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Comparison of humoral and cell-mediated immune responses to cationic PLGA microspheres containing recombinant hepatitis B antigen

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ABSTRACT

Presently available marketed alum adsorbed hepatitis B vaccine used for prophylactic immunization, can effectively elicit humoral immunity but is poor inducer of cell-mediated immunity (CMI). Besides, conventional alum-adjuvant vaccines require multiple injections to achieve long-lasting protective immune responses. Therefore, as a result of insufficient immunization, infections are still the leading killer among diseases. The present investigation was therefore, aimed at developing "single-shot" HBsAg adsorbed microspheres of poly (DL)-lactide-co-glycolide (PLGA) (L/G 50:50 and 75:25) and their capability to stimulate the cell mediated immune response against hepatitis B surface antigen. These microspheres were characterized *in vitro* for their size, shape polydispersity index, percentage HBsAg adsorption efficiency and *in vitro* release profile. The immune-stimulating activities were also studied following subcutaneous injection of HBsAg adsorbed PLGA microspheres (single-dose on day 0) and compared with alum adsorbed vaccines (two-doses on 0 and 28 days) in Balb/c mice. Specific cell-mediated immune responses such as lymphocyte transformation assay (stimulation-index) including release of interferon-gamma (IFN-γ), interleukin-2 (IL-2) and nitric-oxide were determined. Cellular responses in case of alum adsorb HBsAg vaccine was very low. These studies demonstrate the potential of cationic polymeric microspheres based vaccine in stimulating cell mediated immune response along with humoral response against hepatitis B.

1. Introduction

Hepatitis B is an enigmatic disease in which the host's own immune response to persistent viral infection may bring about host destruction through antiviral inflammatory responses. Alum adsorbed marketed hepatitis B vaccine (licensed in 1981) is the simple solution to hepatitis B, which prevents both infection and late sequelae of liver cirrhosis and hepatocarcinoma (Hilleman M.R., 2003).

Alum adsorbed vaccines can effectively stimulate humoral immunity but are poor inducers of cell-mediated immunity. Besides, conventional alum adjuvanted vaccines require multiple injections to achieve long-lasting protective immune responses. The most commonly used immunization schedule for hepatitis B vaccine is three injections given at 0, 1 and 2 months or at 0, 1 and 6 months to get protective antibody levels. More than 95% of vaccines against hepatitis B have been reported to be effective after primary and secondary booster immunization. But it is very difficult, especially in developing countries to maintain a periodic immunization rate in case of multiple administration immunization programme. Therefore, as a result of incomplete immunization, infection is still the leading killer. A major step towards better vaccination coverage is therefore, thought to be the introduction of single shot vaccine over number of booster administrations (Feng et al., 2006).

Biodegradable microparticles based on lactide and glycolide polymers have the capacity to elicit strong long-lasting antibody and T-cell proliferative response to the adsorbed antigens. The microencapsulation may create mechanical, thermal and chemical stresses that affect the antigenicity of the protein. In addition, the moisture, acidity, temperature and un-physiological salt concentration of the microenvironment created during the course of degradation and release may cause the destabilization and aggregation of the encapsulated proteins (Feng et al., 2006). Typically, protein adsorption to surfaces and the formation of polymer degradation products can affect the observed amount of antigen release, as well as its stability (Johansen et al., 2000). Absolom et al. reported that the greatest amount of adsorption occurs on those substrates, which have lowest surface tension and more hydrophobicity (Absolom et al., 1987). The number and nature of the charged groups on certain peptides may be favorable for adsorption onto

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empty microparticles by electrostatic interaction, thereby giving rise to adjuvant effects. Polypeptide adsorption onto polymer surface is also known to be influenced by pH, ionic strength and composition of the incubation medium (Moynihan et al., 2002). Protein adsorption from particulate substrates is governed by a complex interaction involving polar forces e.g. hydrogen bonding, hydrophobic interactions via the hydrophobic regions of protein molecules and weaker van der Waals forces (Coombes et al., 1996).

Major challenges in the development of new generation prophylactic or therapeutic vaccines against chronic hepatitis B virus infection are the induction of potent cellular immune responses and efficient targeting of the specifically induced immune effector cell to the liver (Bos et al., 2004). Delivery of antigen to dendritic cells in a particulate form has been found to up-regulate cytokine gene transcription compared with soluble antigen. Thus the delivery of antigen in a particulate form per se can enhance immunogenicity (Lavelle et al., 1999). Vaccination strategies favoring the induction of stable cell-mediated responses might protect against subsequent viral infections. The cytolytic and γ-interferon producing $CD8^+$ T cell subset is an attractive candidate to clear acute, chronic and persistent infections with intracellular pathogens (Schirmbeck et al., 1995). Production of NO has also been directly correlated with the host's ability to suppress microbial proliferation and infection (Ghaffari et al., 2006).

The present investigation was therefore, aimed at developing "single dose" HBsAg vaccine adsorbed on to poly (lactic-co-glycolic acid) microspheres (different lactide and glycolide ratio viz. 50:50 and 75:25) and its ability to stimulate humoral and cell mediated immunological responses after single administration in the rodent host, Balb/c mice.

