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Cationic liposomes formulated with a novel whole *Leishmania lysate* (WLL) as a vaccine for leishmaniasis in murine model

Iman Jafari[a], Vahid Heravi Shargh[a], Maryam Shahryari[a], Azam Abbasi[a], Mahmoud Reza Jaafari[a,b,c,*], Ali Khamesipour[d], Ali Badiee[a,c,*]

[a] Nanotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran
[b] Biotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran
[c] Department of Pharmaceutical Nanotechnology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[d] Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Although there have been numerous attempts to develop a successful vaccine against leishmaniasis, based on the clinical trial in this field, no vaccine against *Leishmania* in routine way can be found for globally effective vaccination in human. Amongst, first generation vaccines consisting of parasite fractions or whole killed *Leishmania* showed more successful results in clinical trials. It seems that the main reason for the low efficacy of these vaccines is lack of a suitable adjuvant. In this study, a crude extract of detergent-solubilized *L. major* promastigotes as a novel developed antigen (whole *Leishmania* lysate (WLL)) was formulated in liposomal form. The cationic liposomes consisting of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were used to deliver WLL. Liposomes formulations containing different WLL concentrations (prepared from 10^3, 10^4, 10^5, 10^6 and 10^7 parasites) were prepared and characterized for particle size, surface charge, proteins, DNA and phospholipids contents. Moreover, to explore the type of immune response generated and extend of immunization, *in vivo* and *in vitro* tests including evaluation of lesion development, parasite burden in the foot and spleen, Th1 and Th2 cytokine analysis, and titration of IgG isotypes before and after the challenge were used. The maximum immunization was provided by WLL06 as depicted by the reduction of footpad swelling andparasite load, increase in anti-*Leishmania* IgG2a production, though no significant difference was observed between mice which received WLL05 *vs* WLL06. While maximum immunization was seen in WLL06 group, most of the liposomal WLL formulations induced a mixed Th1/Th2 response. Hence, a more protective immune response is expected to be induced when an immune potentiator adjuvant such as CpG ODNs would be co-deliverd in WLL liposomal formulations.

1. Introduction

Leishmaniasis as one of the most important vector-borne diseases of humans continues to have a major impact on much of the world's population. Currently, leishmaniasis occurs in four continents and is endemic in 88 countries, mostly in developing ones and some are among the least developed. A population of 350 million is at risk with a global incidence of 1–1.5 million cases of cutaneous and 500,000 cases of visceral leishmaniasis. The clinical manifestation of the disease depending upon *Leishmania* species and host genetic background varies from ulcerative skin lesions developing at the site of sand fly bite (localized cutaneous leishmaniasis) and multiple non-ulcerative nodules (diffuse cutaneous leishmaniasis) to destructive mucosal inflammation (mucosal leishmaniasis) and disseminated visceral infection (visceral leishmaniasis). The key control measures mainly rely on early case detection and chemotherapy which has been hampered by the increased resistance to first line drugs (pentavalent antimonials) or toxicity and side-effects associated with second line drugs (pentamidine, amphotericin B). Control of reservoir host and vector is difficult due to operational difficulties and frequent relapses in the host. Therefore, the development of effective and affordable vaccine against leishmaniasis represents a reasonable perspective considering the fact that individuals recovered from cutaneous leishmaniasis (CL) induced by natural infection or leishmanization were protected against further infection and showed strong immune responses against *Leishmania* antigens (Modabber, 1995; Khamesipour et al., 2006; Nagill and Kaur, 2011). The control of leishmaniasis is regulated by cell mediated immune response since the parasites escape the humoral immune response by

* Corresponding authors at: Nanotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran.
E-mail addresses: jafarimr@mums.ac.ir (M.R. Jaafari), badieea@mums.ac.ir (A. Badiee).

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residing in the phagolysosomes of macrophages. The proliferation of Th1(T helper) cells confers immunization through leishmanicidal cytokines including IL-12, IFN-γ and TNF-α while Th2 cell expansion exacerbates the disease (Cunningham, 2002; Sacks and Noben-Trauth, 2002).

First-generation *Leishmania* vaccine comprising killed parasites or live attenuated *Leishmania* parasites is considered a promising alternative for leishmanization which was discontinued due to safety issues (Silvestre et al., 2008). Parasite fractions or whole killed *Leishmania* of this category, with or without adjuvant, reached to phase 3 clinical trials (Khamesipour et al., 2006). Yet, the results of these trials have not been conclusive, and in general the induced immune response has been suboptimal due to the limited Th1 inducer adjuvant for use in human (Noazin et al., 2009). Several other pan-antigenic extracts such as ALM, KLM, LAg, SLA were recently used but to date there has been no study using whole *Leishmania lysate* (WLL) which is a mixture of proteins, phospholipids and DNA produced through parasite detergent lysis. In the current study, for the first time to the best of our knowledge, we have used WLL as a novel first generation *Leishmania* antigen in liposomal form and explored the type of induced immune response in murine model of leishmaniasis.

