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Biodegradable poly-*l*-lactide based microparticles as controlled release delivery system for filarial vaccine candidate antigens

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ABSTRACT

Modern recombinant vaccines are less immunogenic than conventional vaccines which require adjuvants to enhance the effect of a vaccine. Alum is being used as a standard adjuvant for protein based vaccines to augment immune response in several diseases. However, the problem associated with alum is it requires multiple doses at specific time intervals to achieve the adequate level of immunity. Currently the adjuvanticity of Poly-*l*-lactide microparticles as single dose immunization was explored to overcome multiple immunization and reported to be effective for several diseases. In this regard we adsorbed filarial recombinant chimeric multivalent vaccine candidates such as TV and FEP on to PLA by double emulsion method and analyzed the characterization of PLA encapsulated microparticles and evaluated its immune responses in mice. The efficacy of single dose of PLA encapsulated proteins was investigated in comparison with single dose of alum or protein alone. In mice, single dose of PLA encapsulated antigens such as TV and FEP elicited significantly high antibody titer of 50,000 and 64,000 respectively than single dose of alum adsorbed TV/FEP (6000/9000) and single dose of protein TV/FEP (3000/4000) alone. Further PLA encapsulated antigens induced higher levels of cellular proliferation together with significant ($P<0.0001$) levels of cytokine response [PLA-TV induced high levels of IL-4 (Th2) and IFN-γ (Th1) cytokines whereas PLA-FEP showed high levels of IL-5(Th2) and IFN-γ (Th1)] indicating a balanced response elicited by PLA antigens. Overall strong humoral and cellular responses were observed for PLA encapsulated antigens compared with single dose of alum adsorbed or protein alone.

1. Introduction

Lymphatic Filariasis, a mosquito borne parasitic infection caused by three closely related nematode worms, *Wuchereria bancrofti, Brugia malayi* and *Brugia timori* which damage the lymphatic system leading to painful swelling and disfigurement. Filariasis is the second largest cause of permanent and long-term disability in the world (Gyapong et al., 2005). It has a major social and economic impact in many tropical and subtropical countries. Current antifilarial drugs and exposure control measures have several limitations (Bockarie and Deb, 2010; Hoerauf et al., 2011; Taylor et al., 2010) and hence there is a need for additional strategy for elimination of lymphatic filariasis. In recent times, several recombinant vaccine candidate antigens have been reported to elicit promising results (Thirugnanam et al., 2007; Vanam et al., 2009a; Anand et al., 2008, 2011; Dakshinamoorthy et al., 2013) which provided an excellent hope for developing vaccine against lymphatic filariasis. Although the recombinant protein vaccines elicited significant protection, they require adjuvant to induce optimum immune response. Currently, alum (U.S. FDA approved adjuvant) is widely used to increase immune response for protein based vaccines (Schmidt et al., 2007). However, multiple injections at specific time intervals are the general requirements to achieve the adequate level of immunity for alum adsorbed antigens, moreover it is a poor stimulator of cellular (Th1) immune responses, which are also important for protection (Schmidt et al., 2007; Petrovsky and Aguilar, 2004). In developing countries, limited access to medical care, lack of awareness and of floating population plays a crucial role in following the multiple immunization coverage to obtain desired response. Hence, an injectable formulation should be designed to ensure long-lasting immunity in a single dose vaccine which releases an antigen in a controlled manner as booster dose would be an improved strategy for immunization coverage.

Currently, biodegradable microparticles based on poly-*l*-lactide (PLA) an FDA-approved polymer, is used for controlled-release

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delivery of protein-based vaccine antigens. The role of PLA as adjuvant has been reported by several investigators (Coombes et al., 1999; Gupta et al., 1998; Witschi and Doelker, 1998) and was reported to elicit strong and long-lasting immune response in single immunization. PLA is advantageous over other FDA approved polymer *e.g.* Poly(lactide-*co*-glycolide) (PLGA) because of its hydrophobicity and very low surface tension which are essential for efficient antigen adsorption (Absolom et al., 1987; Coombes et al., 1996) and its particulate form helps for more efficient antigen delivery to dendritic cells (Lavelle et al., 1999). These polymers undergo hydrolysis when injected into the body and forming biologically compatible and metabolizable moieties (lactic acid) due to their polyester nature. Finally they are removed from the body through the citric acid cycle. Recently, we have reported protective efficacy of filarial recombinant chimeric multivalent vaccine candidate namely TV (a 39 kDa fusion protein which comprising two filarial key vaccine candidate antigens such as Thioredoxin (TRX) and Venom Allergen Homologue (VAH)] (Anugraha et al., 2013) and another recombinant chimeric mutiepitope vaccine candidate such as Filarial epitope protein (FEP). FEP, a multi epitope protein (synthesized and expressed as 25 kDa protein) comprising immunodominant epitopes from multistage antigens such as Thioredoxin (TRX) (Madhumathi et al., 2010a), Transglutaminase (TGA) (Vanam et al., 2009b; Madhumathi et al., unpublished data), and Abundant Larval Transcript-2 (ALT-2) (Gregory et al., 1997; Madhumathi et al., 2010b, 2010c) and proved as potential vaccine candidate (Anugraha et al., unpublished data). In this present study we proposed to explore the use of PLA as a delivery system for filarial antigens and characterized the effective immune response for a single administration of PLA encapsulated antigens. To analyze this possibility adsorption of the recombinant Fusion protein (TV) and Filarial epitope protein (FEP) on to PLA and the immune responses of the same were studied in comparison with single dose of alum adjuvant or protein alone in mice model.