2. Materials and methods

2.1. Materials

Polymers such as poly (DL-lactide-co-glycolide; 50:50), mol wt 40,000–75,000 and poly (DL-lactide-co-glycolide; 75:25), mol wt 66,000–107,000 were used for the preparation of the microspheres. The hepatitis B surface antigen (HBsAg; source genetically modified yeast cells) was obtained as gift samples from the Serum Institute of India Ltd., Pune. The BCA kit was purchased from Genei (Bangalore, India). The reagents used in SDS–PAGE were obtained from Sigma, USA. All other chemicals and reagents were of analytical grade and purchased from local suppliers.

2.2. Preparation of empty microspheres

PLGA (50:50 and 75:25) microspheres were prepared aseptically by a double emulsion solvent evaporation method (W/O/W) at room temperature as reported by Singh et al. (Singh et al., 2000) and Stivaktakis et al. (Stivaktakis et al., 2005) with slight modifications. In brief, 1 ml of phosphate buffer saline (pH 7.4) containing cetyl trimethyl ammoniumbromide (CTAB) (0.2%, w/v) was suspended in 10 ml of 4% w/v PLGA solution in ethyl acetate and sonicated for 1.0 min at 50 W in an ice-bath (Soniweld, Mumbai, India). This water-in -oil (w/o) emulsion was added into 20 ml of 2% w/v aqueous polyvinyl alcohol and mixed at high speed (4000 rpm) using mechanical stirrer (RQ-122, Remi Motors, Mumbai, India). The organic solvent was allowed to evaporate and the microspheres formed were washed and collected through mild centrifugation (4000 rpm, 5 min).

2.3. Aseptic microsphere preparation

The PLGA polymer solution including buffer(s) was filtered through 0.22 μm aseptically and the PLGA microsphers were manufactured under aseptic conditions (class 100 area).

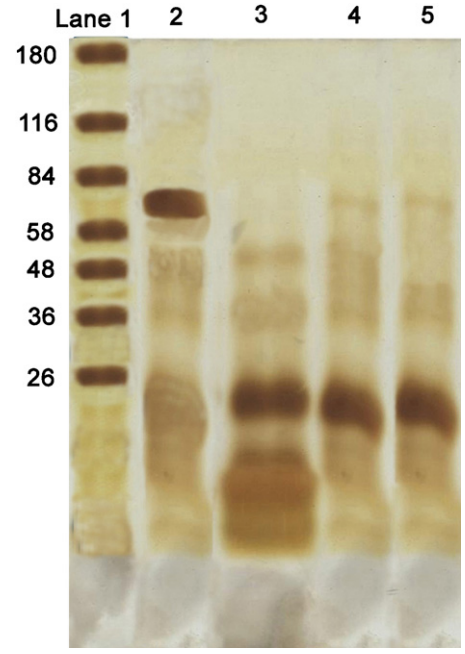

Fig. 1. SDS–PAGE showing bands for different formulations: Lane 1: molecular weight markers, Lane 2: bovine serum albumin, Lane 3: recombinant hepatitis B surface antigen, Lane 4 and 5: *In vitro* released hepatitis B surface antigen from PLGA (50:50) and PLGA (75:25) microspheres respectively.

To challenge the process, a colony of *Bacillus subtilis* (ATCC 6633, HiMedia, India) was suspended in 0.5 ml water, which was mixed with 20 ml of a 5% (w/w) polymer solution in ethyl formate. The mixture was ultrasonicated (50 W, 30 s) to yield a W/O-emulsion. The emulsion was sterile filtrated through a 0.2 μm regenerated cellulose membrane filter. The subsequent process steps are as described above. Further sterility test was performed using soyabean casein digest.

2.4. Characterization of HBsAg

Molecular weight of HBsAg in known concentration was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using 5% stacking gel and 15% resolving gel. The band was visualized by using silver stain (Jaganathan et al., 2004; Saraf et al., 2006; Jaganathan and Vyas, 2006).

2.5. Characterization of microspheres

The average size and polydispersity index of PLGA (50:50) and (75:25) microspheres were studied by Dynamic Light Scattering method (DLS), in a multimodal mode using a computerized inspection system (Malvern UK, Malvern Zetasizer, ZEM 5002) after mixing the particles with the medium (PBS, pH 6.5). The surface morphology was studied using scanning electron microcopy (SEM; Leo 430 Germany). The samples for SEM were prepared by sprinkling the powder form of microspheres on a double adhesive tape that stucked to an aluminum stub. The stub was coated with gold using sputter coater. The samples were then randomly scanned and photographed (Saraf et al., 2006; Venkataprasad et al., 1999; Jabbal-Gill et al., 1999) (Fig. 1).

