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A *Leishmania* hypothetical protein-containing liposome-based formulation is highly immunogenic and induces protection against visceral leishmaniasis

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ABSTRACT

Leishmania proteins have been evaluated as vaccine candidates against leishmaniasis; however, most antigens present low immunogenicity and need to be added with immune adjuvants. A low number of licensed adjuvants exist on the market today; therefore, research conducted to produce new products is desirable. The present study sought to evaluate the immunogenicity and protective efficacy of a recombinant *Leishmania* hypothetical protein, namely LiHyR, administered with saponin or liposomes in BALB/c mice. Immunological and parasitological parameters were evaluated, and results showed significant protection against *Leishmania infantum* infection produced by both compositions in the immunized animals; however, this was not identified when the antigen was used alone. In addition, the liposomal formulation was more effective in inducing a polarized Th1 response in the vaccinated animals, which was maintained after challenge and reflected by lower parasitism found in all evaluated organs when the limiting dilution technique and RT-PCR assay were employed. The protected animals showed higher levels of protein and parasite-specific IFN-γ IL-2, IL-12, GM-CSF, and TNF-α, which were evaluated by capture ELISA and flow cytometry, in addition to a higher production of anti-protein and anti-parasite IgG2a antibodies, both before and after challenge. The Lip/rLiHyR combination induced higher IFN-γ production through both $CD4^+$ and $CD8^+$ T cell subtypes. Results indicate the possibility of using the LiHyR, containing a liposomal formulation, as a vaccine candidate against visceral leishmaniasis.

1. Introduction

Leishmaniasis is a neglected disease compound caused by protozoan parasites of the *Leishmania* genus. It is endemic in 98 countries, with 350 million people at risk of infection. An estimated 900,000 to 1.3 million new cases occur each year while 20,000–30,000 deaths are registered annually [1]. Visceral leishmaniasis (VL) is the most fatal clinical form of disease, with around 200,000–400,000 new cases occurring in the world. About 90% of all cases of this disease are registered in Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan [2]. The epidemiology of disease is dynamic and complex, and the manifestation of the disease depends mainly on the environment, nutritional conditions, and epidemiological aspects of the hosts, as well as their immune response against parasites [3–5].

The treatment against VL presents problems, since drugs are old, toxic, and/or have a high cost [6–8]. In addition, the diagnosis of disease is difficult to perform due to the variable sensitivity and/or specificity of the tests, mainly when samples from asymptomatic patients or from those presenting cross-reactive diseases are evaluated. As a consequence, prophylactic vaccination could contribute to controlling

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the disease, as well as present a low cost, when compared to the discovery of new antileishmanial compounds [9].

Immunity against *Leishmania* depends on the development of specific $CD4^+$ and $CD8^+$ T cell-mediated responses by the infected hosts, and is characterized by the production of pro-inflammatory cytokines, such as IFN-γ, IL-2, IL-12, GM-CSF, TNF-α, among others [10,11]. On the other hand, susceptibility to infection is linked to the development of Th2 response, with the production of anti-inflamatory cytokines, such as IL-4, IL-10, IL-13, among others. More specifically, the Th1 cell-mediated response is associated with macrophage activation, host resistance, and protection, while the Th2 cell-mediated response is associated with the inhibition or down-regulation of macrophage activation and disease progression [12,13].

In recent decades, parasite recombinant proteins have been evaluated as vaccine candidates against VL. However, the use of adjuvants has been also necessary, since most antigens induce a low immunity if they are administered alone in the vaccinated hosts [14–16]. As a consequence, combinations between parasite antigens and adjuvant products have resulted in the development of immunogens that have proven to be more effective in inducing protection against *Leishmania* infection. In this context, IL-12 [17], montanide ISA 720 [18], monophosphoryl lipid A [19], CpG oligodeoxynucleotides [20], saponins [21], among others, have been used for this purpose.

Liposomes have been used as delivery systems and/or adjuvants for peptides, proteins, and DNA vaccines against diseases [22–24]. These products present important advantages, since they are biodegradable, atoxic, simple to prepare, and cheaper. Moreover, their composition can be manipulated to obtain more efficient antigen-liposome combinations [25,26]. Liposomes can selectively interact with antigen presenting cells (APCs) and elicit a Th1 response through both major histocompatibility complex (MHC) class I and class II pathways, which activates $CD8^+$ and/or $CD4^+$ T cells, respectively, to produce cytokines, such as IFN-γ, IL-12, among others [27,28].

In a recent immunoproteomics study, hypothetical proteins were identified in *Leishmania infantum* antigenic extracts, which were expressed in the amastigote and promastigote forms of the parasites [29]. By recognition of these molecules by antibodies present in VL dog sera, one could speculate that such antigens are antigenic during active disease and can act in the infectivity, drug resistance, and/or parasite intracellular survival in mammalian hosts [30].