2. Materials and methods

2.1. Preparation and characterization of polymer particles

The poly lactide (M.W. 103 kDa) (Sigma-Aldrich, Bangalore, India) microparticles were prepared using double emulsion (w/o/w) solvent evaporation method as described by Katare and group (Katare et al., 2005) with optimized modifications. Briefly, recombinant proteins (TV and FEP) were dissolved in 0.5 ml of an aqueous solution of 10% (w/v) polyvinyl alcohol (M.W. 30 to 70 kDa; 87–90% hydrolyzed). This primary aqueous phase was vigorously mixed through sonication, with an oily phase consisting of 250 mg of PLA dissolved in 5 ml of dichloromethane (50 mg/ml of dichloromethane). The resultant water-in-oil emulsion was then added, during vigorous agitation, to 75 ml of 5% (w/v) polyvinyl alcohol. The microparticles formed through overnight solvent evaporation in a magnetic stirrer were washed and harvested by centrifugation (40,695*g*, 20 min), and lyophilized to obtain the form of free-flowing powder. Size and surface morphology of the particles were analyzed using scanning electron microscope (Carl Zeiss, Germany).

2.2. Estimation of protein encapsulation efficiency in microparticles

To estimate the protein content of particles, accurately weighed particles were dissolved in acetonitrile to solubilize the polymer (10 mg/ml). The suspension was centrifuged at 9037*g* for 10 min and the resultant pellet was dried at 37 °C. After the addition and incubation (1 h at 37 °C) of 100 μl PBS buffer to the pellete, the reaction mixture was centrifuged at 9037*g* for 10 min and the supernatant were collected. The procedure was repeated with 100 μl of 0.1 M NaOH. The protein content of the supernatant was estimated by BCA (Bicinchoninic acid) assay (Smith et al., 1985) which is a more sensitive assay for estimation. 10 μl of protein sample was added to 190 μl of BCA working reagent (Pierce, Rockford, USA) and incubated at 37 °C, in a dark room for 30 min and the absorbance was measured at 540–590 nm. The encapsulation efficiency was calculated as the percentage weight of antigen per unit weight of polymer (μg antigen/mg of PLA particles).

2.3. In vitro release assay from microparticles

The release of proteins from microparticles was determined by incubation of accurately weighed particles (approx. 10 mg) in 100 μl PBS buffer at 37 °C in continuous shaking. The suspension was centrifuged and the supernatant was removed for analysis. At weekly intervals fresh PBS buffer (100 μl) was added to the pellet and resuspended by gentle vortexing and returned to the shaker. The process was repeated for up to 3 months. Released samples were estimated by BCA method as described earlier.

2.4. Protein stability study

The integrity of the antigens (TV and FEP) was analyzed in SDS-PAGE after encapsulation in comparison with purified proteins (TV and FEP). The known amount of antigen encapsulated with PLA microparticles were incubated (separately) with 0.1% (w/v) SDS-phosphate buffered saline (PBS; 0.01 M; pH 7.4) at 37 °C for 3 h with gentle shaking and then centrifuged at 18,074*g* for 25 min at 4–8 °C (Saini et al., 2011). The supernatants were checked in SDS-PAGE gel.

2.5. Immunological studies in mice

2.5.1. Immunization protocol

Six to eight weeks old BALB/c (H-2^d) mice were purchased from King Institute, Chennai, Tamilnadu, India. All the experiments were followed as per 'Indian Animal Ethics Committee' regulations. For PLA encapsulated antigen (mentioned as PLA-TV and PLA-FEP) groups (5 mice/group), were immunized with 50 μg (an optimum concentration for microsphere encapsulated proteins (Madhumathi et al., 2010a) of protein equivalent in microspheres suspended in 100 μl of PBS buffer. Other groups were immunized with 50 μg of TV and FEP protein mixed with an equal volume of alum (Sigma-Aldrich, Bangalore, India) (mentioned as Alum+TV and Alum+FEP) and as well as antigens without alum [TV and FEP in Phosphate buffered saline (PBS) alone]. Three control groups were kept for this study. One group received alum alone, another received PBS alone and the third group with PLA microparticles alone. All groups were injected with single dose of respective antigens via subcutaneous route. A small amount of blood (approximately 50 μl) was taken from tail vein of immunized animals at weekly intervals up to three months since microsphere encapsulation provides long-lasting immunity due to sustained release of antigen and serum were separated to analyse the antibody levels by ELISA.

2.5.2. Analysis of total IgG and Isotype antibody levels

Protein specific IgG levels in the sera were observed by ELISA. The 96-well microtiter plates (Nunc, Maxisorp, Nalge Nunc International, Denmark) were used and each wells were coated with 100 ng of TV and FEP proteins prepared in 100 μl of coating buffer separately (Prince et al., 2013). The plates were incubated over night at 4 °C or for 4 h at 37 °C. After washing and blocking with 5% skim milk powder, a serial dilution of antisera from all groups was carried out. The color was developed using *p*-nitrophenyl