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Evaluation of the immunoprophylactic potential of a killed vaccine candidate in combination with different adjuvants against murine visceral leishmaniasis

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ABSTRACT

Despite a large number of field trials, till date no prophylactic antileishmanial vaccine exists for human use. Killed antigen formulations offer the advantage of being safe but they have limited immunogenicity. Recent research has documented that efforts to develop effective *Leishmania* vaccine have been limited due to the lack of an appropriate adjuvant. Addition of adjuvants to vaccines boosts and directs the immunogenicity of antigens. So, the present study was done to evaluate the effectiveness of four adjuvants i.e. alum, saponin, cationic liposomes and monophosphoryl lipid-A in combination with Autoclaved *Leishmania donovani* (ALD) antigen against murine visceral leishmaniasis (VL). BALB/c mice were immunized thrice with respective vaccine formulation. Two weeks after last booster, challenge infection was given. Mice were sacrificed 15 days after last immunization and on 30, 60 and 90 post infection/challenge days. A considerable protective efficacy was shown by all vaccine formulations. It was evident from significant reduction in parasite load, profound delayed type hypersensitivity responses (DTH), increased IgG2a titres and high levels of Th1 cytokines (IFN-γ, IL-12) as compared to the infected controls. However, level of protection varied with the type of adjuvant used. Maximum protection was achieved with the use of liposome encapsulated ALD antigen and it was closely followed by group immunized with ALD + MPL-A. Significant results were also obtained with ALD + saponin, ALD + alum and ALD antigen (alone) but the protective efficacy was reduced as compared to other immunized groups. The present study reveals greater efficacy of two vaccine formulations i.e. ALD + liposome and ALD + MPL-A against murine VL.

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1. Introduction

Visceral leishmaniasis (VL) is a vector-borne parasitic disease targeting tissue macrophages. It is caused by *Leishmania donovani* complex and transmitted to humans by the bite of infected sand flies [1]. The parasite targets reticulo-endothelial system causing irregular bouts of fever, weight loss, enlargement of the liver, spleen, and anaemia [2]. Current treatment is based on chemotherapy which is expensive and difficult to administer for extensive use in developing countries [3]. Vaccination remains the best hope for control of all forms of the disease and considerable efforts have been made to formulate vaccine against leishmaniasis. Recently, Leish-F3 has qualified as a first vaccine to reach the human phase I clinical trials for VL [4].

Although current strategy for vaccination relies on the use of recombinant proteins or purified fractions, killed vaccines still remain a reliable perspective considering their stability, cost and safety [5]. These vaccines have been the subject of many investigations over several decades and have undergone phase 3 clinical trial evaluation [6]. Various studies have been carried out with autoclaved *Leishmania major* and *L. donovani* antigens along with BCG or CpG oligonucleotides against leishmaniasis and encouraging results were observed in primates and murine models by many workers [7–11]. However, conclusive results were not seen in case of human trials [12–14]. Concurrently, an immunotherapy trial of alum-ALM + BCG with sodium stibogluconate showed great efficacy for treatment of post kala azar dermal leishmaniasis (PKDL) in Sudan [15]. Various leishmanial antigens have been shown to induce protection when used with IL-12 as an adjuvant in an animal model of leishmaniasis. Limitations in using IL-12 justify searching for an appropriate adjuvant to accelerate induction of a Th1-type of immune response and protection against this disease [16].

Alum has been the most widely used adjuvant for over 80 years [17]. Although there have been searches for alternative adjuvants, aluminium-containing adjuvants are still in use due to their ease to produce on large scale, good track record of safety, low cost and adjuvanticity. An additional advantage is stabilization of alum with a variety of antigens [18]. As far as its mode of action is concerned, it is now clear that alum can function through both NLRP3 dependent and independent pathways to induce type 2 immune responses [19]. In relation to leishmaniasis, alum has been used alone or in combination with BCG or IL-12 and autoclaved *L. major* (ALM) to protect against experimental murine CL [20,21].

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Saponin derived from the bark of *Quillaja saponaria* (Quil A) is a known immunostimulator whose adjuvant properties were recognized in the beginning of 1900 [22]. Purified fractions of saponin such as QS21 are known to promote both humoral and CMI and have been successfully used in a number of vaccination attempts in hamsters, canines and murine VL [23]. QS21 formulation with FML (Leishmune) has been used in Brazil for immunization of dogs, which induced significant protection in various trials [24].

MPL is thought to interact via TLR4 (toll like receptor) found on antigen presenting cells (APCs), consequentially releasing pro-inflammatory cytokines which activate the adaptive arm of the immune system and is therefore classified as a pathogen associated molecular pattern. It is a Th1 promoting adjuvant [25,26]. Formulations of adjuvant based on MPL have been evaluated in various clinical trials with vaccines against malaria [27], tuberculosis [28], leishmaniasis [29] and cancer [30]. These studies have established the safety and efficacy of this promising adjuvant. Liposome vaccine technology has advanced in recent years and now several vaccines containing liposome-based adjuvants have been approved for human use or have reached late stages of clinical evaluation [31]. Studies have shown that leishmanial antigens encapsulated in cationic liposomes induce protection against murine VL [32–34].

