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Evaluation of DNA/DNA and prime-boost vaccination using LPG3 against *Leishmania major* infection in susceptible BALB/c mice and its antigenic properties in human leishmaniasis

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ABSTRACT

One of the main issues in vaccine development is implementation of new adjuvants to improve the antigen presentation and eliciting the protective immune response. Heat shock protein (HSP) molecules are known as natural adjuvants. They can stimulate the innate and adaptive immune response against infectious diseases and cancer. Lipophosphoglycan 3 (LPG3), the *Leishmania* homologous with GRP94 (glucose regulated protein 94), a member of HSP90 family, is involved in assembly of LPG as the most abundant macromolecule on the surface of *Leishmania* promastigotes. In the present study as a primary step, we tested LPG3 as a vaccine candidate in two regimens, DNA/DNA and prime-boost (DNA/Protein), against *Leishmania major* infection in BALB/c mice model. Our results showed that LPG3 and its fragment (rNT-LPG3) are highly immunogenic in BALB/c mice and can stimulate the production of both IgG1 and IgG2a. In prime-boost immunization strategy, the level of antibody response was higher compared with DNA/DNA immunization. The levels of IFN-γ in the supernatant of splenocytes from mice immunized with DNA/DNA and prime-boost regimens were significantly higher when compared to control groups. In fact, immunization with prime-boost vaccination has higher ratio of IFN-γ/IL-5, suggesting a shift towards a Th1 response.

In addition, sera reactivity against LPG3 in visceral leishmaniasis (VL) patients was significantly higher in comparison with cutaneous leishmaniasis (CL) patients. Therefore, we recommend further investigations on the usage of LPG3 co-delivery with candidate antigens for vaccine development against leishmaniasis.

1. Introduction

Leishmaniasis is one of the most important infectious diseases caused by intracellular protozoan parasite of the genus *Leishmania*. Depending on the parasite species and the immunological status of the host, the disease manifestations may range from chronic cutaneous lesions which heal spontaneously, to visceral leishmaniasis, which is fatal if left untreated (Murray et al., 2005). Currently, 12 million people in 88 countries are infected, and 350 million people are at risk of infection. In addition, about 2 million new infections occur each year, and the disease causes about 60,000 deaths annually (Ghosh and Bandyopadhyay, 2003; Croft et al., 2006; Stebut, 2007).

WHO has designated leishmaniasis as a category-1 (emerging and uncontrolled) disease with prevention focused on vector control, control of animal reservoirs, and research on potential vaccines (Roberts, 2006). It has been shown that Th1 response in animal models of *Leishmania major* infection leads to controlling parasite replication and curing the animal, while Th2 response exacerbates the infection. Thus, during the past decades, extensive efforts have been put into search for an effective *Leishmania* vaccine which is able to induce Th1 responses.

Many vaccination strategies have been designed and examined against leishmaniasis, including killed or live attenuated parasites, recombinant *Leishmania* proteins or DNA encoding leishmanial proteins, and immunomodulators from sand fly saliva. Although up to now, there is no effective vaccine against leishmaniasis, a few vaccine preparations (e.g. Leish-111f and AG-702) are at advanced stages of clinical trials (Kedzierski et al., 2006).

In addition, an increasing number of antigens from *Leishmania* have been identified and evaluated for both their protective efficacy and serodiagnostic application (Roberts, 2006; Kedzierski et al., 2006). Remarkably, many of them belong to evolutionary conserved protein families. Among them, heat shock proteins (HSPs) are important not only as pathogen-derived immunogens, but also as potential carrier adjuvants. HSPs are family of the most highly conserved proteins found in eukaryotes (even in plants) and

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prokaryotes (Bolhassani and Rafati, 2008). They constitute a superfamily of several distinct proteins, which can be classified into different families based on their molecular weight, such as small HSPs, HSP40, HSP60, HSP70, HSP90, and HSP110 (Bolhassani and Rafati, 2008; Fink, 1999). Their function is essential for cell survival since they act as molecular chaperones (Hartl and Hayer-Hartl, 2002). Under normal conditions, they help to achieve suitable protein folding and subunit assembly. In case of stress, HSP synthesis is increased to protect cells; they prevent aggregation of misfolded proteins and initiate their refolding or their proteolytic degradation if the misfolding is irreversible.

In addition to their cellular function, it is recognized that they possess immunologic properties. HSPs are involved in eliciting a potent, specific, cellular adaptive immune response, which have been suggested to be dependent on their ability to chaperone with a large variety of peptides (Singh-Jasuja et al., 2001; Binder et al., 2001; Zugel and kaufman, 1999).

There are some specific receptors on antigen-presenting cells (APCs), which enable HSP proteins to initiate signaling pathways and enter APCs. Among these receptors, CD91 (Binder et al., 2000), CD40 (Becker et al., 2002), several scavenger receptors such as CD36 (Panjwani et al., 2002) and LOX-1 (Delneste et al., 2002), and Toll-like receptors (TLR-2 and TLR-4) (Asea et al., 2002) are well-characterized. They can prime T cells by increasing antigen display via major histocompatibility complex (MHC) class I and II molecules (Roberts, 2006).

