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RESEARCH PAPER

Particle Size Influences the Immune Response Produced by Hepatitis B Vaccine Formulated in Inhalable Particles

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ABSTRACT

Purpose To test the hypothesis that particle size influences the magnitude of immune response produced by hepatitis B surface antigen (HBsAg) encapsulated in poly (lactic-*co*-glycolic acid) (PLGA) microspheres.

Methods Microspheres were prepared by a double-emulsion-solvent-evaporation method, and the particles were characterized for size, morphology, porosity and antigen content. Immunogenicity of encapsulated antigen and safety were studied in rats. Uptake of fluorescent-labeled particles by rat alveolar macrophages was studied by confocal microscopy.

Results With increasing internal aqueous phase (IAP) volume of the microsphere, an increase in particle size and a decrease in particle density were observed. Particles with varying geometric diameters showed aerodynamic diameters between 1 and 6 μ. Addition of poly vinyl alcohol to the IAP resulted in particles with a porous surface. The integrity of HBsAg was maintained upon encapsulation in microspheres. Continuous release of the antigen was observed for formulations incubated in phosphate-buffered saline for 28 days. Immunogenicity increased as a function of particle size upon pulmonary administration. HBsAg encapsulated in ~5 μm particles elicited a significantly higher immune response compared to that encapsulated in ~12 μm particles. Similar to *in vivo* immune response data, fluorescent-labeled microspheres of smaller size were taken up more efficiently by rat alveolar macrophages compared to those of larger size. No significant increase in either tumor necrosis factor alpha level in bronchoalveolar lavage fluid or wet lung weight, indicators of inflammation, was observed in rats that received optimized formulations via the pulmonary route.

Conclusions HBsAg delivered in PLGA microspheres elicited an increase in immunogenicity, and the magnitude of immune response was more pronounced with smaller particles. Inhalable particles of HBsAg could be a viable approach to needle-free vaccination.

KEY WORDS bronchoalveolar lavage · hepatitis B surface antigen · immunogenicity · microspheres · pulmonary delivery

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INTRODUCTION

A variety of polymeric particulate carriers have been studied as substrates for vaccine delivery, including polyanhydrides, hyaluronic acid, chitosan, and poly (lactic-*co*-glycolic acid) (PLGA) (1,2). Importantly, biodegradable PLGA microspheres have shown tremendous promise as an antigen delivery system because they are similar in size to the pathogens that attack the immune system (3,4). Furthermore, PLGA-based particles are efficacious in eliciting immune response similar to universally acceptable aluminum-based adjuvants, and for this reason, PLGA microspheres have even been proposed as an alternative to alum. In fact, the adjuvanticity of PLGA microspheres has been documented in several published reports (5). A single injection of hepatitis B surface antigen (HBsAg) entrapped in PLGA microparticles, for example, elicited an antibody response comparable to that observed with three doses of intramuscular injections of alum-adjuvanated vaccine (6). PLGA-based microparticles have also been studied as carriers for mucosal immunization (7). Recently, PLGA microspheres of HBsAg were shown to induce strong systemic as well as mucosal immune responses after intranasal administration (8).

