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Sufficiency of a single administration of filarial antigens adsorbed on polymeric lamellar substrate particles of poly (L-lactide) for immunization

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ABSTRACT

A majority of antigens require repeated administration to ensure development of adequate humoral and cell mediated immune response. To minimize the number of administrations required, we investigated the utility of biodegradable polymeric lamellar substrate particles of poly (L-lactide) (PLSP) as adjuvant for filarial antigen preparations. PLSP was prepared and characterized and *Brugia malayi* adult worm extract (BmA) and its SDS-PAGE resolved 54–68 kDa fraction F6 were adsorbed on to PLSP. Swiss mice received a single injection of PLSP-F6, PLSP-BmA, FCA-F6, FCA-BmA and two doses of the plain antigens. Specific IgG, IgG1, IgG2a, IgG2b and IgE levels in serum, IFN-γ, TNF-α and nitric oxide (NO) release from cells of the immunized animals in response to antigen challenge were studied. The average size of PLSP particles was <10 μm and its % antigen adsorption efficacy was 60.4, 55.2 and 61.6 for BSA, BmA and F6, respectively. Single injection of PLSP-F6 or PLSP-BmA produced better immune responses compared to one injection of FCA-F6/BmA or two injections of plain F6 or BmA. Moreover, PLSP-F6 produced much better response than PLSP-BmA. These data demonstrate for the first time that PLSP is a superior immunoadjuvant for enhancing the immune response to filarial BmA and F6 molecules and obviates the need for multiple immunization injections.

1. Introduction

Human lymphatic filariasis (LF), a mosquito-borne disease of the tropics, is caused by the nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. It affects 120 million people worldwide, of which 40 million people show the chronic debilitating disease manifestations: elephantiasis and hydrocele and a further one billion people are at risk of infection. The disease is the second leading cause of permanent and long-term disability worldwide (Molyneux, 2003).

Currently available methods to control and prevent the infection include administration of the antifilarials DEC and ivermectin/albendazole and exposure control programmes. In recent years identification of several parasite antigens raised hopes for developing vaccines (Babayan et al., 2006; Gregory et al., 2000; Krithika et al., 2005; Ramachandran et al., 2004; Shakya et al., 2009; Vedi et al., 2008). Recently we reported that BmAFII, a Sephadex G-200 eluted fraction (Dixit et al., 2004, 2006) and F6, a SDS-PAGE (employing denaturing conditions) resolved 54–68 kDa fraction of *B. malayi* adult worm extract (BmA) (Sahoo et al., 2009), stimulates release of proinflammatory mediators and protects pre-immunized host from the parasite via Th1/Th2 type responses. BmAFII also cross-protected hamsters from *Leishmania donovani* infection via upregulation of NO and cellular proliferative response (Murthy et al., 2008). Earlier too, our group (Bhattacharya et al., 1997; Shakya et al., 2009) and other investigators (Chenthamarakshan et al., 1997) have shown antibody responses to denatured filarial antigens and sizeable protection against infective larval challenge in immunized host by generating a Th1 biased milieu in the host. In these studies traditional adjuvant (FCA/FIA) was employed to immunize the animals and found that an efficient immunization could be produced only by administering 3–4 injections. For future human use, it is however necessary to use an adjuvant that is safe, biodegradable and which does not require repeated administration to produce the desired result. Currently, alum is the only adjuvant approved by the U.S. FDA for clinical use to increase immune responses to protein-based vaccines (Schmidt et al., 2007) but unfortunately, alum is a poor stimulator of cellular (Th1) immune responses, which are important for protection (Petrovsky and Aguilar, 2004; Schmidt et al., 2007).

Biodegradable microparticles based on lactide and glycolide polymers have recently been used as adjuvants by several investigators (Coombes et al., 1999; Gupta et al., 1998; Witschi and Doelker, 1998) because they have the capacity to elicit strong

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and long lasting humoral and T-cell proliferative response. Among the polymers poly (L-lactide) has attracted significant attention 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 that helps in more efficient antigen delivery to dendritic cells (Lavelle et al., 1999). We have recently reported the excellent adjuvant effect of the polymer poly (L-lactide) for immunization against HBsAg (Saini et al., 2009).

In the present study we investigated whether particles of poly (L-lactide) can be used as more efficient delivery system for filarial antigens and whether a single administration of the antigen adsorbed on to poly (L-lactide) is sufficient to induce the desired immune response. For this purpose we adsorbed BmA- or F6 on to polymeric lamellar substrate particles (PLSPs), characterized it and administered to mice. Specific IgG and its subtypes, lymphocyte proliferation, interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) release were determined. The data were compared with one dose of BmA- or F6 emulsified with Freund's complete adjuvant (FCA).

2. Materials and methods

2.1. Materials

L-Poly-L-lactide (L-PLA; molecular weight: 360–380 kDa: Purac Biochem, Netherlands) was obtained as gift sample. The reagents used in sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and FCA were procured from Sigma–Aldrich Corporation, India. All other chemicals and reagents were of analytical grade and purchased from local suppliers.

2.2. Preparation of parasite antigens from B. malayi

2.2.1. Isolation of parasites

Adult worms were collected from peritoneal cavity of jirds harbouring 4–5 months old *B. malayi* infection (Dixit et al., 2004).

2.2.2. Isolation of F6 fraction

Soluble somatic extract of freshly collected adult worms (BmA) was prepared and SDS-PAGE resolved F6 (54–68 kDa) was isolated (Sahoo et al., 2009). Proteins from gel strips were electro eluted (Electroeluter, Millipore, India), concentrated (Centricon of 10 kDa cut off; Millipore, India), and contents estimated (Bradford, 1976). Plain BmA and F6 were then stored in aliquots at −20 °C till use. It is estimated that F6 proteins constitute about 10% of BmA.

2.3. Preparation of blank PLSP

PLSPs were prepared strictly aseptically by non-solvent induced precipitation at room temperature as described earlier with some modifications to suit our conditions (Jabbal-Gill et al., 2001; Saini et al., 2009). Briefly, 2% (w/v) polymer solution (5 ml) in dioxane was mixed in a magnetic stirrer and then filtered through membrane filter of 0.22 μm pore size (PTFE membrane, Millipore, USA). 10 ml of distilled water was injected (drop-wise) into 5 ml of polymer solution in dioxane which was stirred vigorously with a magnetic stirrer. The mixture was then gently stirred for 16 h with a magnetic stirrer under ambient conditions to evaporate the solvent. The PLSP thus formed were separated by centrifugation followed by 2–3 washes with sterile distilled water and stored at 2–8 °C.

2.4. Characterization of BmA and F6 (determination of molecular weight)

Molecular weight of BmA and F6 in known concentration was confirmed in SDS-PAGE using 12% resolving gel and 5% stacking gel. The band was visualized by Coomassie brilliant blue stain (Saini et al., 2009).