LPG3 is the *Leishmania* homolog of the mammalian endoplasmic reticulum (ER) chaperone GRP94. It have been reported that LPG3 has an essential role in synthesis of the lipophosphoglycan (LPG), the most abundant surface molecule identified on promastigotes of all *Leishmania* species (Beverley and Turco, 1998; Descoteaux et al., 2002). LPG is a multifunctional virulence factor that is required for parasite survival and development in both sand flies and mammals. In fact, LPG is implicated by parasite in binding to and release from the midgut wall of sand fly, inhibition of midgut proteases, attachment and entry into the host macrophages, resistance to complement molecules, manipulation of signal transduction pathways and gene expression in macrophages, resistance to oxidative stress, and allowing establishment of durable infection (Beverley and Turco, 1998; Descoteaux et al., 2002; Kamhawi, 2006; Ilg, 2000).

Descoteaux et al. reported that LPG3/GRP94 in *Leishmania* is one of the class II LPG genes and belongs to GRP94/HSP90 family (Descoteaux et al., 2002). Based on this finding, it was proposed that the role of LPG3 in virulence should be exerted through its function in the synthesis of LPG, GPI-anchored proteins (such as GP63) and other phosphoglycan-bearing molecules (Descoteaux et al., 2002). GRP94 homolog from the related species *Leishmania infantum* was identified and shown to be highly antigenic and is a valuable molecule for diagnostic purposes and a promising candidate for eliciting protective immunity (Larreta et al., 2000, 2002).

In the present paper, the recombinant proteins of LPG3, NT-LPG3 and CT-LPG3 (rLPG3, rNT-LPG3 and rCT-LPG3) were expressed in *Escherichia coli* and seroreactivities of patients suffering from visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) were examined against these recombinant proteins as diagnostic markers. We have also evaluated the protective immunity using *L. major* LPG3 by different vaccination strategies (DNA/DNA and DNA/Protein) in the susceptible BALB/c mice.

2. Materials and methods

2.1. Mice and parasites

Inbred 6–8-week-old female BALB/c mice were obtained from the breeding stocks maintained at Pasteur Institute of Iran. *L. major* (strain MHRO/IR/75/ER) parasites were kept in virulent state by continuous passage in BALB/c mice. Promastigotes were cultured at 26 °C in NNN medium or 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. *L. major* stationary-phase promastigotes were used to infect the animals. To prepare SLA (soluble *Leishmania* antigen) of *L. major*, promastigotes were harvested by centrifugation (3000 rpm for 15 min at 4 °C), washed one time in phosphate-buffered saline (PBS, 8 mM $NaHPO_4$, 1.75 mM KH_2PO_4, 0.25 mM KCl and 1.37 mM NaCl) and resuspended at a concentration of 2×10^8 parasites/ml. Then this preparation was frozen and thawed ten times in liquid nitrogen and 37 °C water bath, respectively, centrifuged (4000 rpm for 10 min at 4 °C), and the supernatant was kept as SLA. Protein concentration was determined by bicinchoninic acid reagent (BCA, pierce, Rockford, USA) and SLA was kept at −70 °C until use.

2.2. Human sera

All CL sera were collected from endemic areas in Iran including the city of Kerman, Kashan and Mashhad. These samples were grouped as active and recovered CL cases. Active cases consisted of 15 newly diagnosed CL patients without prior treatment (subjected by detecting parasite in the tissue smears and/or NNN culture). We had also sera from 18 recovered individuals with positive Leishmanin Skin Test (LST) and scar.

The active and cured VL patients were selected from different villages around Meshkinshahr area, in the north-west of Iran, where no CL have been reported. Nineteen sera samples were obtained from active VL individuals ranging from 1 to 3 years in age whom were diagnosed based on detection of amastigotes in their bone marrow aspirate and clinical evaluation (fever, splenomegaly). The cured cases consisted of 19 individuals ranging from 5 to 15 years old. Thirteen healthy individuals living in non-endemic areas with the negative LST were selected as controls. All detailed information is shown in Table 1.

2.3. Cloning of *L. major LPG3* in eukaryotic expression vector and large scale preparation of endotoxin-free plasmid

The complete open reading frame of *L. major lpg3* gene (GeneDB Accession Number LmjF29.0760; 2316 bp) was amplified from genomic DNA and cloned into the *Kpn I* and *Afl II* sites of pGEM2 vector (Promega). PCR was performed using the primer set designated as follows:

LPGF: 5′-GCGGAATTC**GGTACC**ATGGCGACCTCGAGCTTG-3′
LPGR: 5′-GG**CTTAAGT**TACAGATCGCCCTCGTCCACTGC-3′

Kozak sequence is underlined and the *Kpn* I restriction site in forward primer and *Afl* II restriction site in reverse primer are in bold.

The condition for PCR was as follows: a 50 μl PCR mixture comprised of *L. major* genomic DNA (100 ng); deoxynucleoside triphosphates (dNTPs, 200 μM of each); Taq DNA polymerase (1U, Roche);

Table 1
CL and V L sera of different individuals in the study.

Type	Number of cases	Age (years)	Sex (M/F)	Time of cure/ recovery (Month)
Active CL	15	34.8 ± 21	6/9	NA
Recovered CL	18	47.9 ± 15.1	8/10	17.8 ± 6.2
Active VL	19	1–3	11/8	NA
Cured VL	19	5–15	9/10	5.2 ± 3.5
Normal	13	25–60	13/0	NA

NA: not applicable.