As a mucosal and noninvasive route of administration, the respiratory tract—including the nasal and pulmonary sites—has been studied extensively for delivery of a variety of vaccines. An intranasal influenza vaccine (FluMist® MedImmuneVaccines Inc, Gaithersburg, MD) is already available commercially. However, the small absorptive surface area of the nose, the mucotoxicity, and pathological conditions in the nose limit the usefulness of this route for drug administration. Being needle-free, the pulmonary route of immunization would facilitate mass immunizations, in addition to offering many immunological benefits. The pulmonary epithelium plays an important role in host defense by removing inhaled pathogens and releasing chemokines and cytokines in the mucous layer covering the airway surfaces. By secreting these mediators, the pulmonary epithelium is capable of recruiting and activating cells of the innate immune system, killing pathogens and initiating an adaptive immune response (9,10). The presence of two professional antigen presenting cells (APCs)–macrophages and dendritic cells—located in the interstitium of the lungs and the linings of conducting airways is critically important for both innate and adaptive immunity. The large surface area of the lungs also enables extensive interactions between antigens and immunoreactive cells located either in the lumen (mostly macrophages) or embedded in epithelium (mostly dendritic cells) (9,11). Moreover, pulmonary immunization can induce a mucosal immune response by producing secretory immunoglobulin antibodies (sIgA) that can prevent entry of pathogens through mucosal sites (10). Indeed, the immunological features of the respiratory epithelium indicate that the pulmonary route could provide substantial benefits as an avenue for vaccination against many infectious diseases, including hepatitis B, which affects about one third of the world population. Hepatitis B infection remains a major global health problem despite considerable efforts to combat the disease because the currently available vaccines and delivery methods have a variety of limitations: 1) unsafe use of needles during vaccination has been associated with hepatitis B transmission; 2) a cold chain is required for storage and transportation of the vaccines; and 3) the vaccines fail to induce mucosal immunity. In fact, estimates by WHO indicate that ~8 million infections annually result from the unsafe use of needles for vaccination in developing countries (12). Furthermore, currently available intramuscular vaccines use aluminum hydroxide (alum) as an adjuvant, which can cause the formation of nodules, granuloma and erythema at the injection site.

However, very little research has been conducted to examine the feasibility of PLGA-based respirable particles for hepatitis B vaccination. HBsAg encapsulated in PLGA and lipid microparticles has previously been shown to generate increased systemic and mucosal immune responses (10). Nevertheless, there are scant data on the use of PLGA-based particles as a carrier for delivery of HBsAg via the pulmonary route, an attractive means of mucosal immunization. Neither is it known how size and porosity of particulate carriers might influence the immunogenicity of HBsAg administered as an aerosol. Recently, we showed that it is feasible to administer recombinant hepatitis B vaccine formulated in PLGA particles (13) or tetradecylmaltoside (14) via the pulmonary route, and that particle surface charge influences the immunogenicity of hepatitis B vaccine (13). Thus, the present study was designed to test the hypothesis that immune response produced by HBsAg entrapped in PLGA microspheres varies depending on the particle size of the inhaled formulations and the *in vivo* immune response is a function of particle uptake by alveolar macrophages.

MATERIALS AND METHODS

Materials

Poly (D, L-lactide-co-glycolide) polymers, a) 50:50 ratio (inherent viscosity 0.55–0.75 dl/g) and b) 50:50 (inherent viscosity 0.15–0.25 dl/g) having average molecular weights of 43.5 kDa and 4.5 kDa, respectively, were purchased from Durect Corporation (Lactel Absorbable Polymers, Pelham, AL). Hepatitis B surface antigen in phosphate buffer at a concentration of 2 mg/ml was received as a gift from Shantha Biotechnics (Hyderabad, India). Polyvinyl alcohol (PVA) and horseradish-conjugated goat anti-rat IgG were purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO). Fluorescein isothiocyanate-conjugated bovine serum albumin (BSA-FITC) and tetramethylrhodamine isothiocyanate-phalloidin (TRITC-phalloidin) was purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO). DRAQ5 (Biostatus Ltd., Leicestershire, UK) was provided by Dr. Ulrich Bickel of Texas Tech University Health Sciences Center, Amarillo, TX.

