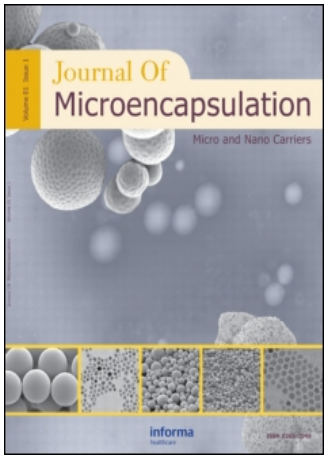

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Enhancement of immune response of HBsAg loaded poly (L-lactic acid) microspheres against Hepatitis B through incorporation of alum and chitosan

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Enhancement of immune response of HBsAg loaded poly (L-lactic acid) microspheres against Hepatitis B through incorporation of alum and chitosan

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Abstract

Purpose: Poly (L-lactic acid) (PLA) microparticles encapsulating Hepatitis B surface antigen (HBsAg) with alum and chitosan were investigated for their potential as a vaccine delivery system.

Methods: The microparticles, prepared using a water-in-oil-in-water (w/o/w) double emulsion solvent evaporation method with polyvinyl alcohol (PVA) or chitosan as the external phase stabilising agent showed a significant increase in the encapsulation efficiency of the antigen.

Results: PLA-Alum and PLA-chitosan microparticles induced HBsAg serum specific IgG antibody responses significantly higher than PLA only microparticles and free antigen following subcutaneous administration. Chitosan not only imparted a positive charge to the surface of the microparticles but was also able to increase the serum specific IgG antibody responses significantly.

Conclusions: The cytokine assays showed that the serum IgG antibody response induced is different according to the formulation, indicated by the differential levels of interleukin 4 (IL-4), interleukin 6 (IL-6) and interferon gamma (IFN-γ). The microparticles eliciting the highest IgG antibody response did not necessarily elicit the highest levels of the cytokines IL-4, IL-6 and IFN-γ.

Keywords: *Hepatitis B surface antigen, vaccine, microsphere, poly (L-lactic acid), chitosan, alum, cytokine*

Introduction

Vaccines formulated using polymeric microspheres provide a promising carrier system for delivery of antigens and several investigations have been carried out in recent years into the pharmaceutical potential of such formulations (Langer and Folkman 1976;

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Mathiowitz et al. 1990; O'Hagan et al. 1993a; Alpar et al. 1994; Coombes et al. 1996; Kersten et al. 1996; Singh et al. 2006). Theoretically, single shot vaccines should be able to deliver the vaccine at a pre-programmed rate in a controlled release manner, upon administration of a single dose, thereby conferring lifelong protective immunity (Raghuvanshi et al. 2002). Moreover, they should be stable and highly immunogenic. To achieve these objectives, several potential approaches have been explored and current research focuses on the use of biodegradable polymers in conjunction with alum to formulate antigen encapsulated microspheres. Microencapsulated antigens are capable of eliciting both humoral and cellular immune responses in naïve animals, probably as a result of targeting of the antigen to antigen presenting cells upon release from the microspheres (Johansen et al. 2000). By careful optimisation of process variables, antigens can be encapsulated in polymer microspheres and, by tailoring parameters such as degradability of the polymer, microsphere size and loading capacities, the microspheres can be formulated to release antigen over extended periods (Gilding and Reed 1979). Poly-(L-lactic acid) (PLA) is a biodegradable polymer that possesses the ideal qualities of a polymeric biomaterial, i.e. biodegradability and biocompatibility, and has proven safety and a non-toxic profile (Gilding and Reed 1979). Encapsulation studies using PLA for formulating microspheres for vaccine delivery have included several antigens including HBsAg (Singh et al. 1997) and PLA microspheres have been found to provide adjuvant activity in inducing immune responses (Raghuvanshi et al. 2002). Antigens such as tetanus toxoid (TT), *Yersinia pestis* V antigen and HBsAg have been encapsulated previously in PLA microspheres (Gilding and Reed 1979; Nellore et al. 1992; Almeida et al. 1993; Raghuvanshi et al. 2002; Eyles et al. 2003) and these have provided encouraging results. The main problems that have been faced are antigen degradation during processing, low protein loading in the microspheres and generation of relatively weak immune responses. The incorporation of adjuvants to enhance immune responses has been investigated and a number of immunostimulatory adjuvants evaluated (O'Hagan et al. 1993b; Diwan et al. 2001; Raghuvanshi et al. 2002; Tobio et al. 2002; Bramwell et al. 2003; Moschos et al. 2006). Aluminium hydroxide (alum) has been widely used as an adjuvant and is in use currently an adjuvant in many human vaccine formulations (Iyer et al. 2004; Moschos et al. 2006). The mechanism of enhancement of immune responses by alum is due to the triggering of humoral immunity by selective stimulation of type 2 T-helper (Th2) responses (Cox and Coulter 1997). The effect of incorporation of alum in TT encapsulated PLA (45 kDa) microspheres was studied by Raghuvanshi et al. (2002). Results of this study indicated that alum adsorbed TT microspheres elicited significantly higher anti-TT antibody titres, as compared to plain TT microspheres. In the case of the HBsAg, there is substantial published data that demonstrates that there is considerable potential for the development of single dose HBsAg vaccines using controlled release biodegradable polymeric microspheres and there is extensive information about the release kinetics of the encapsulation (Nellore et al. 1992; Singh et al. 1997; Shi et al. 2002). However, very little has been reported on the effect of co-encapsulation of alum and antigen on the physicochemical properties of the microspheres and immune responses. In view of this, the overall purpose of this study was to study the effect of co-encapsulation of alum adsorbed HBsAg on microsphere characteristics such as size, surface charge, loading level, as well as on immune responses. HBsAg encapsulated PLA microspheres were prepared by a conventional double emulsion solvent evaporation technique and characterisation of physiochemical properties of the encapsulated microspheres were then carried out, followed by comparison of immune responses among encapsulated alum adsorbed HBsAg, HBsAg in plain PLA microspheres and HBsAg in chitosan coated microspheres in the mouse model.