2.6. Adsorption of hepatitis B surface Antigen (HBsAg)

One part of HBsAg (1.5 mg/ml in PBS = 7.4) was incubated with 10 parts of microspheres overnight at room temperature in a

shaking incubator. Microspheres were centrifuged and washed once with distilled water to remove unbound antigen. The supernatant was collected and analyzed for antigen content using a bicinchoninic acid (BCA) protein assay. A calibration curve was constructed from a serial dilution of HBsAg and the amount of protein adsorbed on the particles was determined by subtraction (Stivaktakis et al., 2005; Venkataprasad et al., 1999; Jabbal-Gill et al., 1999; Coombes et al., 1998)

2.7. In vitro release of antigen from HBsAg adsorbed cationic microspheres formulation

Microspheres with adsorbed HBsAg were incubated in phosphate buffered saline (PBS) release medium (0.1 M, pH 7.4, 25 °C) containing 0.02% sodium azide as a bacteriostatic agent. Sample vials containing approximately 50 mg particles dispersed in 5 ml PBS (pH 7.4) were shaken intermittently to retain the particles in suspension on a water-bath shaker (Julaboo). A vial was withdrawn after 1 day, the content was centrifuged at 8000 *g* for 10 min and fresh medium was added to the sample. This process was repeated at 3, 5, 7 11, 15, 21, days interval up to 4 weeks. Protein content was determined using a BCA protein assay kit (Stivaktakis et al., 2005; Venkataprasad et al., 1999; Jabbal-Gill et al., 1999; Coombes et al., 1998).

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 were incubated 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 supernatant was then proceeded for sample preparation by heating with sample buffer at 95 °C in water bath. The extracted antigen was concentrated and loaded on to a 5% stacking gel and subjected to electrophoresis on a 15% separating gel at 200 V (Electrophoresis apparatus, miniprotean 3 cell assembly Gel Dryer, BIO-RAD, India) until the Commassie dye stained protein bands reached to the bottom of the gel (Jaganathan et al., 2004; Saraf et al., 2006).

2.9. Immunization of animals

Balb/c mice (4–6 weeks old) were used in all experiments for the present study. All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals (IAEC number: 89/08/Para/IAEC). Throughout the study, the animals were housed in climate (23 ± 2 °C; RH: 60%) and photoperiod (12 h light–dark cycles) controlled animal quarters. They were fed standard rodent pellet supplemented with grain and had free access to drinking water.

The study included five groups with six animals in each. Animals of group 1 were treated with PBS (pH 7.4) and served as control. Groups 2 and 3 received pure recombinant HBsAg and alum adsorbed HBsAg, respectively. Each animal was administered with 10 μg antigen on day 0 and 28. Animals of groups 4 and 5 were administered subcutaneously once (day 0) with PLGA (50:50) and PLGA (75:25) microspheres adsorbed with same concentration of the antigen. Animals were sacrificed one week after the secondary immunization. Lymphocyte transformation and release of IFN-γ, IL-2 and NO were determined.

2.10. Sera collection

Blood sample was collected from retro orbital plexus of the mice before immunization (day 0) and thereafter on days 7, 15, 27 and 42 of primary immunization. Sera collected were stored at −20 °C until analyzed.

2.11. Determination of IgM and IgG

HBsAg specific IgM (day 7) and IgG antibody levels on days 0, 7, 15, 27, 42 in the serum were determined by indirect ELISA as per method described by Dixit et al. (Dixit et al., 2006). Briefly, each well of 96-well microtiter plate (Nunc Immuno Plate® Fb96 Mexisorp, NUNC) was coated with 100 μl of HBsAg (10 μg/ml in bicarbonate-buffer). The plate was incubated at 4 °C over night and then washed three times with PBS-T_{20} (0.01% v/v Tween-20 in PBS). The remaining free sites of each well were blocked with 150 μl of 3% w/v gelatin in PBS-T_{20} (blocking buffer). The plate was again incubated for 90 min at 37 °C and washed thrice with PBS-T_{20}. 100 μl of serum sample at 1:400 dilutions (diluted with 1.5% w/v gelatin PBS-T_{20}) was added to each well and incubated for 90 min at room temperature. Plate was washed three times with PBS-T_{20}. 100 μl of horseradish peroxidase (HRPO) conjugated goat anti-mice IgG antibodies at 1:1000 dilutions with 1.5% w/v gelatin PBS-T_{20} was incubated again for 90 min. For IgM, 100 μl biotinylated IgM (at 1:20,000 dilutions) was added to each well. Streptavidine conjugated with HRPO (1:1000) was added (additional step) after washing with PBS-T_{20} and incubated again for 90 min. The plate was washed six times and 100 μl of O-phenylenediamine dihydrochloride (20 mg OPD in citrate phosphate buffer, pH 4.5 containing 40 μl H_2O_2) was added as substrate. Since the OPD is sensitive to light, the plate was covered properly. After 15–20 min 25 μl of 2.5 M H_2SO_4 was added to stop the reaction and color produced was measured using ELISA plate reader (Biotek Power Wave X) at 492 nm.

2.12. Cytokine determination

For cytokine assay, HBsAg (5.0 μg/ml) stimulated culture supernatants, from 48-well plate was collected at 48 h post stimulated for estimation of IFN-γ, IL-2 (BD Pharmingen™). Mouse monoclonal antibodies against the cytokine were used in a paired antibody sandwich ELISA following the manufacture's instructions with some modification to suit our conditions. Triplicates of each sample were run separately. The concentration of the cytokines was calculated using optical density readings for measured amount of standards (Dixit et al., 2006; Vordermeier et al., 1995; Elias et al., 2005).