Preparation of HBsAg Encapsulated in Porous PLGA Microparticles

Seven sets of PLGA particles of HBsAg were prepared by a water-in-oil-in-water (w/o/w) emulsion and evaporation method. An aqueous solution of HBsAg (internal aqueous phase, IAP) was emulsified in 5 ml dichloromethane (organic phase, OP) containing PLGA polymer (50 mg/ml) by homogenization (Ultra-Turrex T25 basic, IKA, Wilmington, DE) at 15,000 rpm for 3 min. First, three sets of formulations of varying porosity and particle size (MS-1, MS-2 and MS-3 in Table I) were prepared using three different IAP volumes—160 µl, 250 µl and 500 µl. The resulting water-in-oil (w/o) emulsion was then poured into

Table I Composition and Entrapment Efficiency of the Microsphere Formulations

Formulations	IAP:OP:EAP (v/v/v)	IAP composition (w/v)	Inherent viscosity of PLGA 50:50 (dl/g)	Entrapment efficiency (%)	Drug loading (%)
MS-1	0.160:5:25	–	0.55–0.75	38.02	0.12
MS-2	0.250:5:25	–	0.55–0.75	88.45	0.65
MS-3	0.500:5:25	–	0.55–0.75	97.97	1.4
MS-4	0.250:5:25	1% PVA	0.55–0.75	99.03	0.3
MS-5	0.250:5:25	–	0.55–0.75	72.44	0.22
MS-6	0.250:5:25	1% PVA	0.15–0.25	79.2	0.24
MS-7	0.250:5:25	–	0.15–0.25	74.84	0.22
MS-8*	0.250:5:25	–	0.55–0.75	NA	NA
MS-9*	0.250:5:25	–	0.55–0.75	NA	NA

IAP Internal aqueous phase, *OP* Organic phase, *EAP* External aqueous phase. MS-1, MS-2 and MS-3 formulations were prepared by increasing the IAP volume by homogenization in the primary emulsion procedure; MS-4, MS-5, MS-6 and MS-7 formulations were prepared by using probe sonifier in the primary emulsification procedure. Average molecular weights of PLGA (0.55–0.75 dl/g) is 43.5 kDa and PLGA (0.15–0.25 dl/g) is 4.5 kDa. *Fluorescent microparticles used in the macrophage uptake study

an aqueous solution of 25 ml PVA (external aqueous phase, EAP) and emulsified by homogenization at 11,500 rpm for 10 min (Table I). The w/o/w emulsion thus obtained was stirred overnight at room temperature to evaporate the solvent, dichloromethane. The polymeric particles were then washed three times and lyophilized to obtain free-flowing powder. An additional four HBsAg-entrapped particles were prepared to investigate the influence of core-modifying agents, use of probe sonication during primary emulsification, and the polymer's hydrophilicity or lipophilicity on particle size, porosity and release properties. For the MS-4 and MS-5 formulations (Table I), we used a fixed volume of IAP (250 µl) in the absence or presence of 1% PVA as a core-modifying agent in the IAP. The primary emulsification of these formulations was performed using a probe sonifier (Branson Sonifier 450, Branson Ultrasonics Corporation, Danbury, CT). Similar to MS-4 and MS-5, the final two formulations (MS-6 and MS-7) were prepared using 250 µl formulations in the absence or presence of 1% PVA, except that the PLGA was more hydrophilic compared to that of the other formulations. The other processing parameters, including homogenization of the secondary emulsions, solvent evaporation, and drying, were similar to those MS-1, MS-2 and MS-3 formulations. A blank microspheric formulation without HBsAg was also prepared. For the macrophage uptake studies, fluorescein isothiocyanate-conjugated bovine serum albumin (BSA-FITC) was encapsulated in porous PLGA microspheres. Fluorescent microspheres of two different size ranges were prepared by varying the energy input during the primary emulsification as well as varying the homogenization speed during the secondary emulsification. The average size of one set of particles was 10–15 µm (MS-8) and that for the other was 4–5 µm (MS-9). All the formulations and the control groups contained 2% sucrose and 2% magnesium hydroxide [Mg $(OH)_2$] in the IAP as protein stabilizers.

Characterization of PLGA Microspheres

A series of experiments was performed to characterize the particles for density, size, surface morphology and antigen content.

