promising strategy to improve immunogenicity and DNA vaccine potency.

1. Introduction

Leishmaniasis is a group of vector-borne diseases caused by obligate intracellular protozoan parasites belonging to the genus *Leishmania*. Clinical manifestations range from self-healing cutaneous lesions to fatal visceral disease. Leishmaniasis is prevalent in six continents and considered endemic in 88 countries (Sharma and Singh, 2009). A prophylactic vaccination would prove to be the most effective strategy to control the infection and spreading of diseases (Rodriguez-Cortes et al., 2007). However, despite substantial effort made in developing a vaccine, there is currently no licensed vaccine against human leishmaniasis (Reithinger et al., 2007). Different vaccination strategies have been tested including the use of heat-killed, genetically modified or live-attenuated parasites as well as several subunit- and DNA- based vaccines with adjuvant (Palatnik-de-Sousa, 2008). Immunization with plasmid DNA encoding *Leishmania* antigens represents a promising approach for vaccination against leishmaniasis since it has intrinsic adjuvant properties, induces both humoral and cell-mediated immune responses, and results in long lasting immunity (Dunning, 2009). The ~33.6 Mb genome (~8300 protein coding genes) of *Leishmania major (L. major)* has been sequenced (Ivens et al., 2005) and provided a rich source of potential vaccine candidates. Furthermore, *Leishmania* gene products that show preferential expression in the intracellular amastigote stage could represent promising candidates for vaccine development (Palatnik-de-Sousa, 2008).

Amastin developmentally regulated proteins belong to one of the larger families of surface proteins in *Leishmania* (Rochette et al., 2005) and show high similarity to the amastin proteins in *Trypanosoma cruzi*. The members of the amastin gene family, approximately 45 in average in all species, are dispersed throughout the genome of *Leishmania*. Amastin genes usually code for small proteins of about 200 amino acids (Rochette et al., 2005) and are expressed specifically in the amastigote stage of the parasite (Rochette et al., 2005; Wu et al., 2000). Due to their relatively hydrophobic sequence and localization in the plasma membrane, it is hypothesized that amastin proteins function in proton or ion traffic across the membrane to adjust the cytoplasmic pH. However, the role of the amastin gene family in the virulence of *Leishmania* is still unclear (Rochette et al., 2005). All amastin proteins possess two predicted extracellular domains. The first domain, located between transmembrane helices 1 and 2, is 55–60 aa long and contains a highly conserved sequence of amino acids at

* Corresponding author. Fax: +98 21 66496560.
E-mail addresses: sima-rafatisy@pasteur.ac.ir, s_rafati@yahoo.com (S. Rafati).
[1] Equally participated as first author.

0014-4894/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2011.01.012

positions 52–62, which is present in all of the *Leishmania* and *Trypanosoma* amastin homologs and corresponds to the amastin signature sequence (Rochette et al., 2005). There is limited information about the immunogenic and protective role of amastin in literature. Recently, we showed that amastin signature is recognized by both cutaneous (CL) and visceral leishmaniasis (VL) patients and could act as relevant biomarker for the serodiagnosis of VL not CL cases (Rafati et al., 2006a,b). In addition, a report by Stober et al. demonstrated that among 100 randomly selected amastigote expressed genes as DNA vaccine, 14 protective novel vaccine candidates were identified. The best novel antigen was amastin-like gene that maps to a 17 gene tandem array on *Leishmania* chromosome 18 (Stober et al., 2006).

However, the actual function of the amastin signature sequence is not yet determined. Due to low efficiency of naked DNA uptake by cells, various strategies are under evaluation to enhance the immunogenicity of DNA vaccines (Hellgren et al., 2004). A variety of peptides called protein transduction domains (PTDs) or cell penetrating peptides (CPPs) have been characterized for their ability to translocate into live cells. Cell penetrating peptides have low toxicity and a high yield of delivery (Javer and Langel, 2004; Wagstaff and Jans, 2006). The most commonly studied CPPs are homeodomain transcription factors such as Antennapedia, the herpes simplex virus (HSV) type1 protein VP22, and the human immunodeficiency virus (HIV-1) transactivator TAT protein (Wagstaff and Jans, 2006). VP22 has been claimed to exit the cell in which it is synthesized via an uncharacterized (golgi-independent) secretory pathway and to enter adjacent cells by a non-endocytic mechanism. This protein is one of the most abundant tegument proteins in the HSV virion particle and has been conserved through the evolution of the alpha herpes viruses (Perkins et al., 2005). Antigenic peptides may be delivered to cells by fusion to VP22 in two ways; the recombinant fusion protein could be applied either exogenously to cells or by transfection of cells with a DNA vector (Elliott and O'Hare, 1997). Furthermore, the enhanced green fluorescent protein (EGFP), which is used widely as a novel marker gene product, can influence the immune response and stimulate specific cytotoxic T cell activity in different models such as humans, rhesus macaque monkeys, and mice (Re et al., 2004; Rosenzweig et al., 2001; Gambotto et al., 2000; Skelton et al., 2001; Tian et al., 2003). In this study, both the VP22 and EGFP ORFs were incorporated within a DNA vaccine harboring amastin as the target antigen. We showed here that co-administration of VP22 and EGFP could be an effective strategy to augment antigen immunogenicity.