2.13. Lymphocyte transformation test (LTT)

Spleens were aseptically removed on 4–11 days after the second immunization. The spleen cells were then released into RPMI incomplete medium by pressing the fragment of spleen and the single cell suspension was made by passing through strainer (100 μm nylon filter). The erythrocytes were lysed by the treatment of cells with RBC lytic buffer. After washing, the contaminated erythrocytes were removed and then the cells were suspended in complete RPMI Medium 1640 (10% FCS) at density of 1×10^6 per ml prior to challenge with same recombinant antigen. Each well of 96-well flat bottomed micro plate received 100 μl cells (1×10^5 cells), plus 100 μl of either complete medium, ConA (10 μg/well) or the same recombinant pure antigen (5 μg/well). Cultures carried in triplet were incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 72 h the cells were then pulse labeled with [^{3}H] thymidine (1 μci/well) and harvested (Dynatech multimash 2000). After 18 h of [^{3}H] thymidine incorporation, radioactivity was counted in a liquid scintillation-beta counter (1217-reckbeta, Finland). The results were expressed as a stimulation index (SI), Calculated by dividing mean counts per minute (CPM) of stimulated culture with CPM of unstimulated culture. Radio active material

used in the study was handled in the 'Radio isotope laboratory' in the Division of Biochemistry, Central Drug Research Institute (CSIR), Lucknow, under the supervision of Radiological Safety Officer of the Institute.

2.14. Nitric oxide determination

NO determination was carried out according to Dixit et al. (2006). Prior to the collection of peritoneal macrophage, abdominal portion of the animals were cleaned with 70% ethyl alcohol. Aseptically 4–5 ml Dulbecco's modified Eagle's medium (DMEM) containing EDTA (0.1%) and antibiotics (penicillin: 100 U/ml; streptomycin: 100 μg/ml) were injected into peritoneal cavity of the animals and lavage was collected. Peritoneal cells thus obtained, were washed thoroughly with the medium, suspended in the medium containing 10% fetal bovine serum at a concentration of 0.5 million/800 μl/well, and dispensed into sterile 48-well tissue culture plates (Nunc-Rosklide, Denmark). After overnight incubation at 37 °C in 5% CO_2 atmosphere, adherent cells were replenished with fresh medium and stimulants were added (HBsAg: 0.5; LPS: 1.0 μg/ml) followed by incubation at the same atmosphere. The presence of nitrite in culture supernatants of 48 h post-stimulation (PS) was using Griess-reagent at 550 nm.

2.15. Statistical analysis

Statistical analysis was performed on the data obtained in the *in vitro* and *in vivo* studies by one-way analysis of variance (ANOVA) with Tukey multiple comparisons post test using Graph Pad prism 3.0 software. Throughout, the level of significance was chosen as less than 0.05 (i.e., $P < 0.05$). The post-hoc test was performed only if findings of the ANOVA were significant.

3. Results

3.1. Antigen characterization

The SDS–PAGE showed a distinct band at around 24 kD position, which corresponds to the S subunit of HBV envelop indicating that HBsAg consisted mainly of S polypeptide (non-glycosylated form) (Fig. 1).

3.2. Microspheres-HBsAg characterization

3.2.1. Physical characterization of microspheres formulation

The PLGA microspheres were prepared by double emulsion solvent evaporation method were spherical in shape. PLGA (50:50) microspheres had smooth surface, whereas PLGA (75:25) microspheres had slightly rough surface upon morphological examinations using scanning electron microscopy (SEM) (Fig. 2(A and B)). The actual size for PLGA (50:50) microspheres and PLGA (75:25) microspheres were found to be 7.35 ± 1.1 and 5.08 ± 1.5 respectively (Table 1). The polydispersity of PLGA (50:50) microspheres and PLGA (75:25) microspheres were found to be 0.313 and 0.220, respectively.

The percentage antigen adsorption efficiency of HBsAg for PLGA (50:50) microspheres and PLGA (75:25) microspheres were 62.66 ± 1.26% and 68.34 ± 1.22% respectively as determined by micro BCA assay. Microspheres were earlier standardized with the help of a selected model antigen, i.e., bovine serum albumin (BSA) to optimize process parameters and to obtain uniform and reproducible batches of microspheres.

The HBsAg adsorbed microspheres could not be sterilized by filtration. Terminal sterilization of the microspheres (UV-irradiation) also led to degradation of the antigen. Thus, microspheres were prepared aseptically, which assured the sterility of the final formulation that was able to protect integrity and activity of HBsAg.