Materials and methods

Materials

Hepatitis B surface antigen (HBsAg) in phosphate buffer at a concentration of 1.68 mg mL^{-1} was provided as a gift by Shantha Biotechnics (India). The antigen was a recombinant yeast derived HBsAg from portions of a commercial batch (Lot #19 D04). Poly (L-lactic acid) (LACTEL®, 147 kDa,) was from Birmingham polymers Inc (USA). Alum in the form of aluminium hydroxide gel (3% m/v) was obtained from Superfos Biosector (Denmark). Chitosan (high viscosity) was from Fluka (Germany). RPMI and foetal bovine serum were from Gibco (UK). L-glutamine and penicillin/streptomycin were from Sigma (UK). Horseradish peroxidase conjugated goat anti-mouse isotype specific immunoglobulin was from Oxford Biotechnology (UK). Polyvinyl alcohol (87–89% hydrolysed, 13–23 kDa) was from Sigma-Aldrich (UK). QuantiPro Bicinchoninic acid assay kit was from Sigma (UK). SilverQuest® silver staining kit was from Invitrogen (USA). DuoSet sandwich ELISA kits were from R&D Systems (UK). 2,2′ azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) was from Sigma (UK), dichloromethane was from BDH laboratory suppliers (UK). All other reagents used were of analytical grade.

Methods

Preparation of microspheres. HBsAg-loaded microspheres were prepared by a *w/o/w* emulsification solvent evaporation method, this technique has been used extensively for microencapsulation of many antigens including HBsAg (Yan et al. 1994; Shi et al. 2002). The theoretical loading of HBsAg for all formulations was 1% m/m of the polymer. Briefly, PLA was dissolved in 4 mL dichloromethane. HBsAg was mixed with 0.5 mL of aqueous solution containing 10% m/v PVA. In the case of the alum containing formulation, the antigen was initially adsorbed to aluminium hydroxide gel (3% m/v) and then mixed with 0.5 mL of 10% m/v PVA. The primary emulsion was formed by dispersing the two immiscible solutions using an ultra-turrax (T25 Janke & Kunkel, IKA-Labortechnik, Germany) for 2 min at 24 000 rpm. This emulsion was added dropwise to 30 mL of 1.25% m/v PVA solution in deionized water or 0.75% m/v chitosan solution in 1% acetic acid and homogenised for 6 min at 10 000 rpm with a high shear homogenizer (Silverson L4RT, Silverson Machines, UK). The resultant *w/o/w* emulsion was magnetically stirred at room temperature overnight to evaporate the organic solvent. The microspheres were collected by centrifugation (Beckman J2-21, USA) for 15 min at 20 000 rpm and washed once with deionised water. The resultant suspension was freeze-dried (Virtis, UK) to obtain a free flowing powder.

Characterization of microspheres. Particle size analyses were performed on microsphere formulations using a Malvern Mastersizer 2000 (Malvern Instruments, UK). Three measurements were performed on each sample. The results were analysed by the Fraunhofer model, mean volume diameters (MVD) and standard deviations (SD) were calculated.

For morphology and surface characteristics, prepared microspheres were coated with gold in a 20 nm thickness using a sputter coater (Emscope SC500, Ashford, UK) under vacuum in an argon atmosphere. The surface morphology of the microspheres was then studied by scanning electron microscopy (SEM) (Philips/FEI XL30, Philips Co, The Netherlands).

The zeta potential of microspheres was determined using an electrophoretic light-scattering technique (ZetaMaster, Malvern Instruments, UK). The microspheres

were dispersed ($2\,mg\,mL^{-1}$) in 10 mM potassium chloride solution. This dispersion was then added to the ZetaMaster electrophoresis cell, the electrophoretic mobility was measured and the data converted to zeta potential values. All analyses were carried out in triplicate.