In the present study, one of these hypothetical proteins identified in the previous immunoproteomics study, namely LiHyR (XP_001469544.1), was cloned, purified, and evaluated as an immunogen against *L. infantum* infection. This recombinant antigen (rLiHyR) was used to immunize BALB/c mice alone or in combination with saponin or liposomes as adjuvants. The ability of rLiHyR to protect mice was evaluated by distinct parasitological and immunological parameters in the infected and/or vaccinated animals, and a comparison between the use of saponin and liposomes was performed.

2. Material and methods

2.1. Ethics, mice, and parasites

The study was approved by the Committee on the Ethical Handling of Research Animals (CEUA) from the Federal University of Minas Gerais (UFMG, Belo Horizonte, Minas Gerais, Brazil), logged under protocol number 333/2015. Female BALB/c mice (8 weeks of age) were obtained from the breeding facilities of the UFMG Department of Biochemistry and Immunology, Institute of Biological Sciences, and were maintained under specific pathogen-free conditions. Experiments were performed using *L. infantum* (MHOM/BR/1970/BH46). Parasites were grown at 24 °C in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was supplemented with 20% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4. The soluble *Leishmania* antigenic (SLA) extract was prepared from 10^9 stationary promastigotes, as described elsewhere [31].

2.2. Production of the recombinant LiHyR (rLiHyR) protein

The LiHyR (XP_001469544.1) gene was cloned from *L. infantum* genomic DNA using the following primers: 5́-GGCTAAGCTTACCATGG GATCGGCCACGCACGCACT-3‘ (*forward*) and 5́-TGATGGATCCCTAATC CTTCATCATCGG-3́ (*reverse*). The *HindIII* and *BamHI* restriction enzymes were used. The DNA fragment was purified and linked into a pGEM®-T vector system (Promega, USA), and the insert was included into a pET28a-TEV vector to obtain the recombinant plasmid. The protein was induced by incubation with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Promega, Montreal, Canada) for 3 h at 37 °C, at which time cells were ruptured by six cycles of ultrasound, in cycles of 30 s each (38 MHz), followed by six cycles of freezing and thawing. The recombinant protein (~23.5 kDa) was purified onto a HisTrap HP affinity column (GE Healthcare Life Sciences, NJ, USA) connected to an AKTA system. The eluted fractions were concentrated in Amicon® ultra-15 centrifugal filters, with a 10,000 nominal molecular weight limit (NMWL, Millipore, Germany), and further purified on a Superdex™ 200 gel-filtration column (GE Healthcare, USA). The recombinant protein was passed through a polymyxin-agarose column (Sigma-Aldrich, USA) to remove any residual endotoxin content (Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker, MD, USA).

2.3. Liposomes preparation and characterization

1,2-dipalmitoyl-*sn*-3-phosphocholine (DPPC) was supplied by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (CHOL) was obtained from Sigma-Aldrich (USA). rLiHyR-containing liposomes were prepared by the lipid film hydration technique (Bangham, 1965), followed by size calibration by extrusion. Initially, chloroform solutions containing DPPC and CHOL were prepared. Their aliquots (total lipid concentration equal to 10 mM, at 6:4 M ratio, respectively) were then transferred to a round bottom flask and submitted to evaporation under reduced pressure at 30 °C, using a rotative evaporator R-215 and a vacuum pump V-700 (Buchi Labortechnik AG, Flawil, Switzerland). The lipid film was hydrated at room temperature with PBS 1x (phosphate buffer saline 1x), pH 7.4, containing the recombinant protein (1 mg/mL) in a shaker apparatus (model MS1, Staufen, Germany). The resulting multilamellar vesicles dispersion was calibrated by the extrusion (Lipex® biomembrane extruder; Northern Lipids, Burnaby, BC, Canada) of formulation through a polycarbonate membrane with a pore size of 0.4 μm (10 cycles; Millipore, Billerica, USA). Liposomes were purified by ultracentrifugation at 300,000*g* for 120 min at 10 °C (Optima® L-80XP; Beckman Coulter, Indianapolis, USA). Empty liposomes were prepared as described above, but without including proteins. The mean diameter and liposome polydispersity index were evaluated by the dynamic light scattering technique at 25 °C and 90° angle. The zeta (ζ)-potential was evaluated by determining the electrophoretic mobility at 90°. The samples were diluted in PBS 1x pH 7.4, and the measurements were performed by using a Zetasizer Nano ZS90 apparatus (Malvern Instruments, Malvern, UK) [32].

2.4. Evaluation of the in vitro stimulation

In vitro stimulation was performed as described elsewhere [33]. For this, spleen cells (1×10^6) from BALB/c mice (n = 6) were collected and *in vitro* cultured in 24-well plates (Nunc, Nunclon®, Roskilde, Denmark) in RPMI 1640 medium (Sigma-Aldrich, USA), to which was added 20% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4. Cells (5×10^6) were incubated in the absence (medium, control) or presence of liposome or saponin (20 μg, each), for 48 h at 37 °C in 5% CO_2. The IFN-γ, IL-4, and IL-10 production was evaluated in the cell supernatant by using commercial kits (BD