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Alginate microspheres encapsulated with autoclaved *Leishmania major* (ALM) and CpG-ODN induced partial protection and enhanced immune response against murine model of leishmaniasis

Mohsen Tafaghodi [a], Maryam Eskandari [a], Ali Khamesipour [b], Mahmoud R. Jaafari [c,*]

[a] Nanotechnology Research Center, Pharmaceutical Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[b] Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran
[c] Biotechnology Research Center, Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, P.O. Box 91775-1365, Mashhad, Iran

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ABSTRACT

A suitable adjuvant and delivery system are needed to enhance efficacy of vaccines against leishmaniasis. In this study, alginate microspheres as an antigen delivery system and CpG-ODN as an immunoadjuvant were used to enhance immune response and induce protection against an experimental autoclaved *Leishmania major* (ALM) vaccine. Alginate microspheres were prepared by an emulsification technique and the characteristics of the preparation such as size, encapsulation efficiency and release profile of encapsulates were studied. Mean diameter of microspheres was determined using SEM (Scanning Electron Microscopy) and particle size analyzer. The encapsulation efficiency was determined using Lowry protein assay method. The integrity of ALM antigens was assessed using SDS–PAGE. Mean diameter of microspheres was 1.8 ± 1.0 μm. BALB/c mice were immunized three times in 3-weeks intervals with ALM + CpG-ODN loaded microspheres [$(ALM + CpG)_{ALG}$], ALM encapsulated alginate microspheres [$(ALM)_{ALG}$], $(ALM)_{ALG}$ + CpG, ALM + CpG, ALM alone or PBS. The intensity of infection induced by *L. major* challenge was assessed by measuring size of footpad swelling. The strongest protection was observed in group of mice immunized with $(ALM + CpG)_{ALG}$. The groups of mice received $(ALM + CpG)_{ALG}$, $(ALM)_{ALG}$ + CpG, $(ALM)_{ALG}$ and ALM + CpG were also showed a significantly ($P < 0.05$) smaller footpad swelling compared with the group that received either ALM alone or PBS. The mice immunized with $(ALM + CpG)_{ALG}$ or ALM + CpG showed the significantly ($P < 0.05$) highest IgG2a/IgG1 ratio. The IFN-γ level was significantly ($P < 0.0001$) highest in group of mice immunized with either $(ALM)_{ALG}$ + CpG or ALM + CpG. It is concluded that alginate microspheres and CpG-ODN adjuvant when are used simultaneously induced protection and enhanced immune response against ALM antigen.

1. Introduction

Leishmaniasis caused by different species of *Leishmania*. This disease with an annual incidence of about 2 million is a major health problem in some endemic countries (World Health Organization, 2004). Control measures are not always effective and available chemotherapies are expensive, need multiple injections with low efficacy and also drug resistance is emerging. A long lasting protection and strong immune responses induced upon recovery of cutaneous lesions, caused by natural infection or leishmanization, so development of an effective vaccine against leishmaniasis is achievable (Khalil et al., 2000; Khamesipour et al., 2005, 2006; Mauel, 2002; Modabber, 1995; Noazin et al., 2009). Development of Th1 type of immune response plays a major role in recovery and protection at least in animal model of leishmaniasis (Kedzierski et al., 2006; Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002). Recently, several immunoadjuvant like BCG, G-CSF (Follador et al., 2002; Satti et al., 2001) and CpG-ODN (Flynn et al., 2005; Iborra et al., 2005; Jaafari et al., 2007; Tewary et al., 2004; Wu et al., 2006) and also various delivery systems like poly dl(lactide-co-glycolide) (PLGA) microspheres and nanospheres (Coelho et al., 2006) and liposomes (Badiee et al., 2007; Bhowmick et al., 2007; Jaafari et al., 2006; Shimizu et al., 2007) were used to potentiate the immune responses against *Leishmania* antigens in animal models.

Autoclaved *Leishmania major* (ALM) was used in phase 3 trials with a limited efficacy (Khalil et al., 2000; Khamesipour et al., 2006; Noazin et al., 2008). *Mycobacterium bovis* – Bacillus Calmette-Guérin (BCG)– was used as an immunoadjuvant in field efficacy trials of candidate vaccines against leishmaniasis (Armijos et al., 2004; Bahar et al., 1996; Cabrera et al., 2000; De Luca et al., 1999; Kamil et al., 2003; Misra et al., 2001; Mohebali et al., 2004).

* Corresponding author. Fax: +98 511 8823251.
E-mail address: jafarimr@mums.ac.ir (M.R. Jaafari).

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It was shown that ALM mixed with BCG significantly increases the frequency and magnitude of leishmanin skin test response (Alimohammadian et al., 2002). The results of phase 3 clinical trials showed that ALM mixed with BCG induce a Th1 type of immune response which is not strong enough to protect against cutaneous leishmaniasis (Bahar et al., 1996; Khamesipour et al., 2006).

Sodium alginate is a natural polysaccharide polymer which is easily cross-linked into a solid matrix with the addition of di- or tri-valent cations (cross-linking in a water-in-oil emulsion results in the formation of microspheres). Recently, alginate microspheres are used in several studies and showed to be potentially a suitable vaccine delivery system and immunoadjuvant (Fundueanu et al., 1998; Lemoine et al., 1998; Tafaghodi et al., 2006). Immunization of animals by alginate microspheres containing antigenic proteins elicited both humoral and cell-mediated immune responses (Mittal et al., 2000).

Oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as an immuno-adjuvant and shown to induces strong humoral and cellular immune response with a bias towards a Th1 response (Gupta and Siber, 1995; Krieg, 1999; McCluskie and Davis, 1998). There are reports shown that CpG-ODN used as an immunoadjuvant mixed with *Leishmania* antigens induced protection (Jaafari et al., 2007; Mendez et al., 2003; Rhee et al., 2002; Verthelyi et al., 2002).