Actual drug content and encapsulation efficiency. Actual drug content (AC) and encapsulation efficiency (EE) of microspheres were determined by using QuantiPro BCA Assay Kit which can detect 0.5–30 $\mu g\,mL^{-1}$ of protein. In brief, 5 mg of the microspheres were digested in 2 mL of 5% m/v sodium dodecyl sulphate (SDS) in 0.1 M sodium hydroxide solution. The amount of antigen was determined as a percentage according to the manufacturer's instructions. AC and EE were calculated using the equations (1) and (2). All analyses were carried out in triplicate and blank microspheres were used as control.

$$\mathrm{AC}(\%) = \frac{M_{\mathrm{act}}}{M_{\mathrm{ms}}} \times 100 \tag{1}$$

$$\mathrm{EE}(\%) = \frac{M_{\mathrm{act}}}{M_{\mathrm{the}}} \times 100 \tag{2}$$

where M_{act} is the actual HBsAg content in weighed quantity of microspheres, M_{ms} is the weighed quantity of powder of microspheres and M_{the} is the theoretical amount of HBsAg in microspheres calculated from the quantity added in the process.

Assessing the integrity of the antigen. Before and after freeze-drying, samples were assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in order to determine the effects of processing conditions on the integrity of the encapsulated HBsAg. Sample preparation was as follows; 5 mg of microspheres was accurately weighed and dispersed in 100 μL phosphate buffer saline (PBS, pH 7.3) in 1 mL eppendorf tubes and then kept overnight at room temperature for agitation on a plate rocker in order to extract the protein from the microspheres. Prior to loading, the samples were vortexed for 30 s to obtain a homogenous dispersion, and then heated at 95°C for 4 min with the 1 mL treatment buffer (950 μL Laemmli buffer + 50 μL β-mercaptoethanol). Twenty microlitres of each of the samples was then loaded onto 10% polyacrylamide gels along with a molecular weight marker (Bio-Rad precision plus protein standards, Bio-Rad Labs, USA) and a standard HBsAg sample. Gels were visualised using the SilverQuest® silver staining kit and imaged using a UVP gel scanning camera.

Immunisation schedule for HBsAg-loaded microspheres. *In vivo* studies were performed in accordance with the Animal Scentific Procedures Act 1986. Twenty-four female BALB/c mice were randomly divided into six groups of four. In the first, second and third groups, antigen-loaded microspheres without alum (F1), with alum (F2) and with chitosan (F3) which are equivalent to 1 μg/50 μL of HBsAg were administered subcutaneously (s.c.), respectively. Alum-HBsAg and Chitosan-HBsAg solutions (equivalent to 1 μg/50 μL of HBsAg) were applied to the fourth and fifth groups, respectively, and free antigen solution (1 μg/50 μL) was applied as a comparison to the sixth group. Each mouse received 50 μl volume of HBsAg-loaded particle suspension or antigen solution alone as appropriate. The animals were boosted on day 21 with microspheres or solutions which were equivalent to 0.5 μg/50 μL of HBsAg. Tail vein blood samples were taken after 15, 60, 250, 300 and 400 days and antibody responses assessed by ELISA.

In vitro restimulation of spleen cells with soluble HbsAg. For the analysis of HBsAg specific cytokines, spleens were harvested upon termination of the experiment and splenocytes isolated into working media (RPMI supplemented with 10% foetal bovine serum, L-glutamine and penicillin/streptomycin). Splenocytes were stimulated with HBsAg ($5\,\mu g\,mL^{-1}$), concanavalin A as a positive control (Sigma, UK) or working media alone for 48 h and supernatants were taken for analysis of IL-2, IL-4 and IFN-γ using DuoSet sandwich ELISA kits in accordance with the manufacturers instructions.

Enzyme-linked immunosorbent assay (ELISA). Antibody responses in serum of the immunized animals were monitored using a microplate ELISA. The 96-well ELISA microtiter plates (Dynatech, UK) were coated with 100 µl per well of a $10\,\mu g\,mL^{-1}$ HBsAg in PBS solution (pH 7.3) overnight at 4°C. The plates were washed three times with 0.05% v/v solution of Tween 20 in PBS (PBST) using an automatic plate washer. The serum samples were serially diluted (1 in 32) in PBS using flat bottom high binding microtitre plates (Immunolon, UK) and 50 µL of each sample was added to each well of the coated ELISA plates. These were covered and incubated for 1 h at 37°C. The plates were again washed three times with the PBST solution. 50 µL of goat anti-mouse horseradish peroxide conjugate, diluted 1 in 1000 using PBS was added to each well and the plates covered and incubated again for 1 h at 37°C. The plates were again washed three times in PBST and 50 µL of the substrate (2,2′azino-bis) 3-ethylbenzthiazoline-6-sulphonic acid (ABTS), at a concentration of $600\,\mu g\,mL^{-1}$ in a citrate buffer plus 7.5 µL hydrogen peroxide per 50 mL, was added to each well and incubated at 37°C for 20 min for colour development. The optical densities of the plates were then read at 405 nm using the Dynax MRX microplate reader (Dynax Technologies, UK). End-point titres were expressed as the last dilution, which gave an optical density at 405 nm above the optical density of negative control after the 30 min incubation.

Results and discussion

Preparation and characterisation of the microspheres

The fundamental requirements for the success of the use of biodegradable polymers for delivery of vaccine antigens are that the microspheres should efficiently entrap (or adsorb) the antigen; they should be in a suitable size range and possessing appropriate surface properties to interact with and be taken up by antigen presenting cells. This depends upon careful optimisation of process parameters and judicious selection of polymer and adjuvants.