2.5. Characterization of PLSP

The average size, polydispersity index, zeta-potential of blank PLSP was obtained by photon correlation spectroscopy (PCS) using Zeta Plus (Malvern Zetasizer, UK). The surface morphology was obtained using Scanning Electron Microcopy (SEM; Leo 430 Germany). The samples for SEM were prepared by sprinkling the PLSP powder on a double adhesive tape, stucked to an aluminum stub. The stub was coated with gold using sputter coater. The samples were then randomly scanned and photographed. Transmission Electron Microcopy (TEM: Philips CM-10, Eindhoven Netherlands) was used to assess the distribution of proteins (BmA and F6) adsorbed on to PLSP (Saini et al., 2009).

2.6. Adsorption of BmA and F6

For adsorption studies, optimization was done at different concentrations for a limited period of time 2–16 h (Venkataprasad et al., 1999).

2.6.1. Optimization of protein concentration for adsorption

BSA was used as a model protein to study the adsorption isotherm in both formulations. Adsorption isotherm of proteins from phosphate buffered saline (PBS) under different concentration was measured.

2.6.2. Adsorption isotherm for proteins on microparticles

The adsorption efficiency for proteins at increasing concentration was evaluated by adsorbing the protein to blank PLSP at: 0.5, 1.0, 1.5, and 2% (w/w) target load. The level of adsorption was determined by base hydrolysis, followed by bicinchonic acid (BCA) protein assay. The percentage of adsorbed protein(s) i.e. adsorption efficiency at each loading level was calculated (Saini et al., 2009). PLSPs were incubated with optimum amount of proteins for specific time period followed by centrifugation and estimation of protein in supernatant by BCA method. The experiment was repeated three times with BmA, F6 and BSA (model antigen). One part of protein antigen (BSA, BmA or F6) was incubated with 10 parts of microparticles overnight at 4 °C in shaking incubator, centrifuged and washed once with sterile distilled water to remove unbound antigen. The supernatant was collected and analyzed for antigen content by BCA assay. A calibration curve was made from serial dilutions of the amount of protein adsorbed on the particles (Coombes et al., 1998; Jabbal-Gill et al., 1999, 2001; Venkataprasad et al., 1999). The optimized BmA and F6-adsorbed PLSP formulations were used in the present study.

2.7. In vitro release of protein(s) from PLSP

The method of *in vitro* release of protein(s) from PLSPs was broadly followed as described by Coombes et al. (1998) and Stivaktakis et al. (2005). PLSPs with adsorbed proteins were incubated in PBS release medium (0.1 M, pH 7.4, 37 °C) containing 0.02% (w/v) sodium-azide as a bacteriostatic agent. Sample vials containing approximately 50 mg PLSP–protein dispersed in 5 mL PBS were shaken intermittently to retain the particles in suspension. A vial was withdrawn after 1 day, the content was centrifuged at 8000 rpm for 10 min and fresh medium was added to the sample. This process was repeated at 3–5 days interval upto 4 weeks. Protein content in the release medium was determined using a BCA protein assay and stored at 4 °C.

2.8. Stability studies

SDS-PAGE was performed to check the integrity of the observed antigen in the final formulation (in-process stability). The known amount of antigen loaded formulations was incubated (separately) with 0.1% (w/v) SDS-phosphate buffered saline (0.01 M; pH 7.4) at room temperature for 3 h with gentle shaking and then centrifuged at 20,000 rpm for 25 min at 4–8 °C. The supernatants were preceded for sample preparation by heating with sample buffer at 95 °C in water bath (Saini et al., 2009).

2.9. Immunization of animals

Swiss mice (male; 8–9 weeks old, 22–25 g body weight) of CDRI-NLAC animal facility were used for the present study. All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. The animals were housed in climate- (23 ± 2 °C; RH: 60%) and photoperiod- (12 h light–dark cycles) controlled animal quarters and were fed standard rodent pellet supplemented with soaked Bengal gram. The animals had free access to drinking water.

The study included nine groups consisting of six animals each. Group 1 was treated with PBS and served as control. Groups 2 and 3 received two doses of plain BmA (10 μg protein/dose) on days 0 and 15 and single-dose of PLSP-BmA on day 0. Groups 5 and 6 received two doses of plain F6 (5 μg protein/dose) and one dose of PLSP-F6 as above. Groups 7 and 8 received one dose of BmA or F6, respectively, emulsified with FCA and Group 9 was given FCA alone on the same day as above. All the injections were given subcutaneously (s.c.).

Animals were sacrificed on day 35 post first administration (p.f.a.) of antigen or PBS/FCA. Splenocyte preparations from the immunized animals were used for cell proliferation and cytokine (IFN-γ and TNF-α) release while peritoneal macrophages were used for NO release. Antigen (F6/BmA), lipopolysaccharide (LPS) or concanavalin A (Con A) specific responses were determined (Sahoo et al., 2009).

2.9.1. Antibody determination

Filaria specific IgG and its subclasses (IgG1, IgG2a, IgG2b) on day 35 p.f.a. were determined in sera of animals as described by Sahoo et al. (2009). Briefly, Maxisorp ELISA strips (Nunc) were coated with the BmA (1 μg protein/ml) or F6 (0.5 μg protein/ml) prepared in carbonate buffer (0.06 M; pH 9.6). Optimally diluted sera (prepared in 1% BSA in PBS) were added (sera dilution for anti BmA and F6 IgG were: 1:200 and 1:100 respectively, whereas for anti BmA or F6 IgG subclasses, sera were diluted to: 1:50 and 1:25, respectively). After incubation with sera the wells were washed and probed with HRP-conjugated rabbit anti-mouse-IgG (1:1000) and its subclasses (1:15,000) (Sigma Chem. Co, USA). Orthophenylenediamine (OPD) was used as substrate and absorbance was read at 492 nm in an ELISA reader (Power Wave X, BioTek, USA).

IgE antibodies were determined in sera of animals on day 35 p.f.a. as described by Wahyuni et al. (2003). Dilutions used for serum and anti-mouse IgE conjugated with HRP (Bethyl Laboratories Inc.) were 1:8 and 1:1000 (Serotec, USA), respectively. The remaining steps were same as described above.

2.9.2. Cellular proliferation assay

Cellular proliferation assay was carried out as described by Dixit et al. (2006). Briefly, the animals were killed by an overdose of anaesthetic ether, spleen was collected in sterile RPMI-1640. Splenocyte suspension was prepared and adjusted to 2×10^6 cells/ml. Each well of sterile tissue culture plate (Nunc-Rosklide, Denmark) received 4×10^5 cells in 200 μl (96 well) or 2×10^6 cells in one ml (48 well). The cells were stimulated with stimulants (BmA: 1 μg protein/ml; F6: 0.5 μg protein/ml; Con A: 10 μg/ml or LPS: 1 μg/ml) and incubated at 37 °C in 5% CO_2 atmosphere. At 72 h post-stimulation (PS), ^{3}H-thymidine (1 μCi/well) was added to the wells (96 well) and 16–18 h later the cells were harvested, suspended in scintillation fluid and beta-emission was quantified in a scintillation counter (LS Analyzer, Beckman Inc.). The results were expressed as counts per minute (cpm).