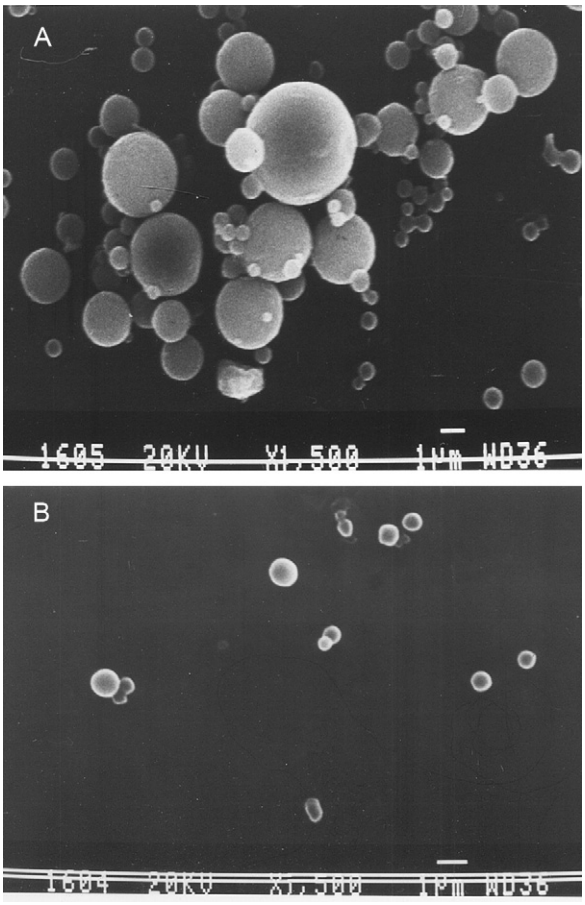

Fig. 2. (A) Scanning electron photomicrograph of PLGA (50:50) microspheres. (B) Scanning electron photomicrograph of PLGA (75:25) microspheres.

3.2.2. In-process stability of the antigen

By performing SDS–PAGE, clearly visible band for BSA and plain recombinant HBsAg (also for extracted HBsAg from PLGA microspheres) was obtained at around 66 kD and 24 kD location, respectively (*in-process* stability studies) (Fig. 1).

3.2.3. In vitro release of antigen

The *in vitro* release rate of HBsAg from PLGA (50:50) microspheres was found to be more (27.63 ± 1.250) than PLGA (75:25) microspheres (22.4 ± 0.200) on day 1. Subsequently, the rate of release was slower but on day 15 about 60–67% of the adsorbed HBsAg was released (Fig. 3).

Table 1
Characterization of PLGA microspheres.

Parameter	PLGA (50:50) microspheres Value (Mean ± SD)	PLGA (75:25) microspheres Value (Mean ± SD)
Mean particle diameter (μm)	7.358 ± 3.163	5.0800 ± 2.559
Polydispersity index	0.373	0.220
Particle shape	Spherical with smooth surface	Spherical with rough surface
Yield of Ms (%)	76.34	68.10
Adsorption efficiency HBsAg (%):	68.34 ± 1.22	62.66 ± 1.26
% Cumulative release of HBsAg in 24 h	27.63 ± 1.250	22.4 ± 0.200

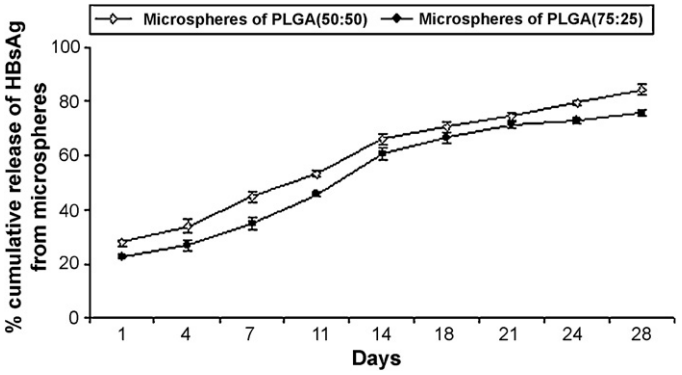

Fig. 3. *In vitro* release of HBsAg from PLGA microspheres at different time intervals. Values are mean ± SD of data from three experiments.

3.3. Immune responses of animals to Ms-HBsAg

3.3.1. Antibody response

The immunogenicity of HBsAg adsorbed microspheres (Ms-HBsAg) was compared with plain recombinant HBsAg and conventional alum-adsorbed vaccine in Balb/c mice. On day 7 after primary immunization IgM antibody titer for alum adsorbed HBsAg vaccine was higher than HBsAg adsorbed PLGA microspheres (50:50) and (75:25), ($P > 0.05$ and $P < 0.01$, respectively). On days 7 and 15 (after primary immunization) IgG antibody titer was comparatively higher in alum adsorbed HBsAg than other formulations. However, on day 27 (after primary immunization) IgG antibody titer against alum adsorbed HBsAg vaccine was significantly lower as compared to HBsAg adsorbed PLGA 50:50 and 75:25 microspheres formulations ($P < 0.001$ and $P < 0.01$, respectively). Booster injection of alum adsorbed HBsAg vaccine given on day 28, increased the IgG antibody titer (on day 42) which was comparable to the response produced by both HBsAg adsorbed PLGA microspheres formulations administered once only ($P > 0.05$). These results indicate that a single injection of Ms-HBsAg formulations produced comparable antibody response elicited by two doses of alum-adsorbed HBsAg vaccine (Fig. 4(a)).