In this study, the formation of chitosan or alum co-encapsulated microparticles was achieved by a *w/o/w* double emulsion solvent evaporation method that involved the incorporation of either the positively charged polysaccharide or the negatively charged alum in the microparticle formation medium. PLA was selected for the formulation of microspheres for this study as there have been several previous studies in which it was shown that PLA was a suitable polymer for encapsulating a variety of proteins (Coombes et al. 1996; Lee at al. 1997; Raghuvanshi et al. 2002). Prior to this study a preliminary study was carried out using Bovine serum albumin as a model antigen, the preparation method was therefore standardised and process parameters were optimised for this study (data is not shown). There have been previous attempts at encapsulating the HBsAg in microparticles and HBsAg encapsulated in PLGA microspheres is known to elicit good immune responses in several investigations (Lee et al. 1997; Singh et al. 1997; Shi et al. 2002).

Table I. The composition of microsphere formulations.

Formulation	Antigen	Oil phase (DCM) amount (mL)	Internal aqueous phase (m/v)	External aqueous phase (mL)
F1	HbsAg	5	PVA (10%)	PVA (2.5%)
F2	HBsAg adsorbed alum	5	PVA (10%)	PVA (2.5%)
F3	HBsAg	5	PVA (10%)	Chitosan (0.75%)
F4	HBsAg	10	PVA (10%)	PVA (2.5%)
BF1	–	5	PVA (10%)	PVA (2.5%)
BF2	–	5	PVA (10%)	PVA (2.5%)
BF3	–	5	PVA (10%)	Chitosan (0.75%)
BF4	–	10	PVA (10%)	PVA (2.5%)

The characteristics of the HBsAg encapsulated microspheres fabricated here are summarised in Table I.

The size of the microspheres is an important criterion in the induction of an antibody response, since they are in the size range of 1–2 μm and, therefore, in a range suitable for phagocytosis and uptake by the antigen presenting cells (Kanke et al. 1986; Yamaguchi and Anderson 1993; Morris et al. 1994). The surface morphology of the particles depends upon factors such as the nature of the polymer, concentration of the stabiliser and fabrication parameters (Rafati et al. 1997). All the microsphere preparations produced in this study are smooth and spherical, with no aggregation (Figure 1).

Microspheres have a particle size smaller than 2 μm diameter and the mean particle size of microspheres ranged from 1.33 ± 0.02 to 1.93 ± 0.03 μm for empty microspheres (BF1–BF4) and from 1.13 ± 0.07 to 1.98 ± 0.16 μm for HBsAg encapsulated microspheres (F1–F4). Incorporation of the antigen or the inclusion of alum and chitosan didn't change significantly the particle size and distribution of microspheres ($p > 0.05$) (Table II).

The surface charge in terms of the Zeta potential of the microspheres is an important determinant for the *in vivo* behaviour of microspheres and determines their degree of interaction with the cell surface and their uptake by phagocytotic cells. Surface charges of the microspheres (Table II) were negative for all formulations except microsphere formulations containing chitosan (F3 and BF3). Surface charge values between −19.5 and +27.5 mV for empty microspheres (BF1–BF4) and between −30.6 and +21.5 mV for antigen-loaded microspheres (F1–F4) were measured. Incorporation of the antigen or the inclusion of alum reduced the surface charge of microspheres. Alum adsorbed microspheres displayed a slightly higher negative charge than HBsAg-loaded microspheres, suggesting that the adsorption of the antigen on alum has caused a shift in charge towards negative. Using chitosan as a stabiliser in the external aqueous phase instead of negatively charged PVA significantly increased the surface charge of microspheres when comparing these formulations (F3: +21.5 mV; BF3: +27.5 mV) ($p < 0.01$). The zeta potential values measured, as expected, were positive (due to the amine groups of the chitosan molecule) for the formulations where chitosan was used as the external phase stabiliser. This indicates that the outer surface of F3 and BF3 formulations consist largely of chitosan.

Yield, actual antigen content and encapsulation efficiency

The production yield, actual antigen content and encapsulation efficiency of microspheres are given in Table II.