For cytokine determination 48 h culture supernatants were collected from the 48-well plate.

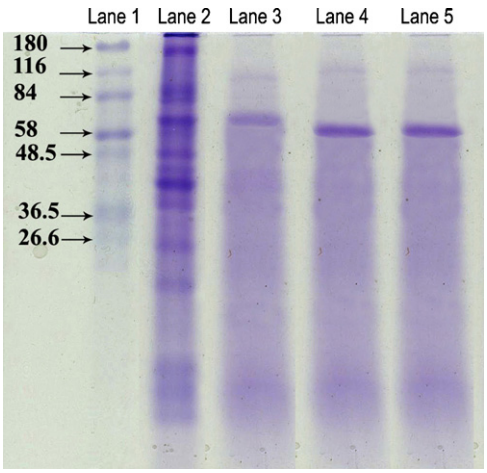

Fig. 1. SDS-PAGE resolved bands of proteins. Lanes (1) Molecular weight markers, (2) *Brugia malayi* adult worm extract, (3) bovine serum albumin, (4) F6 fraction of *B. malayi* adult worm extract, and (5) *in vitro* release of F6 from adsorbed PLSP-F6.

2.9.3. Cytokine assay

IFN-γ (Pierce, endogen) and TNF-α (BD Pharmingen™) were determined in supernatant using paired mouse monoclonal antibodies to cytokines in a sandwich ELISA following the manufacturer's instructions with some modifications (Sahoo et al., 2009). Standards suitable for the paired antibodies obtained from the same sources were simultaneously used for calculation of conc. of the cytokines in the supernatants.

2.9.4. NO determination

NO release in the form of nitrite in 48 h PS culture supernatants was determined in peritoneal macrophages as described by Sahoo et al. (2009).

2.10. Statistical analysis

Results were presented as mean ± SD of two experiments conducted in six animals/group and the data were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison tests in GraphPad prism 3.03. Differences with $P < 0.05$ were considered to be significant.

3. Results

3.1. Antigen characterization

Fig. 1 shows SDS-PAGE profile of BmA and F6. BmA showed bands between 10.0 and >180 kDa and F6 between 54 and 68 kDa, respectively.

3.2. Physical-characterization of PLSP

Blank PLSP were irregular/lozenge/diamond shaped with lamellae as shown by SEM (Figs. 2 and 3). The actual size and polydispersity index for PLSP was found to be 6.30 μm (mean

Table 1
In vitro characterization of polymeric lamellar substrate particles (PLSPs) of poly (L-lactide) bearing *Brugia malayi* adult worm extract (BmA)- or its fraction F6 proteins.

Parameters	PLSP of poly (L-lactide) Value (mean ± SD)[a]
Mean particle diameter (μm) of PLSP (blank)	6.302 ± 1.84
Polydispersity index	0.0137 ± 0.12
Particle shape	Irregular/lozenge/diamond shaped with lamellae
% Adsorption efficiency of BSA, BmA and F6 proteins onto PLSP	BSA: 60.373 ± 2.208, BmA: 55.168 ± 0.815 and F6: 61.564 ± 6.272
Zeta-potential (mv) of protein adsorbed PLSP	−23.00 ± 5.0 (BSA), −26.667 ± 1.901 (BmA) and −20.467 ± 1.724 (F6)
Cumulative release of F6 proteins from PLSP-F6 in 24 h (%)	−15.4 ± 1.6 (BSA), 13.2 ± 0.6 (BmA) and −17.60 ± 1.724 (F6)

[a] Mean of three experiments.

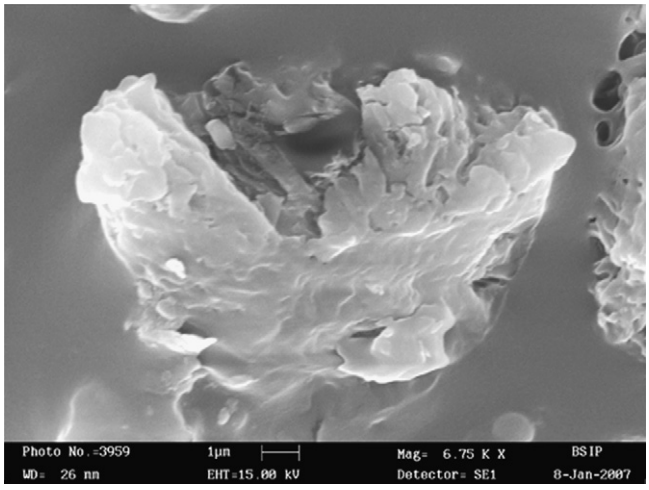

Fig. 2. Scanning electron photomicrograph of blank polymeric lamellar substrate particles (PLSPs).

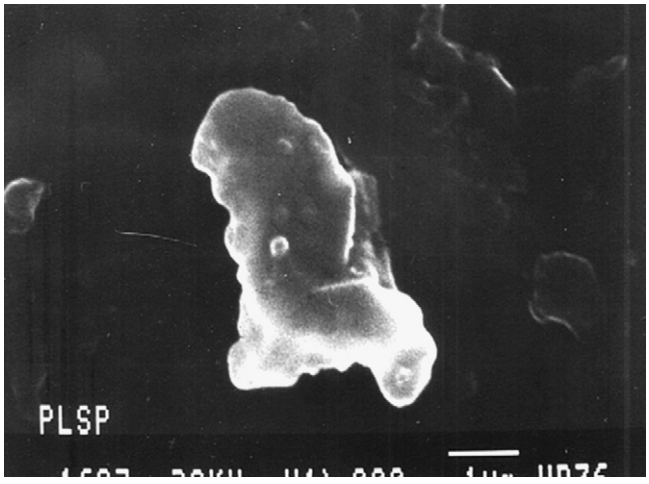

Fig. 3. Scanning electron photomicrograph of a blank PLSP.

particle diameter) and 0.01, respectively. Zeta-potential of blank PLSP was found to be −38.7 mV. PLSPs were earlier standardized with the help of a selected model antigen, BSA to optimize process parameters to obtain uniform and reproducible batches of PLSP (Table 1).