These promising reports encouraged us to carry out the present study to assess the protective efficacy of Autoclaved *Leishmania donovani* antigen along with different adjuvants against experimental challenge of *L. donovani* in BALB/c mice.

2. Materials and methods

2.1. Parasite culture

L. donovani promastigotes of strain MHOM/IN/80/Dd8 were used for the present study. They were maintained in modified Novy, McNeal and Nicolle's (NNN) medium by serial subcultures in Minimum Essential Medium (MEM) after every 48–72 h.

2.2. Animals

Female inbred BALB/c mice weighing 20–25 g were acquired from the Institute of Microbial Technology, Chandigarh, India and then maintained in central animal house of Panjab University, Chandigarh. They were housed in clean cages and fed with water and mouse feed ad libitum.

2.3. Ethical clearance

Experiments were carried out according to the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA, Registration No. 45/1999/CPCSEA). The ethical clearance for conducting the experiments was obtained from the Institutional Animal Ethics Committee (IAEC), Panjab University, Chandigarh, India (Approval No. IAEC 284-295/3.09.2012). Mice were handled according to their guidelines.

2.4. Preparation of Autoclaved Leishmania donovani antigen

Promastigotes in the stationary phase of growth were harvested from NNN medium. These were washed thrice with phosphate buffered saline (PBS). The dead promastigotes were counted in Neubauer's chamber and then concentration was adjusted to 10^8 parasites per ml in sterile PBS (pH-7.2). The parasite suspension (10^8/ml) was autoclaved at 15 lbs for 30 min [7]. 0.1 ml of this suspension containing 10^7 parasites was used for immunization of mice.

2.5. Preparation of vaccine formulations

Five vaccine formulations were prepared to immunize the animals. Each animal was immunized with ALD antigen (10^7) alone or mixed with respective dose of adjuvant in 0.1 ml PBS. The adjuvant dosages were 100 μg saponin, 40 μg of Monophosphoryl lipid A (MPL-A) and 100 μg alum for each mice. For preparation of positively charged liposomes commercially available kit from Sigma Aldrich (USA) was used. 63 μmol of phosphatidylcholine, 9 μmol of cholesterol and 18 μmol of stearylamine in the ratio of (7:1:2) were used for the preparation of positively charged liposomes. Encapsulation of antigen in liposomes was carried out by the method described by Afrin and Ali [35]. The protein content entrapped in the liposome was estimated by the method described by Lowry et al. [36], with bovine serum albumin as the standard, in the presence of 0.8% sodium dodecyl sulfate and appropriate blanks. The amount of protein content entrapped per mg of liposome was found to be 43.3 μg.

2.6. Experimental design

BALB/c mice were immunized on the thigh region by subcutaneous route. Three immunizations were carried out at an interval of two weeks. Animals immunized with PBS only and challenged with 10^7 promastigotes of *L. donovani* served as infected controls. Normal control mice received only PBS. Two weeks after last booster mice were challenged intravenously with 10^7 freshly transformed stationary phase promastigotes [37]. Each group comprised of twenty four mice and various parasitological and immunological studies were carried out by sacrificing six mice from each group after 15 days of last immunization and on 30, 60 and 90 post infection/challenge days. Details of groups are mentioned in Table 1.

2.7. Assessment of parasite burden

For assessment of infection, the liver and spleen of all animals were removed and weighed. Impression smears were made on clean glass slides. The smears were air dried, fixed in methanol and stained with Giemsa. The parasite load was assessed in terms of Leishman Donovan Units (LDU) by the method prescribed by Bradley and Kirkley [38] and calculated as follows:

Number of amastigotes / number of macrophages × weight of organ (in mg).

2.8. Delayed type hypersensitivity (DTH) responses to leishmanin

For measurement of DTH responses, two days before the day of sacrifice, 40 μl of leishmanin (prepared in PBS) was injected intradermally in the right and PBS in the left foot pad. Leishmanin was prepared by the method already described by Sachdeva et al. [37]. After 48 h, the thickness of right and left foot pad was measured using a pair of vernier

Table 1
Groups used in the present study.

Group 1	Normal controls (PBS only)
Group 2	Infected controls (infection with 10^7 promastigotes/0.1 ml PBS)
Group 3	Mice immunized with Autoclaved *Leishmania donovani* (ALD) antigen (0.1 ml)
Group 4	Mice immunized with ALD + alum (dose of alum 100 μg/mice in 0.1 ml of ALD antigen)
Group 5	Mice immunized with ALD + saponin (dose of saponin 100 μg/mice in 0.1 ml of ALD antigen)
Group 6	Mice immunized with ALD + MPL-A (dose of MPL-A 40 μg/mice in 0.1 ml of ALD antigen)
Group 7	Mice immunized with ALD + liposome

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