3.3.2. Cytokine release

Studies were carried out to evaluate the effect on IFN-γ and IL-2 release pattern from spleen cells of animal immunized with plain rHBsAg or alum adsorbed HBsAg with booster doses and Ms-HBsAg (single injection) in response to HBsAg challenge *in vitro*. Levels of cytokine release (IFN-γ and IL-2) were estimated in spleen cells after 42 days of primary immunization with different formulations. A significantly elevated level of both IFN-γ and IL-2 was observed in mice immunized with HBsAg adsorbed PLGA microspheres formulations as compared to alum adsorbed HBsAg formulation in response to HBsAg challenge *in vitro* ($P < 0.001$) (Fig. 4(b)).

3.3.3. Cellular proliferative response

A significantly high cellular proliferative response (as indicated by stimulation index; SI) was observed in mice immunized with HBsAg loaded PLGA 50:50 and 75:25 microspheres formulations as compared to alum-adsorbed HBsAg ($P < 0.001$). SI was found to be more for PLGA (50:50) microspheres than PLGA (75:25) microspheres ($P < 0.01$) (Fig. 4(c)).

3.3.4. Nitric oxide release

Peritoneal macrophages of HBsAg loaded PLGA (50:50 and 75:25) microspheres immunized animals released higher NO as compared to alum-adsorbed HBsAg ($P < 0.001$ and $P < 0.01$ respectively). NO release was found to be comparatively more for

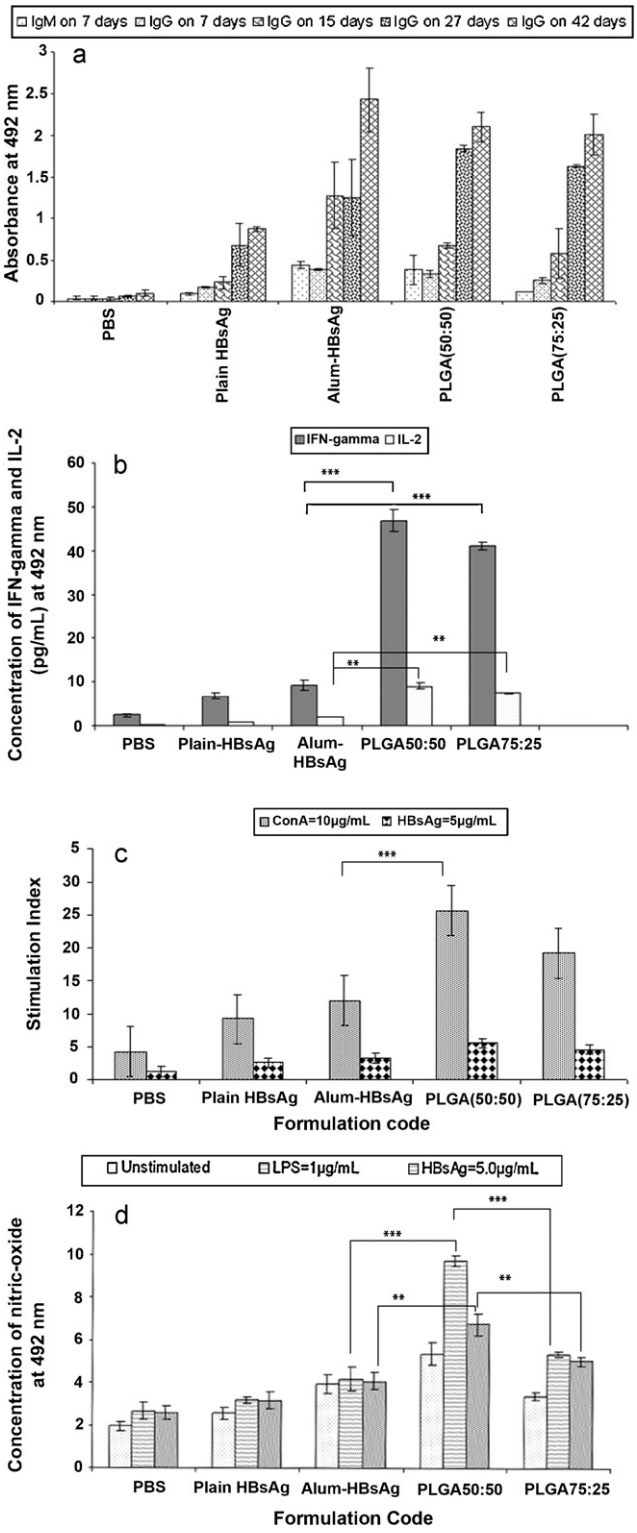

Fig. 4. (a) Serum anti-HBsAg IgM level of mice immunized with different formulations. Serum anti-HBsAg IgG levels of mice immunized with different formulations of recombinant hepatitis B surface antigen on days 7, 15, 27 and 42. (b) Level of cytokine (IFN-γ and IL-2) release in spleen cells from animals immunized with different formulations of recombinant hepatitis B surface antigen on day 42 after primary immunization. (c) Proliferative responses (stimulation index) of cells from animals immunized with different formulation of recombinant hepatitis B surface antigen *in vitro* stimulated with different concentration of Con A (10 μg/ml) and HBsAg (5.0 μg/ml). (d) Nitric-oxide release in response to LPS and HBsAg challenge *in vitro* in peritoneal macrophages of immunized animals. Values are mean ± SD of data from 5 to 6 animals in two experiments.