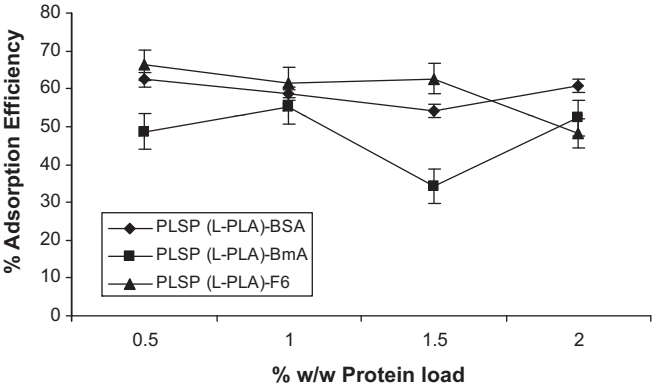

Fig. 4. Adsorption isotherm of proteins of bovine serum albumin (BSA), *B. malayi* adult extract (BmA) and its fraction F6 on PLSP.

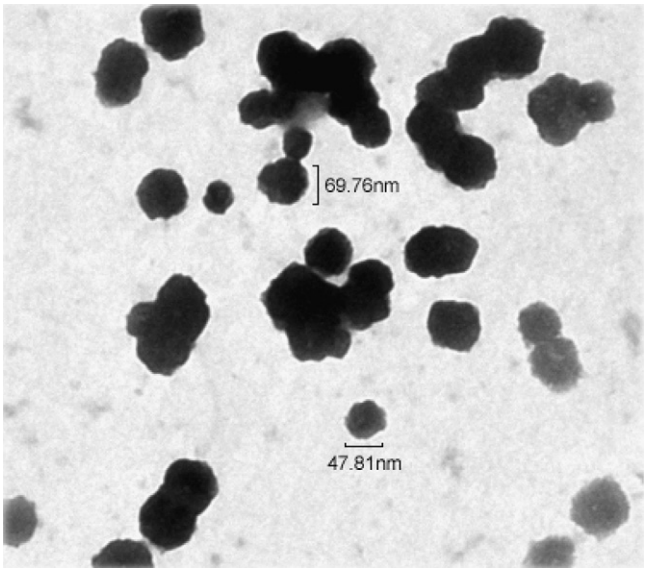

Fig. 5. Transmission electron photomicrograph of BmA adsorbed PLSP.

3.3. Adsorption studies

The percentage adsorption efficiency of BSA, BmA and F6 adsorbed PLSP was found to be 60.4, 55.2 and 61.6, respectively (Table 1).

3.3.1. Adsorption isotherm

For BmA adsorbed PLSP maximum adsorption efficiency was found to be 55.2 (protein input 1.0, w/w). For BSA and F6 adsorbed PLSP maximum adsorption efficiency was observed to be 60.4 and 61.2 respectively (protein input 0.5, w/w) as determined by micro BCA assay. After adsorption of BSA, BmA or F6 zeta-potential of PLSP was found to be −23.00 mV, −26.67 mV and −0.47, respectively (Fig. 4, Table 1).

3.4. Transmission electron microscopy (TEM)

TEM photographs showed distribution of adsorbed proteins onto PLSP (Figs. 5 and 6).

3.5. In vitro release of antigen

On day 1, the *in vitro* release rate of BSA, BmA or F6 from PLSP was found to be 15.4, 13.2 and 17.6%, respectively. Subsequently, by

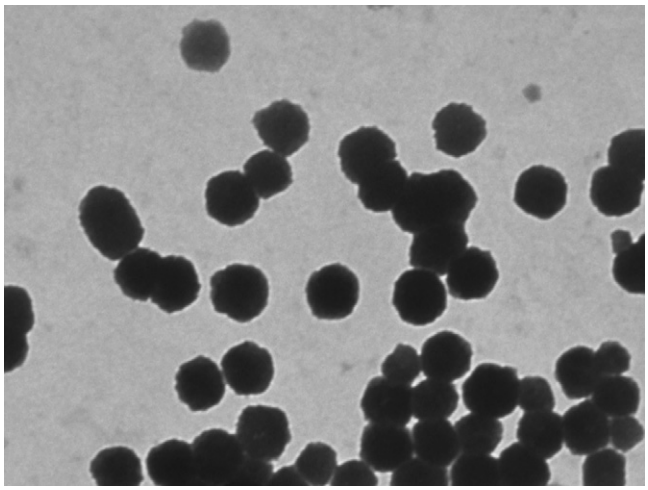

Fig. 6. Transmission electron photomicrograph of F6 adsorbed PLSP.

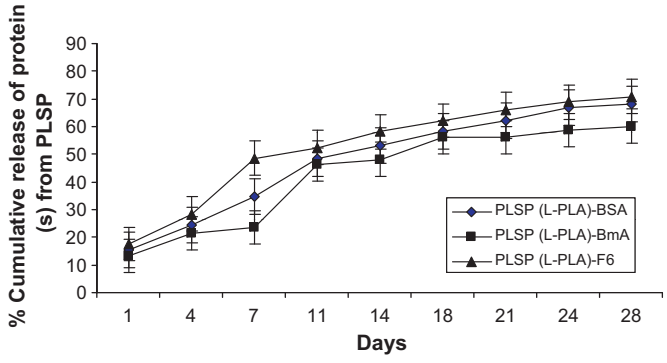

Fig. 7. *In vitro* release of bovine serum albumin (BSA), *B. malayi* adult extract (BmA) and its fraction F6 from PLSP-BSA, PLSP-BmA and PLSP-F6, respectively, at different intervals over a period of 28 days.

day 28 around 60–70% of the adsorbed protein(s) could be released from PLSP (Fig. 7).

3.6. In-process stability studies of the proteins

Fig. 1 shows location of molecular weights of BmA (10–>180 kDa), BSA (67 kDa), plain F6 (54–68 kDa) and *in vitro* released F6 from adsorbed F6 on PLSP in SDS-PAGE.

3.7. IgG and its subclass and IgE responses to protein loaded microparticles

On day 35 p.f.a. sera of all the immunized animals had significantly high ($P < 0.001$) IgG levels over PBS injected animals (control) except BmA-2 immunized animals. IgG level of animals immunized with PLSP-F6 was comparable to the level of F6-2 immunized animals but the level was higher than the PLSP-BmA immunized animals ($P < 0.001$; Fig. 8A). IgG level in F6-2 immunized animals was higher ($P < 0.001$) than that of BmA-2 immunized animals (Fig. 8A).

