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Studies on the protective efficacy of freeze thawed promastigote antigen of *Leishmania donovani* along with various adjuvants against visceral leishmaniasis infection in mice

Ankita Thakur, Harpreet Kaur, Sukhbir Kaur*

Department of Zoology, Panjab University, Chandigarh 160014, India

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ABSTRACT

Visceral leishmaniasis (VL) caused by *Leishmania donovani* persists as a major public health issue in tropical and subtropical areas of the world. Current treatment of this disease relies on use of drugs. It is doubtful that chemotherapy can alone eradicate the disease, so there is a need for an effective vaccine. Killed antigen candidates remain a good prospect considering their ease of formulation, stability, low cost and safety. To enhance the efficacy of killed vaccines suitable adjuvant and delivery system are needed. Therefore, the current study was conducted to determine the protective efficacy of freeze-thawed *L. donovani* antigen in combination with different adjuvants against experimental infection of VL. For this, BALB/c mice were immunized thrice at an interval of two weeks. Challenge infection was given two weeks after last immunization. Mice were sacrificed after last immunization and on different post challenge/infection days. Immunized mice showed significant reduction in parasite burden, enhanced DTH responses with increased levels of Th1 cytokines and lower levels of Th2 cytokines, thus indicating the development of a protective Th1 response. Maximum protection was achieved with liposome encapsulated freeze thawed promastigote (FTP) antigen of *L. donovani* and it was followed by group immunized with FTP+MPL-A, FTP+saponin, FTP+alum and FTP antigen (alone). The present study highlights greater efficacy of freeze thawed promastigote antigen as a potential vaccine candidate along with effective adjuvant formulations against experimental VL infection.

Introduction

Visceral leishmaniasis (VL) is a chronic parasitic disease and imposes a serious threat to public health worldwide. Around 200,000–400,000 new cases of VL are reported across the globe annually (World Health Organization, 2014). Since, the current repertoire of drugs is restricted it is essential to explore other options in the form of vaccine. Till date no antileishmanial vaccine is available against human VL. Nevertheless, immunity to re-infection after recovering from the disease firmly indicates the possibility of vaccination. So, there is enough scientific and epidemiological justification to proceed with the development of antileishmanial vaccines (Kumar and Engwerda, 2014).

Although numerous studies are available on advancement and evaluation of various vaccines against leishmaniasis, limited data is available on the use of freeze thawed promastigotes as vaccine candidates against murine VL. Some studies have been carried out with freeze thawed promastigotes as antigen candidates against VL (Vilela Mde et al., 2007; Grenfell et al., 2010) and results have been good. Most of the studies in past have been performed with autoclaved preparations. For the development of an effective vaccine, it is necessary to identify other protective antigens also. This prompted us to assess the potential of a freeze thawed *Leishmania donovani* antigen against experimental murine VL. Development of an effective vaccine relies not only on the selection of appropriate vaccine candidates but also on selecting the right adjuvant or delivery vehicle. Adjuvants like alum, cationic liposomes, Monophosphoryl lipid A and saponin have been evaluated against different infectious diseases (Bovier, 2008; Descamps et al., 2009; Marrack et al., 2009; Sun et al., 2009) and have shown promising results. It is believed that vaccines may be economical and safer option as compared to the chemotherapeutic options and may provide an encouraging aspect in prevention of visceral leishmaniasis. With this

* Corresponding author. Tel.: +91 172 2534215; fax: +91 172 2541409.
E-mail address: puzoology@yahoo.com (S. Kaur).

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objective, we designed our study to investigate if subcutaneous immunization with freeze thawed promastigotes of *Leishmania donovani* in combination with different adjuvants protects BALB/c mice against experimental VL.

Materials and methods

Parasite culture

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

Chemicals and reagents

IgG1, IgG2a (Serotec Biorad USA), MEM, Na_2CO_3 (Hi-Media, Chandigarh, India), sodium dodecyl sulphate, Albumin bovine (Sisco Research Laboratories, Chandigarh, India), alum, saponin, cationic liposomes, Monophosphoryl lipid A (Sigma–Aldrich, USA), Cytokine ELISA kits (Diaclone, France; Bender MedSystems, Austria), 96 well ELISA plates (Tarson).