PLGA (50:50) rather than PLGA (75:25) microspheres formulation (Fig. 4(d)).

4. Discussion

The adjuvanticity of HBsAg adsorbed different PLGA microsphere formulations was investigated. Various factors including the surface morphology, particle size, polymer composition, viscosity, molecular weight of the polymer and degradation rate of the polymer etc. have been shown to affect the release and immunogenicity of the antigen from PLGA microspheres (Feng et al., 2006; Panyam et al., 2003). Size is one of the important factors for releasing the HBsAg antigen and it finally affects the immunogenicity (Men et al., 1997; Gupta et al., 1997).

The degree of adsorption and the formation of peptide/protein multilayer around PLA/PLGA microparticles are directly related to the hydrophobicity and the concentration of both the polymer and the peptide (Witschi and Doelker, 1998a, b). It is well known that the protein molecules adsorbed directly on the PLA substrate are strongly retained (for at least 4 week) by hydrophobic interaction while the protein–protein interactions in the outer layers are electrostatically controlled and therefore are more sensitive to changes in pH and ionic-strength (Coombes et al., 1999). The higher HBsAg adsorption observed in the present study might be due to its smaller size as compared to BSA (data not shown for BSA).

Despite an increased level of adsorption, extensive HBsAg release occurred from PLGA microspheres over 24 h in PBS at 37 °C indicating the electrostatic nature of HBsAg substrate binding. 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. More protein adsorption and fast desorption on PLGA 50:50 microspheres than PLGA 75:25 microspheres (degrade slowly) might be due to more lactide content of PLGA 75:25 microspheres (Coombes et al., 1996).

Stability in terms of denaturation or deactivation of biological materials/immunological products particularly antigen are very important parameters as they lead to generation of insufficient protective immune response in the subject against the disease. The polymers selected for controlled vaccine formulation are easy to process and stable *in vivo* as SDS–PAGE confirmed retention of adsorbed antigen. The results of the present study indicated that the integrity of antigen was unaltered after adsorbing HBsAg onto microspheres. Rate of degradation of PLGA 50:50 microspheres was found more than PLGA 75:25 microspheres. Glycolic acid, known to render the polymer more hydrophilic, results in a higher water uptake and consequently leads to a lower Tg (glass transition point) (Witschi and Doelker, 1998a, b). During *in vitro* release from W/O/W microspheres, peptide degradation was increased with increasing polymer hydrophilicity and decreasing polymer molecular weight (Witschi and Doelker, 1998a, b) supports faster polymer erosion from PLGA (50:50) microspheres in our studies.

To overcome the problems of HBsAg degradation during microencapsulation and to enhance the amount of HBsAg immediately available to antigen presenting cells (APCs) after cellular uptake of microspheres, we have adopted the strategy of presenting HBsAg on the surface of microspheres. To achieve this, microspheres were prepared that displayed a positive surface charge for HBsAg adsorption, through the inclusion of cationic surfactants in the preparation process. Microspheres play an important role in the interaction with phagocytic cells. Particles smaller than 10 μm injected through subcutaneous route are transported by phagocytic 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 indicated that presence of surface antigen on PLGA microspheres could be important for the induction of strong and long lasting immunity (Rafati et al., 1997). In the present study immune response of mice immunized with HBsAg adsorbed on the microspheres provided support of the above findings. It is well known that once a primary response is generated, a small quantity of lamellae-adsorbed antigen presentation is sufficient to maintain high level of circulating antibodies for extended time periods (Coombes et al., 1999).

In the present study antibody response was produced against different HBsAg adsorbed PLGA microspheres which were given once only, was comparable to that response produced by the alum adsorbed HBsAg formulation, indicates strong adjuvant activity of PLGA microspheres. It has been reported that adjuvant may exert immunomodulatory effect via a number of mechanisms including depot formation at the injection site resulting in slow antigen release, stimulation of macrophages and other immune competent cells, activation of compliment system, stimulation of cytokine release and efficient uptake of smaller sized particles (at least less than 10 μm) by antigen presenting cells (Coombes et al., 1999). Lavelle et al., suggested that enhanced phagocytosis of antigen loaded particles led to overloading of phagocytic vesicles which allowed some of the antigen to access the cytoplasm where it could associate with newly synthesized MHC class I molecule. The shift in T-cell response from Th2 to Th1 by administration of microspheres associated antigen might have facilitated uptake and presentation of particulate antigen by phagocytic cells which are considered as antigen presenting cells for Th1 responses (Lavelle et al., 1999).