IgG1, IgG2a and IgG2b levels were found to be increased in PLSP-F6 immunized animals as compared to F6-2 immunized animals ($P < 0.001$; Fig. 8A). The levels in PLSP-F6 immunized animals were also high ($P < 0.01$–0.001) over PLSP-BmA immunized animals. However, levels of IgG subclasses in sera of animals immunized with F6-2 were comparable to the levels in sera of animals receiving BmA-2. Sera of PLSP-BmA immunized animals showed significantly elevated ($P < 0.01$) level of IgG2a. Interestingly IgG2a in PLSP-F6

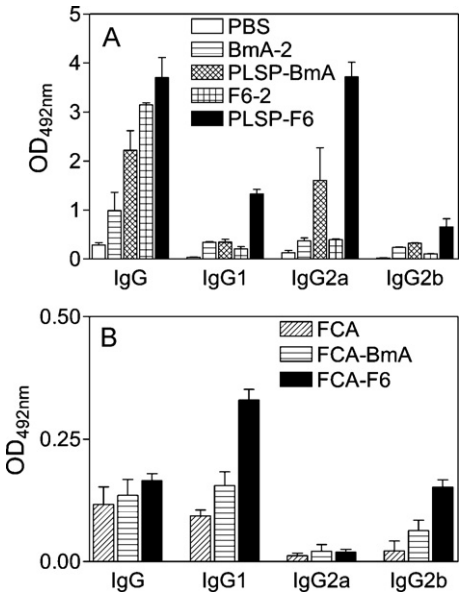

Fig. 8. Antifilarial IgG, IgG1, IgG2a, IgG2b antibody levels in serum of Swiss mice immunized with (A) one dose of PLSP-BmA or PLSP-F6 and 2 doses of plain BmA or F6 (BmA-2/F6-2) of *B. malayi* or PBS; (B) one dose of BmA or F6 of *B. malayi* emulsified with FCA or FCA alone. Values are mean ± SD of data of two experiments using six animals. The animals were sacrificed on day 35 post first administration (p.f.a.) of antigen formulations or PBS/FCA alone. Statistics: Newman–Keuls multiple comparison tests [significance levels for data in part (A), IgG: NS: (PLSP-F6 vs F6-2), $^{***}P < 0.001$ (PBS vs F6-2/PLSP-BmA/PLSP-F6, PLSP-BmA vs BmA-2, BmA-2 vs F6-2, PLSP-F6 vs PLSP-BmA); IgG1: $^{**}P < 0.01$ (F6-2 vs PBS), $^{***}P < 0.001$ (PLSP-BmA/F6 vs PBS, PLSP-F6 vs PLSP-BmA, PLSP-F6 vs F6-2); IgG2a: $^{**}P < 0.01$ (PLSP-BmA vs BmA-2), $^{***}P < 0.001$ (PLSP-BmA/F6 vs PBS, PLSP-F6 vs PLSP-BmA, PLSP-F6 vs BmA-2/F6-2); IgG2b: $^{**}P < 0.01$ (PLSP-BmA vs PBS,); $^{***}P < 0.001$ (PLSP-F6 vs PLSP-BmA, PLSP-F6 vs PBS, PLSP-F6 vs F6-2); part (B), IgG1: $^{*}P < 0.05$ (FCA-BmA vs FCA), $^{***}P < 0.001$ (FCA-F6 vs FCA); IgG2b: $^{**}P < 0.01$ (FCA-F6 vs FCA)].

immunized animals was found remarkably increased ($P < 0.001$) compared to PLSP-BmA and BmA-2/F6-2. Besides, the level of IgG2a in PLSP-F6 immunized animals was more than 5 folds and around 3 folds greater than IgG2b and IgG1 levels of the animals, respectively. IgG1 response in PLSP-BmA immunized animals was found to be almost similar to BmA-2 immunized animals.

In general IgG and its subclass levels in FCA-BmA/F6 immunized animals were remarkably low as compared to PLSP-BmA/F6 immunized animals. IgG1 levels were found to be elevated in FCA-F6 ($P < 0.001$) or BmA ($P < 0.05$) immunized animals compared to FCA alone. IgG2b was increased in FCA-F6 immunized animals but not in FCA-BmA immunized animals (Fig. 8B).

In summary, specific IgG1, IgG2a and IgG2b subclass levels were significantly high in animals immunized with PLSP-F6 or PLSP-BmA with IgG2a being the maximum. The subclass levels in FCA-BmA/FCA-F6 immunized animals were remarkably low compared to PLSP-F6 or PLSP-BmA immunized ones.

IgE levels in all the immunized animals remained unaltered compared to non-immunized ones (data not shown).

3.8. Cellular proliferative response

Cells of PLSP-F6/BmA immunized animal challenged with BmA or F6 showed upregulation of cellular proliferation significantly ($P < 0.01$–0.001) over BmA-2/F6-2 immunized animals (Fig. 9A). However there was no significant difference in proliferative responses between cells of PLSP-F6 immunized and PLSP-BmA or plain BmA-2 and F6-2 (Fig. 9A). PLSP-F6 immunized animal cells when challenged with Con A *in vitro* showed upregulation of proliferation significantly ($P < 0.001$) as compared to F6-2 or PLSP-BmA immunized animals (Fig. 9B). In summary, the results showed that