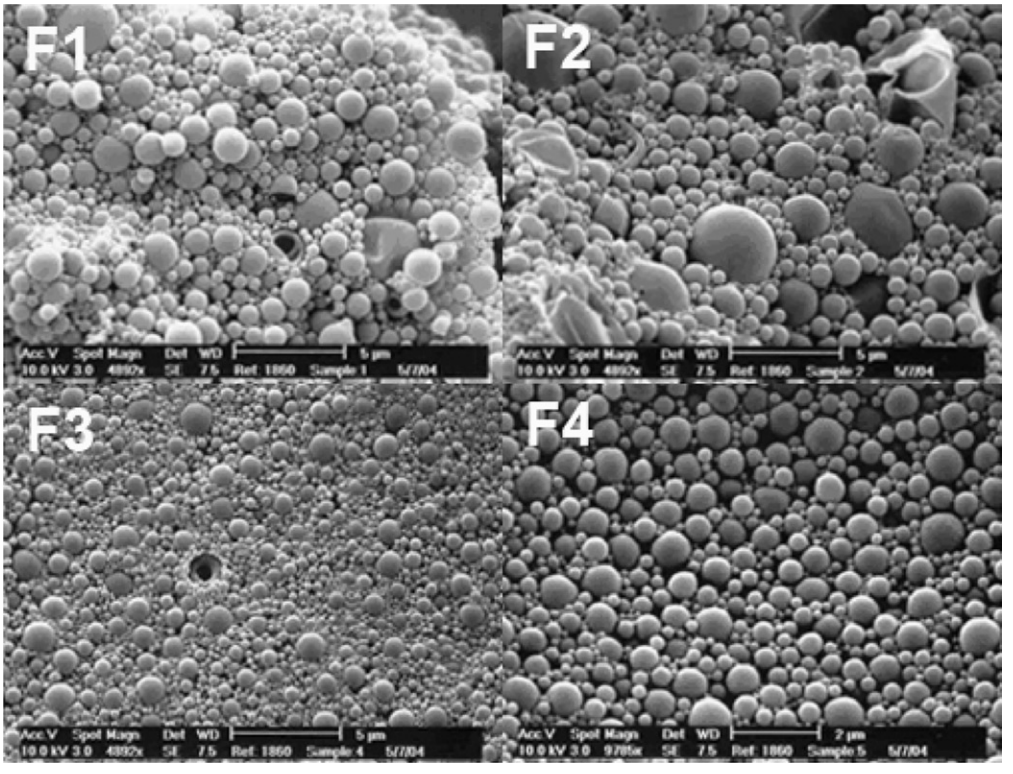

Figure 1. Scanning electron micrographs of the HBsAg encapsulated PLA microspheres prepared using *w/o/w* emulsion solvent evaporation method after freeze drying. F1, F2, F3 and F4 formulations are HBsAg encapsulated PLA microspheres (oil phase amount is 5 mL), alum adsorbed HBsAg encapsulated PLA microspheres, HBsAg encapsulated chitosan coated PLA microspheres and HBsAg encapsulated PLA microspheres (oil phase amount is 10 mL), respectively.

Table II. Production yield, actual antigen content, encapsulation efficiency, mean volume diameter and zeta potential of HBsAg loaded microparticles ($n = 3$).

Formulation	Yield (% ± SD)	Actual antigen content ((μg mg^{-1}) ± SD)	Encapsulation efficiency (%m/m ± SD)	Mean volume diameter (μm ± SD)	Zeta potential (mV ± SD)
F1	62.8 ± 1.12	1.97 ± 0.11	12.34 ± 1.02	1.36 ± 0.02	−23.9 ± 1.0
F2	67.2 ± 1.34	14.62 ± 0.26	98.24 ± 1.28	1.56 ± 0.18	−30.6 ± 3.1
F3	68.8 ± 2.13	12.55 ± 0.21	86.36 ± 2.14	2.12 ± 0.16	21.5 ± 2.2
F4	63.2 ± 2.87	7.11 ± 0.22	44.96 ± 2.09	1.13 ± 0.07	−24.8 ± 1.5
BF1	68.4 ± 3.08	–	–	1.34 ± 0.03	−11.5 ± 2.0
BF2	73.6 ± 5.13	–	–	1.78 ± 0.03	−19.5 ± 0.9
BF3	79.2 ± 3.89	–	–	2.44 ± 0.14	27.5 ± 0.9
BF4	60.8 ± 2.75	–	–	1.33 ± 0.02	−17.8 ± 0.6

The production yield was between 60.8–79.2% for empty particles and 62.8–68.8% for antigen-loaded micropheres. Antigen loading didn't affect the yield of microspheres significantly ($p > 0.05$). In order for the antigen encapsulated microspheres to function as controlled release delivery systems for the generation of long lasting immune responses, it is

necessary that they should contain suitably high levels of the antigen and, for this to take place, it is necessary to achieve sufficiently high protein loading efficiencies. The actual HBsAg content of microspheres, expressed as a percentage of the amount of antigen entrapped in the microspheres, varied between 1.97–14.62 $\mu g\,mg^{-1}$ (Table II). It was found that the antigen content of microspheres which were prepared with alum (F2) ($14.62 \pm 0.26\,\mu g\,mg^{-1}$) and chitosan (F3) ($12.55 \pm 0.21\,\mu g\,mg^{-1}$) were significantly higher than the microspheres containing antigen alone (F1) ($1.97 \pm 0.11\,\mu g\,mg^{-1}$) ($p < 0.01$).

While the encapsulation efficiency of the F1 formulation was $12.34 \pm 1.02\%$, the use of alum (F2) and chitosan (F3) increased the encapsulation efficiency of formulations to $98.24 \pm 1.02\%$ and $86.36 \pm 2.14\%$, respectively. The actual content and encapsulation efficiency can be enhanced significantly by the use of certain excipients that are known to enhance antigen loading (Yan et al. 1994). Raghuvanshi et al. (2002) have demonstrated that single doses of antigen encapsulated PLA particles exhibit markedly enhanced antibody titres when administered with alum. Alum is used as an adsorbent for antigen in the current HBsAg vaccine formulation and adsorbing first might inhibit the loss of antigen that takes place during several stages of the formulation process such as during emulsification as well as washings, during which a significant portion of the unencapsulated antigen is lost. Iyer et al. (2004) have shown that HBsAg adsorbs strongly onto aluminium hydroxide adjuvant by means of ligand exchange between the HBsAg phospholipids and surface hydroxyls of the adjuvant. The adsorption of HBsAg onto alum adjuvant prior to microencapsulation, therefore, significantly enhanced the antigen loading efficiency of the PLA microspheres. In the case of chitosan, this is a positively charged polymer and protonated amine groups of chitosan are able to form complexes with negatively charged surface antigens. It is suggested that, as a result of complexation between chitosan and HBsAg, the encapsulation efficiency of the F3 formulation was significantly increased.