2. Materials and methods

2.1. Mice and parasites

Female 6–8 week old BALB/c mice were obtained from the breeding stocks maintained at the Pasteur Institute of Iran. *L. major* (MHRO/IR/75/ER) parasites were kept in a virulent state by continuous passage in BALB/c mice. *L. major* promastigotes were grown at 26 °C in M199 medium supplemented with 5% heat inactivated FCS, 40 mM HEPES, 0.1 mM adenosine, 0.5 μg/ml hemin, and 50 μg/ml gentamicin (Sigma). The stationary-phase promastigotes were used for challenge. To prepare frozen-thawed (F/T) antigens of *L. major*, promastigotes were collected by centrifugation (3000 rpm for 15 min at 4 °C, Eppendorf , Centrifuge 5810R, A-4-62), washed three times in PBS, and resuspended in a concentration of 2×10^8 cells/ml. This preparation was then frozen and thawed 10 times using liquid nitrogen and37 °C water bath. For soluble *L. major* antigens (SLA) preparation, F/T antigens were centrifuged at 4000 rpm for 10 min at 4 °C (Eppendorf , Centrifuge 5810R, A-4-62) and supernatant was kept as SLA. Protein concentrations were determined by bicinchoninic acid reagent (BCA, Pierce, Rockford) and antigens were kept at −70 °C until use.

2.2. Synthesis of the N-terminal domain of an L. major amastin gene

The amastin signature peptide of *L. major* (LmjF08.0810) consisting of 52 amino acids with the sequence PIDMFRPHNTSRIGNTP CLTLWGYKSECYSTKYDVRSDDLWANCTDRLLQFR was used for the current study. This sequence shares 48–100% homology to eight other *L. major* amastin homologs (LmjF08.0850, LmjF08.0800, LmjF08.0840, LmjF08.0830, LmjF08.0820, LmjF08.0970, LmjF08. 0960 and LmjF08.0700), as well as 53% homology to one of the *L. infantum* amastin genes (LinJ34.840). The amastin peptide was chemically synthesized and purified (University of Lausanne, Lausanne, Switzerland) as described previously (Rafati et al., 2006a,b). Amino acid analysis, high-pressure liquid chromatography (HPLC) and mass spectrometry were used to determine the purity of the final product. HPLC analysis showed the purity higher than 95%.

2.3. Plasmid constructs and DNA preparation

The C-terminal truncated VP22 (UL49) gene of HSV-1 [CT-VP22] was amplified from purified genomic DNA of HSV-1 (kindly provided by Dr. Mokhtari; Faculty of Health, Tehran University) using the following primers: VP22F (sense: 5' GCA TCT AGA CAT ATG ACC ATG TCG ACG GCG CCA 3') and VP22R (anti sense: 5' TAT AAG CTT GGT ACC TCA CTC GAC GGG CCG 3'). The underlined sites in sense and antisense primers correspond to XbaI and HindIII restriction sites, respectively. The amplified region is 430 bp. PCR mixture was comprised of HSV-1 genomic DNA (100 ng), deoxynucleoside triphosphates (dNTP, 200 μM each), Taq DNA polymerase (5 U/μl, Roche), and 2 μM of each primer. The PCR products of the expected sizes were digested with XbaI and HindIII restriction enzymes, gel purified, and sub-cloned into pGEM-II vector (Promega). In the next step, the open reading frame of the *L. major* amastin gene (LmjF08.0700) consisting of about 600 bps was amplified from genomic DNA (*L. major* Friedlin strain) using primers: AmaF (5′TGC TCT AGA ATGGCGTGCAAGCTC3′), AmaR (5'TCA AAG CTT CTACTCC TGCGCTGC3′). The underlined sites correspond to XbaI and HindIII restriction sites, respectively. The PCR products of the expected size were digested with XbaI and HindIII, gel purified, and subcloned into pGEM-II vector. For obtaining the fusion construct of VP22-amastin, two pairs of primers were designed. The forward and reverse primers for amplification of VP22 and amastin were respectively as follows: VP22F: 5' GCA TCT AGA CAT ATG ACC ATG TCG ACG GCG CCA 3' and VP22R' with an overhang sequence for 5' amastin: 5' CTT GCA CGC CAT CTC GAC GGG CCG TCT GGG 3'; AmaF': 5' CGG CCC GTC GAGATG GCG TGC AAG CTC GGC 3' with an overhang for 3' VP22 and AmaR: 5' TCA AAG CTT CTA CTC CTG CGC TGC 3'. Subsequently, the purified VP22 and amastin PCR products (as template) and the primers VP22F and AmaR were used to prepare VP22-amastin construct in the third PCR reaction as shown in Fig. 1. The PCR product of the expected size (1059 bp) was digested with XbaI and HindIII, gel purified and sub-cloned into pGEM-II vector. All plasmid DNA (pGEM-VP22, pGEM-amastin and pGEM-VP22-amastin) were purified from recombinant clones by an alkaline lysis method (Qiagen Plasmid Mid Kit) verified by restriction enzyme digestion and sequenced using the dideoxy chain termination method on an automated sequencer. After confirmation of VP22 (430 bp), amastin (600 bp), and VP22-amastin (1059 bp) sequences, all genes were excised from their plasmids by XbaI/HindIII and sub-cloned into NheI/HindIII sites of pEGFP-N1 expression vector (Clontech, Palo Alto, CA) for in vivo gene expression studies. The pEGFP-VP22, pEGFP-amastin and pEGFP-VP22-amastin were transformed into the DH5α *E. coli* strain. Plasmid DNA was purified by an alkaline lysis method (Qiagen Plasmid