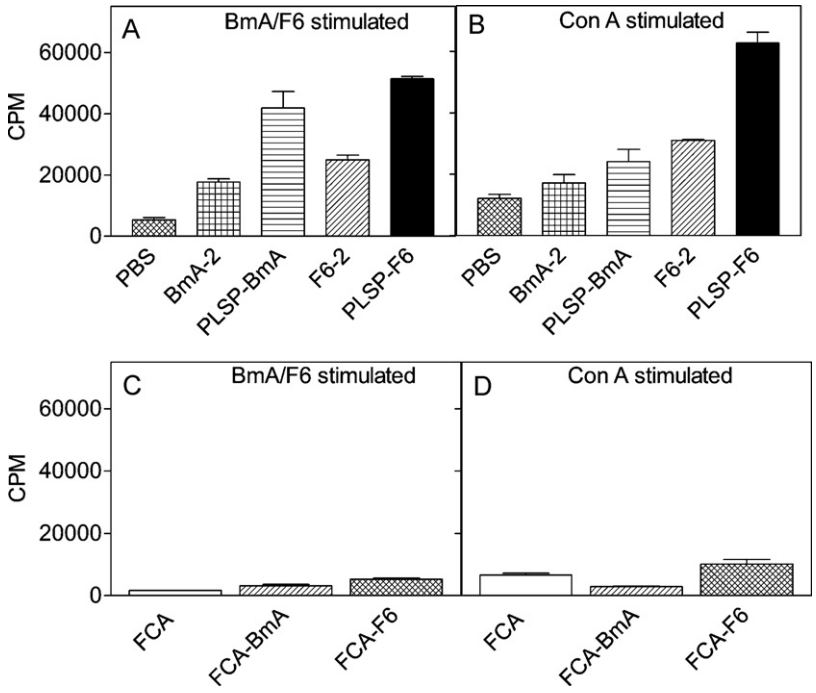

Fig. 9. Proliferative responses (cpm) of splenocytes of animals immunized with one dose of BmA or F6 adsorbed PLSP (PLSP-BmA/F6) or emulsified with FCA, two doses of plain BmA or F6 of *B. malayi* (BmA-2/F6-2) or PBS/FCA alone to BmA/F6 (A and C) and Con A (B and D) stimulation *in vitro*. Values are mean ± SD of data of two experiments using six animals. The animals were sacrificed on day 35 p.f.a. of antigen formulations or PBS/FCA alone. Statistics: Newman–Keuls multiple comparison tests [significance levels for data in part (A), BmA/F6 stimulated: NS (PLSP-F6 vs PLSP-BmA), $^{*}P < 0.05$ (PBS vs F6-2), $^{**}P < 0.01$ (PLSP-BmA vs BmA-2), $^{***}P < 0.001$ (PLSP-BmA/PLSP-F6 vs PBS, PLSP-F6 vs F6-2); part (B), Con A stimulated: $^{*}P < 0.05$ (F6-2 vs BmA-2), $^{**}P < 0.01$ (F6-2 vs PBS), $^{***}P < 0.001$ (PLSP-F6 vs PBS, PLSP-F6 vs F6-2, PLSP-F6 vs PLSP- BmA); part (C), BmA/F6 stimulated: $^{*}P < 0.05$ (FCA-BmA vs FCA), $^{**}P < 0.01$ (FCA-F6 vs FCA), part (D), Con A stimulated: $^{*}P < 0.05$ (FCA-BmA vs FCA-F6/FCA)].

the cell proliferative response was higher in single shot PLSP-F6/BmA immunized animals than 2 doses of the plain antigens.

Specific cellular proliferative responses were upregulated in FCA-BmA/FCA-F6 immunized animals ($P < 0.05$–0.001; Fig. 9C) but Con A response was found downregulated in FCA-BmA immunized animals ($P < 0.05$; Fig. 9D). Taken together, cellular proliferative responses of PLSP-BmA/F6 was much higher (7–10 times) than that of FCA-BmA/F6 immunized animals.

3.9. Cytokine release

3.9.1. IFN-γ and TNF-α release

There was a remarkable upregulation of F6 specific ($P < 0.001$) and more than 3 folds increase in LPS ($P < 0.01$) induced IFN-γ release from cells of PLSP-F6 immunized animals compared to cells of F6-2 immunized ones. However, BmA specific and LPS induced IFN-γ release in cells from PLSP-BmA was comparable to those released by cells of BmA-2 immunized animals (Fig. 10A and B).

Cells from FCA-BmA/F6 immunized animals showed no alteration in BmA or F6 induced IFN-γ release as compared to that of FCA alone immunized ones (Fig. 10C). However, LPS stimulation *in vitro* caused marginal upregulation ($P < 0.05$) of IFN-γ release from cells of FCA-F6 immunized animals and not from FCA-BmA immunized ones (Fig. 10D).

3.9.2. TNF-α release

TNF-α release from cells of PLSP-F6 immunized animals was found to be upregulated significantly when stimulated with F6 ($P < 0.001$; Fig. 11A) *in vitro* whereas LPS stimulated TNF-α release was found marginally increased ($P < 0.05$; Fig. 11B) over F6-2.

Specific TNF-α release from cells of FCA-F6 immunized animals was observed to be marginally upregulated ($P < 0.05$; Fig. 11C) but not from LPS stimulated cells (Fig. 11D) compared to FCA-F6 immunized ones.

In summary, specific IFN-γ and TNF-α release was remarkably increased in cells of PLSP-F6 immunized animals.

3.10. Nitric oxide release

NO release from macrophages of PLSP-F6 or PLSP-BmA immunized animals was found to be significantly increased ($P < 0.001$) when stimulated with BmA, F6 or LPS *in vitro* compared to F6-2/BmA-2 or PBS immunized animals. Interestingly the increase in NO release was also greater in cells of PLSP-F6 immunized animals than in cells of PLSP-BmA immunized animals ($P < 0.01$–0.001; Fig. 12A and B). In summary, the results revealed that immunization with PLSP adsorbed F6 or BmA remarkably increased the antigen specific and LPS induced NO response.

Specific NO release from macrophages of FCA-F6 immunized animals was much greater ($P < 0.01$) than FCA-BmA ($P < 0.05$) immunized animals compared to cells of animals receiving FCA alone (Fig. 12C); LPS stimulated NO release was increased in FCA-F6 immunized animals but not in cells of FCA-BmA immunized ones (Fig. 12D) when compared with FCA alone.

Thus, taken together the findings show that one dose of PLSP-BmA/F6 produced much better immune responses than one dose of FCA-BmA/F6 or two doses of plain BmA or F6.

4. Discussion

One of the most important factors influencing the generation of desired immune response is the stability of the antigen both before and after mixing/formulation with adjuvants. We have recently shown that, as predicted, immunization with SDS-PAGE resolved fraction F6 (denaturing conditions) protects the animals against the infection via Th1 (IFN-γ, TNF-α, IL-6, IgG2a)/Th2 (IgG1, IgG2b) responses (Sahoo et al., 2009). The fraction also reacted with sera of different categories of bancroftian filarial patients detecting

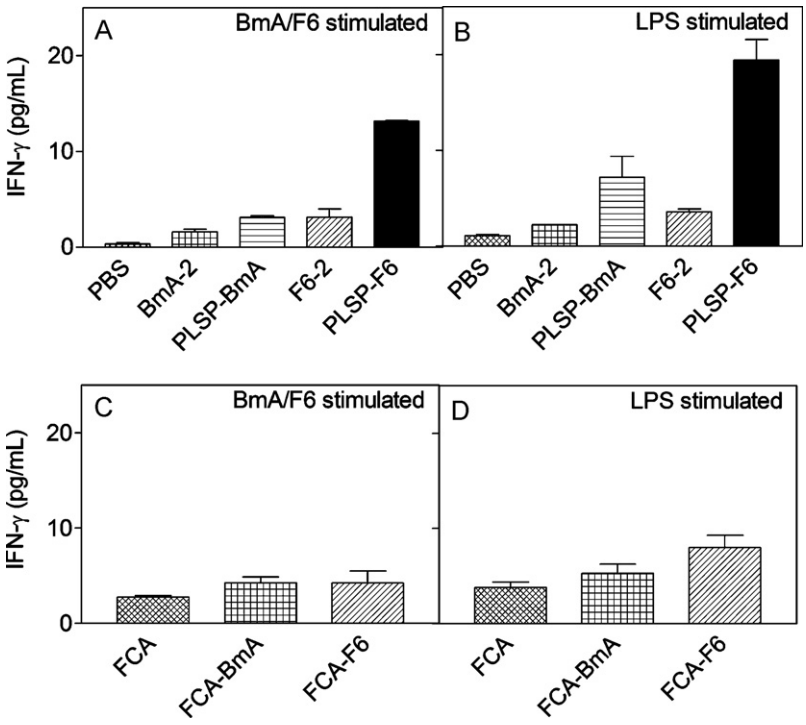

Fig. 10. IFN-γ release from splenocytes of animals immunized with one dose of BmA or F6 adsorbed PLSP (PLSP-BmA/F6) or emulsified with FCA, two doses of plain BmA or F6 (BmA-2/F6-2) of *B. malayi* or PBS/FCA alone in response to BmA/F6 (A and C) or LPS (B and D) stimulation *in vitro*. Values are mean ± SD of data of two experiments using six animals. The animals were sacrificed on day 35 p.f.a. of antigen formulations or PBS/FCA alone. Statistics: Newman–Keuls multiple comparison tests [significance levels for data in part (A), BmA/F6 stimulated: $^{***}P < 0.001$ (PLSP-F6 vs PBS, PLSP-F6 vs F6-2, PLSP-BmA vs PLSP-F6); part (B), LPS stimulated: NS: (PLSP-F6 vs PLSP-BmA); $^{**}P < 0.01$ (PLSP-F6 vs PBS, PLSP-F6 vs F6-2), part (D), LPS stimulated: $^{*}P < 0.05$ (FCA-F6 vs FCA)].