IgM accounts for 5–10% of the total serum immunoglobulin. It is the first immunoglobulin class produced in a primary response to an antigen, and it is also the first immunoglobulin to be synthesized by the neonate. Because of its high valency, pentameric IgM is more efficient than other isotypes in binding antigens with many repeating epitopes such as viral particles and red blood cells (RBCs). It takes 100–1000 times more molecules of IgG than of IgM to achieve the same level of agglutination. A similar phenomenon occurs with viral particles, less IgM than IgG is required to neutralize viral infectivity. Because of its large size, IgM does not diffuse well and therefore is found in very low concentrations in the intercellular tissue fluids (Goldsby et al., 2007). This statement supports the low concentration of IgM in our studies.

Adjuvants not only have the ability to selectively modulate the humoral/cellular immune responses, but also increase the MHC class I or II presentation (Goldsby et al., 2007). The presence of the cationic surfactant (CTAB) on the surface of microspheres also makes an additional contribution to the mechanism, because they may contribute to disruption of endosomes and the release of HBsAg into the cytoplasm. However, this hypothesis remains to be proven and further studies are necessary (Singh et al., 2000).

Venkataprasad et al., reported that IFN-γ and IL-2 play an important role in the protection of hepatitis B viral infection (Venkataprasad et al., 1999). In our study we have found up regulation of IFN-γ and IL-2 in mice immunized with both microspheres HBsAg formulations. These findings suggest that microspheres adsorbed with HBsAg may be useful for inducing Th1 response for complete clearance of the HBV infection. However, immune response produced by the currently used alum adsorbed HBsAg vaccine biases towards Th2-type response. In our study we found no correlation with T-cell proliferation to whole antigen and the magnitude of the anti-HBs antibody response, or the vaccine dose. This reflects a combination of the low frequency of T-cells in the periphery and the efficiency of our system (McDermott et al., 1999). For microspheres based vaccines, MHC antigen-presentation pathways represent a major challenge because the

particles are generally believed to be phagocytosed and degraded in the phagolysosomes and not in the cytoplasm. Consequently, the antigen would not be available for association with the endoplasmic reticulum (ER) and reaction with MHC class I molecules. Nevertheless, MHC class I restricted CTL responses have been observed after *in vivo* treatment with exogenous antigens, even when entrapped in microspheres. Such antigen has been postulated to occur upon release of the exogenous antigen from the phagosome into the cytosol, within early vacuolar compartments or by the direct binding of released antigen to MHC class molecules on the cell surface (Johansen et al., 2000).

A vaccine designed to stimulate the clearance of the virus in chronic HBV infection should stimulate the Th1 component of the immune response (Elias et al., 2005). As shown in the present study, the microspheres based vaccine formulation was able to induce an equilibrated Th1/Th2 response as shown by higher stimulation index (T-cell proliferation) induced by PLGA microspheres HBsAg formulation. In the present study both high Th1 cytokines are indicative of the strong cell mediated immune response elicited by this delivery system. Thus generation of a dominant Th1 cytokine profile may be expected to facilitate elimination of HBV infection and can be utilized for therapeutic purpose in HBV chronic carriers (Jaganathan and Vyas, 2006).

Other mechanism involved in removal of hepatitis B viral load is reactive nitrogen intermediates that are mediated by IFN-γ (Guidotti et al., 2000). IFN-γ plays a critical role in destroying intracellular pathogens through the production of pro-inflammatory mediators such as nitric oxide NO (Eallen and Loke, 2001). NO a pleiotropic free radical with antiviral activity is produced in the liver by the inducible nitric-oxide synthase (iNOS) enzyme. Inducible forms of this enzyme are present in the macrophages and hepatocytes and synthesize high amount of NO upon activation by cytokines such as IFN-γ, TNF-α, and IL-2. One of the most critical functions of a type 1 immune response is the activation of macrophages by interferon IFN-γ. In the present study significant increase in IFN-γ and NO due to microspheres HBsAg formulations supports that IFN-γ and NO mediated role may be useful for suppressing viral infection (Guidotti et al., 2000).

Safety of cationic microspheres for human use should be evaluated. Although PLA/PLGA polymers previously have been widely used in biomedical applications, including the preparation of several drug delivery systems, the effect of inclusion of CTAB in the microspheres needs to be evaluated. Nevertheless, CTAB previously has been widely used in biomedical applications, including use as an antibacterial agent in eye drops. Singh et al., 2000 reported that a low dose of cationic microparticles used for DNA vaccination was found to be safe in guinea pigs. During our experiment all the animals were found to be healthy which indicates that at low concentration (0.2% w/v) CTAB was nontoxic. However, toxicity of cationic microparticles needs further evaluation. The results indicate that the present formulations have potential application in the field of vaccine delivery and could be an appropriate choice for the development of a single-dose vaccine against hepatitis b.

5. Conclusions

The present study carried out with formulations in Balb/c mice showed that a single injection of polymeric microspheres formulations resulted in good humoral as well as cellular immune response, which mimicked booster injection of alum adsorbed HBsAg vaccine. Safety of carrier formulations is essential to justify the further development of cationic microparticulate vaccines. Therefore toxicity issues have to be addressed in future experimental setup before cationic microparticles get due recognition as a single-shot carrier adjuvant for the development of vaccines for other diseases.

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