Liposomes direct peptide antigens into the MHC II or I pathways of APCs, resulting in enhancement of antibody and/or antigen-specific T-cell response based on the formulations (Rao and Alving, 2000). Furthermore, it has been shown that cationic liposomes are superior to neutral and negatively charged liposomes in inducing antigen-specific cytotoxic T lymphocyte (CTL) responses and antibody production (Nakanishi et al., 1997, 1999) and were used as delivery systems for nucleic acids in gene therapy (Wasungu and Hoekstra, 2006; Karmali and Chaudhuri, 2007). There are several reports demonstrating that cationic liposomes induce a selective generation of $CD8^+$ T-cell response, which requires in turn antigen presentation in the context of the MHC I pathway (Nakanishi et al., 1999; Chikh et al., 2001; Hafez et al., 2001). Also, recent studies have shown that cationic liposomes consisting of DOTAP (1, 2-dioleoyl-3 trimethyl ammonium-propane) act not only as vaccine delivery vehicles, but also as tools to increase the poorly immunogenic profiles of peptide/protein antigens, and an inducer of both Th1 and CTL responses (provided by their fusogenic properties) (Christensen et al., 2007).

In this study, we have optimized the load of parasites required to prepare effective dose of WLL in liposomal form against *L. major* infection. Here, we demonstrated that the immunization with liposomes containing WLL exhibit partial protection in BALA/c mice against *L. major* infection.

2. Materials & methods

2.1. Ethics statement

The protocol was approved by the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences (Education Office dated March 31, 2010; proposal code 88527), 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, issued in 2005.

2.2. Animals, parasites and WLL

Female BALB/c mice 6–8 weeks old were purchased from Pasteur Institute (Tehran, Iran). The mice were maintained in animal house of Pharmaceutical Research Center and fed with tap water and laboratory pellet chow (Khorasan Javane Co., Mashhad, Iran). Animals were housed in a colony room 12/12 h light/dark cycle at 21 °C with free access to water and food. Experiments were carried out according to Mashhad University of Medical Sciences, Ethical Committee Acts.

Leishmania major strain (MRHO/IR/75/ER) used in this experiment is the one which was used for preparation of experimental *Leishmania* vaccine, leishmanin, and *Leishmania* for leishmanization (Javadian et al., 1976; Sharifi et al., 1998; Momeni et al., 1999).

For preparation of WLL, the parasites were harvested at stationary phase and washed 3 times using PBS to remove culture media. Then, the number of promastigotes was adjusted to 2×10^n/ml (n = 4–8) in HEPES buffer (10 mM; pH 7.4) solution containing 150 mM Octyl β-D glucopyranoside (OG) and was incubated with gentle shaking for 10 min at room temperature. The resulting clear solution containing WLL plus detergent was sterilized using a 0.22 μm membrane. All these steps were performed under the laminar flow hood in sterile microtubes.

2.3. Liposomes preparation and characterization

Liposomes containing WLL were prepared by detergent removal using batch method with polystyrene beads (Bio-Beads SM-2 adsorbents (Bio Rad, USA)). Briefly, the lipid phase consisting of 1, 2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) (4 μmol/mL; Avanti Polar lipids, USA) and cholesterol (Avanti Polar lipids, USA) (1:1 molar ratio) was dissolved in chloroform in a sterile tube. The solvent was then removed by rotary evaporation (Hettich, Germany) resulting in deposition of a thin lipid film on the tube's wall. The lipid film was further freeze–dried (Taitec, Japan) for 2 h to ensure complete removal of the solvent. The lipid film was then hydrated and dispersed by WLL micellar solutions which were individually prepared at 45 °C. The mixture was briefly vortexed and sequentially subjected to bath-sonication (Branson 5510, USA) for 10 mins at 42 ± 2 °C. Then, Bio-Beads SM adsorbent (0.5 g) was weighed and added to each ml of this mixture. A laboratory tube rotator was used to mix the solution at room temperature for 2 h. The sample was recovered by careful removal using a sterile syringe after a brief centrifugation. This step was repeated 2 more times to ensure complete removal of the detergent and make sure for liposome formation.

Particle size analyzer (Nano-ZS, Malvern,UK) was used to determine the mean diameter and zeta potential of the liposomes. The concentration of proteins encapsulated in liposomes and phospholipid concentrations was quantified using BCA protein assay kit (Thermo Scientific, USA) and Bartlett assay method (Torchilin and Weissig 2003), respectively.

DNA content of each sample was assessed by adding sodium acetate (3 M, pH 5.2) followed by addition of 96% ethanol. The 260/280 nm absorbance was read by the nanodrop spectrophotometer after reconstitution of samples in Tris-EDTA (10 mM:1 mM; pH:7.6) (Maniatis, T, E F Fritsch, and J Sambrook. Molecular Cloning. A Laboratory Manual. NewYork: Cold Spring Harbor Laboratory, 1982).

2.4. SDS-PAGE analysis of liposomal WLL

Analytical SDS-PAGE was carried out to characterize and estimate qualitatively the concentration of WLL encapsulated in the prepared liposomes. The gel was consisted of running gel (10.22% w/v acrylamide) and stacking gel (4.78% w/v acrylamide). The gel thickness was 1 mm. The electrophoresis buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS (pH 8.3). Electrophoresis was carried out at 140 V constant voltage for 45 min. After electrophoresis, the gels were stained with silver for protein detection.

2.5. Immunization of BALB/c mice

Different groups of mice, 8 mice per group, were subcutaneously (SC) immunized in their left hind footpad 3 times at 3 weeks intervals with one of the following formulations: Lip-WLL03 (10^3 parasites lysate/50 μl liposome/mouse), Lip-WLL04 (10^4 parasites lysate/50 μl liposome/mouse), Lip-WLL05 (10^5 parasites lysate/50 μl liposome/