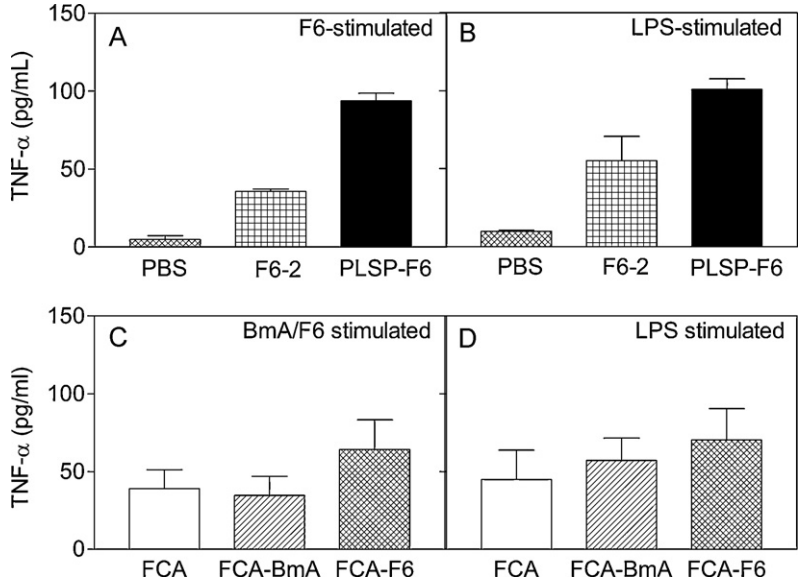

Fig. 11. TNF-α release from spleen cells of animals immunized with one dose of F6 adsorbed PLSP (PLSP-F6) or emulsified with FCA, two doses of plain F6 (F6-2) of *B. malayi* or PBS/FCA alone in response to BmA/F6 (A and C) and LPS (B and D) stimulation *in vitro*. Values are mean ± SD of data of two experiments using six animals. The animals were sacrificed on day 35 p.f.a. of antigen formulations or PBS/FCA alone. Statistics: Newman–Keuls multiple comparison test [significance levels for data in part (A), F6 stimulated: $^{**}P < 0.01$ (F6-2 vs PBS), $^{***}P < 0.001$ (PLSP-F6 vs PBS, PLSP-F6 vs F6-2); part (B), LPS stimulated: $^{*}P < 0.05$ (F6-2 vs PBS, PLSP-F6 vs F6-2), $^{***}P < 0.001$ (PLSP-F6 vs PBS); part (C), BmA/F6 stimulated: $^{*}P < 0.05$ (FCA-F6 vs FCA); part (D), LPS stimulated: NS (FCA-BmA/FCA-F6 vs FCA)].

elevated levels of F6 specific IgG1, IgG2 and IgG3 in endemic normals and chronic symptomatics, and IgG1 and IgG2 in asymptomatic microfilaraemics (Joseph et al., 2011). Earlier too, our group (Bhattacharya et al., 1997; Shakya et al., 2009) and other investigators (Chenthamarakshan et al., 1997) have shown antibody responses to denatured filarial antigens and sizeable protection against infective larval challenge in immunized host by generating a Th1 biased milieu in the host. These findings clearly indicate that the epitopes of interest are intact in F6 in spite of the denaturing conditions of SDS-PAGE. The polymer selected for the study for adsorption of the antigen is easy to process and reported to retain the adsorbed antigen for prolonged period. As evident from the

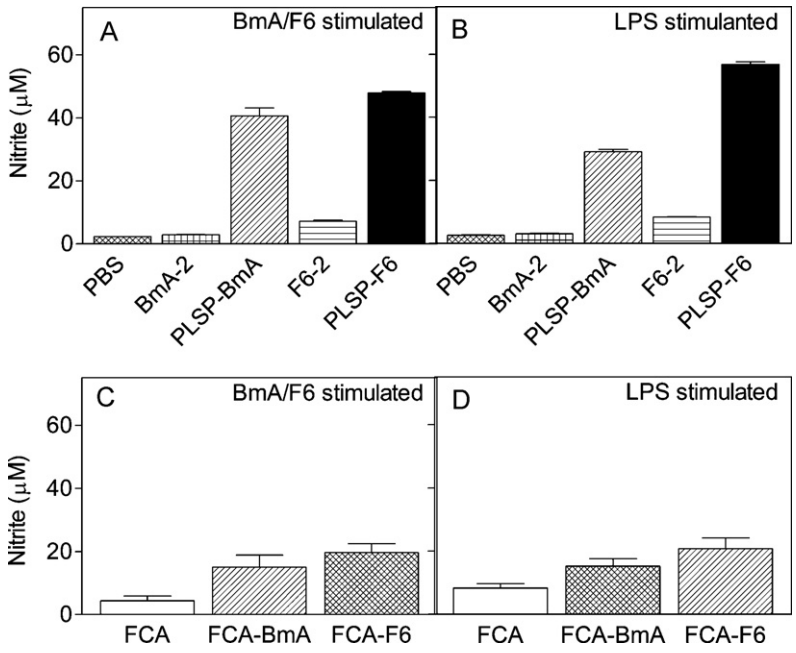

Fig. 12. Nitric oxide release from peritoneal macrophages of animals immunized with one dose of BmA or F6 adsorbed PLSP (PLSP-BmA/F6) or emulsified with FCA, two doses of plain BmA or F6 (BmA-2/F6-2) of *B. malayi* or PBS/FCA alone in response to BmA/F6 (A and C) and LPS (B and D) stimulation *in vitro*. Values are mean ± SD of data of two experiments using six animals. The animals were sacrificed on day 35 p.f.a. of antigen formulations or PBS/FCA alone. Newman–Keuls multiple comparison test [significance levels for data in part (A), BmA/F6 stimulated: $^{*}P < 0.05$ (F6-2 vs PBS), $^{**}P < 0.01$ (PLSP-F6 vs PLSP-BmA), $^{***}P < 0.001$ (PLSP-BmA/PLSP-F6 vs PBS, PLSP-BmA vs BmA-2, PLSP-F6 vs F6-2); part (B), LPS stimulated: $^{***}P < 0.001$ (F6-2/PLSP-BmA/PLSP-F6 vs PBS, PLSP-BmA vs BmA-2, PLSP-F6 vs PLSP-BmA, PLSP-F6 vs F6-2, F6-2 vs BmA-2); part (C), BmA/F6 stimulated: $^{*}P < 0.05$ (FCA-BmA vs FCA), $^{**}P < 0.01$ (FCA-F6 vs FCA); part (D), LPS stimulated: $^{*}P < 0.05$ (FCA-F6 vs FCA)].