Antigen integrity in the encapsulated microspheres

The structural integrity/stability of the encapsulated HBsAg was analysed by SDS-PAGE. Antigen was extracted from microsphere formulations before and after freeze-drying and run on gels with different amounts of HBsAg standard (24 kDa) and, upon visualisation of the gels, it can be observed that the bands seen for the samples are of the same molecular weight as the standard HBsAg samples (Figure 2). The structural integrity of the antigen is a prerequisite for the antigen to generate a suitable immune response, denaturation or inactivation of the protein during processing causes alteration of the native structure of the protein, indicated by a shift in band formation towards higher or lower M_R structures than the standard (Igartua et al. 1998). This is not seen for any of the samples and, therefore, the structural integrity HBsAg in these microspheres does not seem to be affected by processing and has been retained throughout the encapsulation process and storage.

Evaluation of immune responses

Microencapsulated antigens are capable of eliciting both humoral and cellular immune responses in naïve animals, probably as a result of targeting of the antigen to antigen presenting cells upon release from the microspheres (Johansen et al. 2000). Inclusion of additional adjuvants to improve immune responses from polymeric microspheres has been reported previously (O'Hagan et al. 1993a; Diwan et al. 2001; Tobio et al. 2002) and aluminium compounds as adjuvants have been used in combination with liposomes (Alving et al. 1993) and biodegradable polymers (Diwan et al. 1998). The microencapsulation of alum-adsorbed antigens has also been looked at briefly in order to improve the immunity

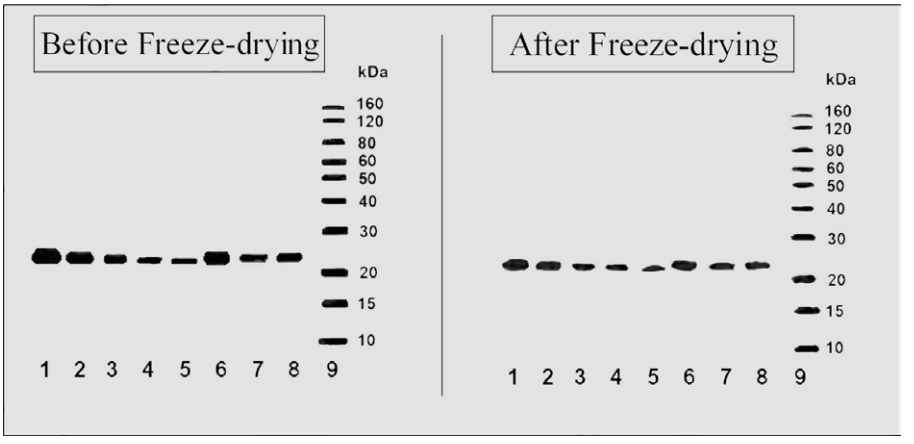

Figure 2. SDS-PAGE analysis of HBsAg following extraction of microspheres taken before and after freeze-drying. Lanes are as follows: (1) HBsAg 50 μg ml^{-1}; (2) HBsAg 25 μg ml^{-1}; (3) HBsAg 12.5 μg ml^{-1}; (4) HBsAg 6.75 μg ml^{-1}; (5) HBsAg extracted from F1; (6) HBsAg extracted from F2; (7) HBsAg extracted from F3; (8) HBsAg extracted from F4; (9) molecular weight marker.

and stabilise the entrapped antigen (Esparza and Kissel 1992; Diwan et al. 1998; Johansen et al. 2000). In addition, alum enhances the hydrophobicity of polymeric microspheres, thereby enhancing uptake by antigen presenting cells and improving the magnitude of the immune response (Raghuvanshi et al. 2002). The enhanced immune responses might be generated as a result of a synergistic effect due to the presence of alum and the PLA microspheres. In a study conducted by Feng et al. (2006) it was shown that a single subcutaneous injection of HBsAg-loaded microspheres with a dose of 7.5 μg of HBsAg per mouse gave comparable serum antibody titres to those elicited by three injections (2.5 μg of HBsAg per dose) of the conventional aluminium adjuvant formulated HBsAg vaccine.