The immunogenicity of an antigen and the potency of an adjuvant are substantially enhanced by co-delivery in biodegradable microspheres (Diwan et al., 2002). In this study the potential of alginate microspheres encapsulated with ALM and CpG-ODN in induction of immune response and protection rate against murine model of leishmaniasis are studied.

2. Materials and methods

2.1. Animals, parasite and SLA

Sodium alginate (low viscosity grade) was purchased from Sigma (St Louis, MO, USA). CpG oligodeoxynucleotide (# 1826, seq (5′–3′): tccatgacgttcctgacgtt) with a nuclease-resistant phosphorothioate backbone was purchased from Microsynth (Switzerland).

ALM is an experimental vaccine produced at Razi Vaccine and Serum Research institute, Hesarak, Karaj, Iran. For preparation of ALM, promastigotes from a seed bank were grown in volume of 50–200 ml RPMI (Gibco, Grand Island, NY, USA) supplemented with 15% Fetal Calf Serum (FCS; Sigma, Saint Louis, USA) at 26 ± 1 °C, in Roux bottles. Fresh medium was added gradually to reach 200 mL on different days. Promastigotes were harvested at stationary phase by daily enumeration usually on days 16–20 by centrifugation at 3200 rpm for 30 min. Parasites were washed five times with saline solution. The parasite pellet then was divided in two parts; one part was freeze/thawed five times and treated with thimerosal (1/10,000 final concentration) and aliquoted in vials containing 11.11 mg/mL. The second part was aliquoted in vials containing 11.11 mg/mL and then was autoclaved for 15 min at 121 °C, (15 PSI) and kept at 4 °C (Khamesipour et al., 2006; Noazin et al., 2008, 2009).

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

L. major strain (MRHO/IR/75/ER) used in this experiment is the same strain which was used for preparation of experimental *Leishmania* vaccine and leishmanization (Alimohammadian et al., 2002; Bahar et al., 1996; Javadian et al., 1976; Kamil et al., 2003; Khamesipour et al., 2006; Mohebali et al., 2004; Noazin et al., 2008).

Soluble *Leishmania* Antigen (SLA) was prepared from promastigotes of *L. major* harvested at log phase (Scott et al., 1987), and aliquoted and stored at −70 °C until use. Protein concentration of SLA was determined using Lowry protein assay.

2.2. Preparation and characterization of alginate microspheres encapsulated with ALM and CpG-ODN

An emulsification method was used to prepare alginate microspheres (Tafaghodi et al., 2006). Briefly, an aqueous solution containing sodium alginate (3.0% w/v) was dispersed in *n*-octanol solution containing a lipophilic surfactant (2.0% w/v, Span-85) by a probe sonicator (Soniprep150, MSE, Sussex, UK). In the case of ALM and CpG-ODN loaded microspheres, 3 mg of ALM and 50 μl of CpG-ODN (5 μg/μl in TE buffer) were added in the aqueous solution containing sodium alginate. The W/O emulsion was rapidly added to a solution of calcium chloride in octanol (60 ml, 0.33% w/v), while stirring the whole medium slowly with a magnetic stirrer. After 10 min, 2 ml isopropyl alcohol was added dropwise to harden the formed microspheres. The microspheres were collected by filtration, washed with isopropyl alcohol and finally dried in a vacuum desiccator.

Optical microscope (Olympus, Germany) and SEM (Leo, Germany) were used to study the morphology of microspheres. Particle size and size distribution of the microspheres were determined by a laser diffraction size analyzer (Shimadzu, Japan). The amount of encapsulated ALM was determined by the Lowry protein assay method (Waterborg, 2002) and amount of CpG-ODN was estimated based on absorbance at 260 nm (Barman et al., 2000).

To study the release profiles of ALM and CpG-ODN from alginate microspheres, 30 mg of microspheres were suspended in 600 μl phosphate buffered saline (PBS, 10 mM, pH 7.4, containing 0.01% sodium azide). The suspensions were then incubated at 37 °C under continuous shaking for 1 week. At various time intervals (0.5, 1, 2, 4, 12, 24, 48 and 168 h), the supernatant (500 μl) was drawn after centrifugation (5000 g for 5 min) and replaced with fresh medium (Diwan et al., 2002; Tafaghodi et al., 2006). The amounts of released ALM or CpG-ODN were determined by above mentioned methods.

The molecular weight integrity of encapsulated ALM was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein bands were visualized by silver nitrate staining (Waterborg, 2002).

2.3. Immunization of BALB/c mice

Different groups of mice, 10 mice per group, were subcutaneously (SC) immunized (three times at 3 weeks intervals) using one of the following formulations: 1– $(ALM + CpG)_{ALG}$ (180 μg ALM + 10 μg CpG-ODN/10 mg microsphere/100 μl PBS/mouse), 2– $(ALM)_{ALG}$ + CpG (180 μg ALM/10 mg microsphere + 10 μg CpG-ODN/100 μl PBS /mouse), 3– $(ALM)_{ALG}$ (180 μg ALM /10 mg microsphere/100 μl PBS /mouse), 4– ALM + CpG (180 μg ALM + 10 μg CpG-ODN/100 μl PBS/mouse), 5– ALM (180 μg ALM/100 μl PBS/mouse), 6– PBS (100 μl).

2.4. Challenge with L. major

The immunized mice (seven per group) were challenged SC into left footpad with 50 μl volume of 1.5×10^6 *L. major* promastigotes harvested at stationary phase, at 3 weeks after the last booster. As a control, right footpads were injected with the same volume of