SDS-PAGE profile the integrity of antigen remained unaltered after adsorbing onto microparticles. Despite an increased level of adsorption, extensive antigen release occurred from PLSP in 24 h at 37 °C indicating electrostatic nature of the antigen substrate binding. Maximum BmA protein adsorption was found at 1.0 mg/ml as it consisted of several proteins with larger size. The existence of maximum binding capacity for adsorption reflects that a monolayer may exist. On the other hand F6 consists of five proteins and out of these five proteins, two proteins show its isoelectric-point at pH 5.5 (Sahoo et al., 2009). It is evident that proteins adsorbing close to their isoelectric point, binding of the primary monolayer appears weaker, perhaps due to reduced electrostatic forces, but subsequent layer formation is more facile as a result of reduced charge repulsion (Chesko et al., 2005). Apart from the electrostatics, additional factors such as non-Coloumbic (London, Van der Waals, hydrophobic interaction) forces might be responsible for the adsorption of the proteins of BmA or F6 used in this study onto PLSP (Chesko et al., 2005). The findings of the study show that charge neutralization at the protein/polymer interface had occurred as a result of protein adsorption as evident from Table 1. Further, the change in zeta-potential following the adsorption of protein is indicative of charge neutralization (e.g., salt bridge formation) at the protein–polymer interface. Thus it shows a general trend of increasing (i.e. less negative) zeta-potential as additional protein adsorbed. The nature of polypeptides might allow time dependent conformational changes leading to multiple attachment sites and irreversible adsorption (Chesko et al., 2005; Coombes et al., 1999). Chesko et al. (2005) reported that polymer surface covered with a protein monolayer may have a reduced affinity to adsorb additional protein as compared to the polymer/surfactant interface with sites unoccupied by protein. Nevertheless the degree of adsorption and the formation of peptide/protein multilayers around PLSP are directly related to the hydrophobicity and concentration of both the polymer and the peptide (Witschi and Doelker, 1998). The higher adsorption of BSA and F6 observed in this study might be due to its smaller size as compared to BmA. Thus, it can be inferred that layer closest to the solid surface being more strongly bound than the outer (protein–protein) layer which are easily removed (burst effect) by washing. Moreover, irregular shape of the PLSP caused more protein(s) adsorption and showed more release of protein(s). SDS-PAGE profile of the PLSP formulation of F6 and BmA did not reveal any cleavage or degradation product of F6 or BmAFI indicating that the denaturing conditions did not affect the antigens. Further, BmA and F6 were found to be stable even after the adsorption onto the surface of PLSP. This is inferred from the absence of any alteration in secondary structure of the protein(s) as seen using Fourier transform infrared spectroscopy (FTIR). In all cases, a typical α-helical confirmation of the protein was observed and the FTIR spectra of the samples showing the C=O stretching vibration (1755–1761 cm^{-1}) of the PLA polymer was well separated from the amide I vibration (1637–1643 cm^{-1}) of the protein(s) (data not shown).

PLSPs play an important role in the interaction with phagocytic cells. It is reported that particles smaller than 10 μm injected through subcutaneous route are transported by antigen presenting cells into draining lymph-nodes for rapid antigen release and antibody response whereas larger than 10 μm size particles act as a depot in releasing the antigen in a second step which are recognized by B cell receptors (Mishra et al., 2006). A more recent study showed that presence of surface antigen on PLG microparticles was important for the induction of strong and long lasting immunity (Rafati et al., 1997). Besides, small quantity of lamellae adsorbed antigen presentation is sufficient to maintain high level of circulating antibodies for extended time periods (Coombes et al., 1999).

IgG1 and IgG2b are Th2 associated whereas IgG2a is indicative of Th1 type responses (Stevens et al., 1988). Specific IgG1, IgG2a and IgG2b subclass levels were significantly high in animals immunized with one dose of PLSP-F6 or PLSP-BmA over two doses of plain F6 or BmA. In addition IgG2a levels were considerably high in comparison to IgG1 and IgG2b level. However IgG1 and IgG2b levels in animals immunized with FCA-BmA or

FCA-F6 were remarkably low compared to PLSP-F6 or PLSP-BmA immunized animals. In our earlier study we have demonstrated that immunization with three doses of F6 + FCA/FIA intensely upregulated IgG1, IgG2b but the level of IgG2a antibodies was not as high as IgG2b. Thus adjuvanticity of PLSP might be due to depot formation at the injection site resulting in slow antigen release and sustained communication with antigen presenting cells (Coombes et al., 1999). By administration of antigen associated with PLSP may facilitate uptake and presentation of particulate antigen by phagocytic cells which are considered as antigen presenting cells for Th1 responses (Lavelle et al., 1999). In filariasis alterations in survival of the parasites has been shown be due to modulation of lymphocyte proliferation (Maizels et al., 2004; Maizels and Lawrence, 1991). We observed that immunization with PLSP-F6/BmA caused 7–10 times greater cell proliferation, elevated levels of specific IFN-γ and TNF-α release and greatly enhanced NO release than that produced by FCA-BmA/F6 immunized animals indicating superior and strong adjuvanticity of PLSP-F6/BmA to produce Th1 immune response. In our earlier study we reported that three doses of F6 injected with FCA/FIA produced predominantly Th1 response (Sahoo et al., 2009) which was correlated with parasite elimination. Thus all the above findings indicate that one dose of PLSP-F6 was sufficient to produce a strong response and which may likely to participate in suppressing parasite establishment and their survival.

5. Conclusion

In conclusion, immunization of mice with PLSP-F6 or PLSP-BmA in single shot upregulated cellular proliferation, IFN-γ, TNF-α and NO production in host's cells and specific IgG and its subclasses specially IgG2a antibodies. These responses were greater than one dose of FCA-F6/BmA or two-dose schedule of plain F6 or BmA. Moreover, PLSP-F6 produced much better response than PLSP-BmA. These data demonstrate for the first time that PLSP is a potent immunoadjuvant and enhances the immune response to filarial F6 molecules and BmA and obviates the need for multiple immunization injections.

Conflict of interest

The authors do not have any commercial or other association that might pose a conflict of interest in this publication.

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