The modification of surface characteristics such as the surface charge and mucoadhesive properties, in the form of chemical modifications of the polymer or in the form of coatings, may profoundly affect the efficiency with which the microparticles target the delivery of bioactive agents to mucosally associated lymphoid tissues and to antigen presenting cells. Many coatings have been used and examples include; chemicals, polymers, antibodies, bioadhesives, proteins, peptides, carbohydrates, lectins (Baras et al. 1999; Delgado et al. 1999; Kaiserlian and Etchart 1999). It is well known that chitosan has mucoadhesive properties and has been used in the past as an adjuvant. Chitosan microparticles have been reported to have immune stimulating activity such as; (i) increasing accumulation and activation of macrophages and polymorphonuclear cells, (ii) suppressing tumour growth, (iii) promoting resistance to infections by micro-organisms, (iv) inducing cytokines, (v) augmenting antibody responses and (vi) enhancing delayed type hypersensitivity and cytotoxic T lymphocyte responses (Ishihara et al. 1993; Tokumitsu et al. 1999). Jaganathan and Vyas (2006) have tested surface modified PLGA microparticles using chitosan chloride *in vivo* and found that these positively charged particles produced humoral (both systemic and mucosal) and cellular immune responses.

In this study, following s.c. delivery of HBsAg encapsulated microspheres (F1, F2 and F3 formulations) and solutions of alum-HBsAg, chitosan-HBsAg and free HBsAg which are equivalent to 1 μg of HBsAg, primary and secondary immune responses were assessed in terms of serum anti-HBsAg IgG levels. Incorporation of chitosan or alum onto/into

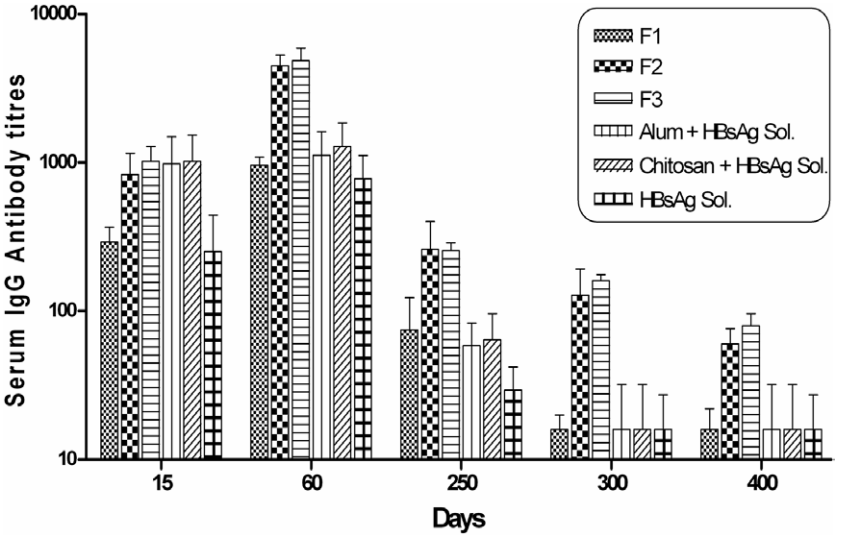

Figure 3. HBsAg-specific serum IgG antibody titres after 15, 60, 250, 300 and 400 days following s.c. delivery of microsphere formulations which were equivalent to 1 μg HBsAg. Mice were boosted with microspheres which were equivalent to 0.5 μg HBsAg on day 21.

microparticles enhanced the serum antibody response to the subcutaneously administered HBsAg. HBsAg-specific IgG antibody titres obtained were significantly higher ($p < 0.05$) for all formulations except F1 than free HBsAg, on the 15th day after immunisation, following one priming dose (Figure 3). The high primary immune responses of the control groups of alum-HBsAg solution and chitosan-HBsAg solution as compared to the microsphere formulations can be attributed to the fact that the microspheres are formulated as controlled release devices and, therefore, the release of the antigen is in a gradual and controlled manner and, therefore, the immune responses too would follow this pattern, whilst in the case of the formulations containing free antigen with alum and chitosan, there would be an enhanced initial response, which may be less likely to be maintained over a longer time scale.

After 60 and 250 days, F2 and F3 formulations displayed enhanced immune responses and antibody titres ~5–6 times higher than PLA microspheres (F1), alum-HBsAg sol, chitosan-HBsAg sol and free HBsAg sol (Figure 3). After 300 days, F2 and F3 formulations gave 8 and 10 times higher antibody titres than other formulations and negative and positive control groups, respectively (Figure 3). The mode of enhancement of immune response to alum adsorbed HBsAg loaded PLA microparticles or PLA-chitosan microparticles may be due to the macrophage activation properties of chitosan or the combination of the depot nature of alum and activation of immune system by chitosan. Thus, the co-encapsulation of alum in microspheres (F2) or the coating of microspheres with chitosan (F3) led to a significant enhancement in the magnitude of the immune response of microspheres, as compared to PLA-HBsAg microspheres.