Animals

Female BALB/c mice were acquired from the Institute of Microbial Technology, Chandigarh, India. Animals were kept in experimental wing of central animal house of Panjab University, Chandigarh. They were housed in clean cages and fed with water and mouse feed ad libitum.

Ethical statement

The ethical clearance for carrying out the experiments was taken from Institutional Animal Ethics Committee (IAEC) of Panjab University, Chandigarh (Approval No. IAEC 284-295/3.09.2012). Animals were handled according to their guidelines.

Preparation of freeze thawed promastigote antigen

Promastigotes in the stationary phase of growth were harvested from NNN medium. These were washed thrice with phosphate buffered saline (PBS). Promastigotes were counted in Neubauer's chamber and a final concentration of 10^8 parasites per ml was prepared in sterile PBS (pH-7.2). Parasites were killed by repeated rounds of freezing and thawing. 0.1 ml of this suspension containing 10^7 parasites was used for immunizing animals.

Preparation of vaccine formulations

Each animal was immunized with freeze thawed promastigote (FTP) antigen alone or along with different dose of adjuvants in 0.1 ml of PBS. Saponin and alum were used at a dose of 100 μg animal and dose of MPL-A used was 40 μg. In total, 100 μl of vaccine (promastigote antigen plus adjuvant) was injected in each experimental animal. For preparation of cationic liposome commercially available kit from Sigma was used. Encapsulation of antigen in liposomes was carried out by the method already described by Afrin and Ali (1997). The protein content entrapped in the liposome was determined by the method given by Lowry et al. (1951), using BSA as standard, in the presence of 0.8% sodium dodecyl sulphate and blanks. The amount of protein entrapped per mg of liposome was found to be 40 μg.

Experimental design

Mice were immunized subcutaneously thrice at an interval of two weeks. Immunized mice were challenged intravenously after two weeks of last booster with 1×10^7 promastigotes. Normal control animals received only PBS. Animals challenged with 10^7 promastigotes of *L. donovani* served as infected controls. Different parasitological and immunological parameters were studied by sacrificing six animals from each group after 15 days of last immunization and on 30, 60 and 90 post infection/challenge days.

Assessment of parasite burden

Hepatic and splenic parasite load was calculated by studying Giemsa stained impression smears of liver and spleen. The parasite burden was determined in terms of Leishman Donovan Units (LDU) by the method prescribed by Bradley and Kirkley (1977) as:

Number of amastigotes/Number of macrophages

× weight of organ (in mg)

Delayed type hypersensitivity (DTH) responses

For evaluation of DTH responses, two days before animal sacrifice, leishmanin (prepared in PBS) was injected (40 μl) in the right and PBS (control) in the left foot pad. Preparation of leishmanin was done by the method explained by Sachdeva et al. (2014). The thickness of right and left foot pad was noted after 48 h using a pair of vernier calliper. Results were expressed as mean ± S.D. of percentage increase in the thickness of the right foot pad as compared to the left footpad of mice (Kaur et al., 2008).

Antibody assay for IgG1 and IgG2a isotypes

For the estimation of IgG1 and IgG2a antibody levels conventional ELISA was carried out by the method described by Ravindran et al. (2004). The absorbance of all samples was read on an ELISA plate reader (Lisa Plus, India) at 450 nm.

Cytokine assays

For the evaluation of cytokine levels, frozen serum samples (−70 °C) were thawed and cytokine levels were assayed using commercial available cytokine ELISA kits. Briefly, 96 well plates precoated with monoclonal antibody of respective cytokine were used. Sera samples were added in the sample wells. All the samples were run in duplicate. After that biotinylated antimouse cytokine specific monoclonal antibodies (anti-mIFN-γ, anti-mIL-2, anti-mIL-4 and anti-mIL-10) were added in respective wells. After incubation, wells were washed with washing buffer followed by addition of streptavidin HRP solution and tetramethylbenzidine (TMB) substrate. The colour intensity in plates was observed and the enzyme-substrate reaction was stopped by using 100 μl of stop reagent (H_2SO_4). The absorbance of wells was read on spectrophotometer at 450 nm. The quantitative evaluation was carried out using standard curve derived from the cytokine standards supplied with the kit.

Statistical analysis

Data analysis was done using two-way analysis of variance (ANOVA) with the help of Sigma plot (12.5) software. Post hoc test used for multiple comparisons was Holm–Sidak method. p values of <0.05 and <0.001 were considered statistically significant.