In vitro *restimulation of spleen cells with soluble HBsAg*

For the analysis of HBsAg-specific cytokines, splenocytes were stimulated with 5 μg mL^{-1} HBsAg. Stimulation with HBsAg successfully elicited IL-4, IL-6 and IFN-γ secretion. Cytokine production was seen to be dependent on the type of adjuvant which had been

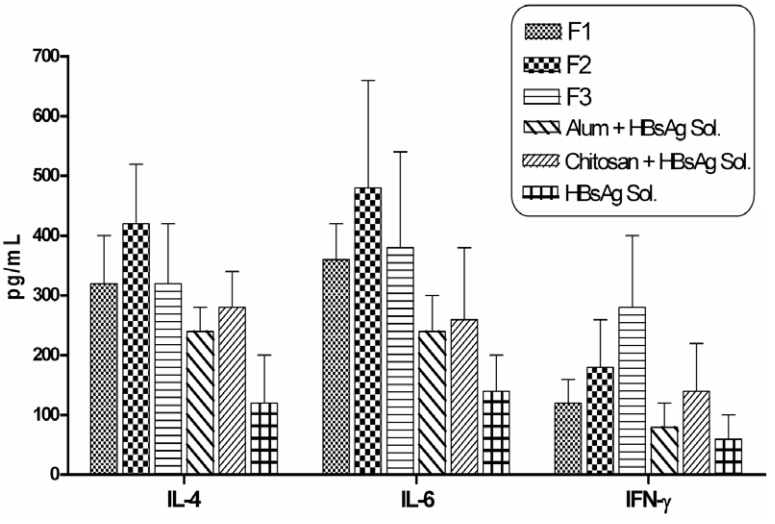

Figure 4. Interleukin 4 (IL-4), Interleukin 6 (IL-6) and Interferon gamma (IFN-γ) production by mixed spleens cells during suspension culture with 5 $\mu g\,ml^{-1}$ concentrations of soluble HBsAg. Cells were derived from immunized mice. Spleens were removed 400 days after s.c. administration of HBsAg in microspheres.

incorporated into/onto particles. These cytokines play potentially important roles in both innate and adaptive immune responses. IL-4 and IL-6 are potentially important cytokines in the Th2 cascade, promoting the proliferation and differentiation of B cells into antibody-producing plasma cells for the production of antibodies. IL-4 promotes IgG_1 and IgE production and decreases IgG_2 and IgG_3 production. IL-6 stimulates immunoglobulin secretion and it can be produced within a few hours of infection by macrophages after ingestion of bacteria (Fraser et al. 1998; Singh et al. 2006). IFN-γ is secreted by effector Th1 cells after appropriate peptidergic stimuli in conjunction with MHC class II (Mosmann and Coffman 1989) and leads via macrophage activation to extensive inflammatory processes that also enable the killing of intracellular pathogens (Bendelac et al. 1997; Singh et al. 2006).

This study shows that the IL-4 and IL-6 responses are higher than IFN-γ responses, indicating a Th2 based response, which can be attributed mainly to the route of administration. Microsphere (F1, F2 and F3) primed spleen cells induced the highest elevated IL-4 and IL-6 productions significantly higher than the solutions of chitosan-HBsAg, alum-HBsAg and free HBsAg primed spleen cells (Figure 4). Incorporation of alum into HBsAg microspheres (F2) has also significantly increased IL-4 and IL-6 production by spleen cells ($p < 0.05$).

The pattern of IFN-γ production by cultured splenocytes was markedly different from those of IL-4 and IL-6, and is shown in Figure 4. The use of chitosan either with microparticles or with free antigen has shown a significant increase in the IFN-γ production by spleen cells when compared with the other formulations.

Conclusion

PLA particles were prepared as microparticulate delivery systems for the delivery of HBsAg by the *w/o/w* emulsion solvent evaporation method with an aim of assessing the effect of the

inclusion of alum as a co-adjuvant and modification of the surface charge of the microspheres by use of chitosan on physiochemical properties of the microspheres as well as potentiation of antibody responses. Particles prepared were found to have comparable uniform size and spherical morphology with high loading efficiencies (where alum and chitosan was used) and retention of antigen integrity. Antigen integrity was also found to be retained following analysis after freeze drying, indicating that by careful optimisation of process parameters and judicious use of polymer and excipients, the structural integrity of the antigen can be preserved during formulation and storage. Characterisation studies indicated that the inclusion of alum or chitosan significantly enhanced the encapsulation efficiency of microspheres. In realisation with the objectives of the study, *in vivo* investigations were carried out to assess the effect of alum or chitosan inclusion in the microspheres upon immune responses. As expected, in analysis of both priming and boosting doses, the highest serum IgG levels are seen in the case of the PLA-HBsAg microspheres, HBsAg-Alum-microspheres and HBsAg-Chitosan-microsphere formulations when compared with the free antigen, free antigen plus alum and free antigen plus chitosan.

Subcutaneous delivery of antigen-loaded microspheres was found to elicit potent primary immune responses in terms of serum IgG, and alum containing HBsAg microspheres displayed higher serum anti-HBsAg IgG titres consistently, demonstrating that admixed alum has a strong impact in enhancing the magnitude of immune responses to PLA microspheres, possibly due to a synergistic immunostimulatory adjuvant effect between the PLA microspheres and alum adjuvant. However, the surface modified microspheres with chitosan have also given slightly higher potency and long lasting immune responses when compared with the alum co-encapsulated microspheres. In addition it was shown that, at least to some extent, these different formulations may have the capacity to alter qualitative aspects of the immune response in terms of antigen specific cytokine responses and, hence, alter the Th1/Th2 characteristics of the immune response. The mode of enhancement of immune response to alum adsorbed HBsAg-loaded PLA microparticles or PLA-chitosan microparticles may be due to the macrophage activation properties of chitosan or the combination of the depot nature of alum and activation of immune system by chitosan.

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