Powder Density and Particle Size

Powder density was estimated from the tapped density as described previously (15). An aliquot (100 mg) of microspheres was transferred to a 10 (±0.05) ml graduated cylinder, and the initial volume was recorded. The cylinder containing the microspheres was then tapped 200 times, and the volume of the particles was again recorded. The tapped density of particles (ρ) was calculated from the ratio between sample weight (g) and the volume (ml) occupied after 200 taps. The particle size analysis was performed by using a MasterSizer (Malvern Instruments, Westborough, MA) that uses a laser diffraction technique, and the size of the particles was reported as mean volume diameter.

Aerodynamic Diameters

The theoretical mass mean aerodynamic diameter ($MMAD_t$) of the particles was calculated using the following equation, as reported earlier (15,16):

$$\mathrm{MMAD_t} = \mathrm{d}(\rho/\rho_0 \mathrm{X})^{1/2;}$$

where d is the geometric mean diameter, ρ is the particle mass density, ρ_0 is a reference density of 1 g/cc, and X is the dynamic shape factor, which is 1 for a sphere.

For determining the mean experimental aerodynamic diameter ($MMAD_e$) of the dry powder formulations, an eight-stage Marc-II Anderson Cascade Impactor (Westech Instruments Inc., Marietta, GA) was used. The formulations were fired into the cascade impactor at a flow rate of 28.3 L/min. The amount of formulation deposited at each stage was determined from the weight of glass fiber filter papers used for each stage. All studies were performed in triplicate, and the data thus obtained were plotted on a semi-log graph to calculate the experimental mass median aerodynamic diameter.

Particle Morphology

The morphology and the surface characteristics of PLGA microspheres were studied using a scanning electron microscope (Hitachi S-3400N, Freehold, NJ). For SEM studies, HBsAg-loaded microparticles were mounted on aluminum stubs using self-adhesive carbon disks and then sputter-coated with a conducting layer of gold under argon (Emitech K550X, Kent, UK); the images were taken at a voltage of 9–16 kV.

Entrapment Efficiency

The amount of the antigen loaded in PLGA microspheres was estimated by the bichinchoninic acid assay (BCA) (Pierce, Rockford, IL). Briefly, 10 mg of HBsAg-encapsulated PLGA microspheres were dissolved in 1 ml of acetonitrile, vortexed and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was removed carefully, and the precipitate was re-dissolved in 1 ml of 1% sodium dodecyl sulfate (SDS) solution. The resulting solution was used to estimate the HBsAg antigen content by using the BCA method. Blank microspheres without HBsAg and containing only sucrose and $Mg(OH)_2$ were used as a control. The entrapment efficiency of the formulations was expressed as the percentage of antigen loaded in the microspheres relative to the actual amount of antigen added during preparation of the microspheres. Actual drug loading is a ratio of amount of antigen encapsulated to the amount of antigen and polymer used in the preparation of the microspheres (15).

Determination of the Integrity of HBsAg in PLGA Microparticles by SDS-PAGE

The integrity of HBsAg entrapped in PLGA particles was studied by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) after slight modification of a previously published procedure reported by Pandit *et al.* (17). The antigen was extracted by dispersing particles containing HBsAg in phosphate-buffered saline (PBS), pH 7.4, followed by overnight agitation at room temperature. The following day, supernatant was collected, mixed with treatment buffer consisting of 950 µl of Laemmli buffer and 50 µl of of β-mercaptoethanol, and heated at 95°C for around 4 min. The sample thus obtained was loaded onto a 12.5% polyacrylamide gel. Precision-plus protein standards were used as molecular weight markers (Bio-Rad Laboratories, Hercules, CA). Standard HBsAg as received from the supplier and the concentrated sample of HBsAg used in the preparation of PLGA particles were used as parallel controls. The SDS-PAGE gel was stained with Coomassie-blue to visualize the bands, and images were taken using a Gel Doc system (Bio-Rad Laboratories, Hercules, CA).

In Vitro Release of HBsAg from PLGA Microspheres

The *in vitro* release studies were performed in phosphate buffer. An aliquot (20 mg) of freeze-dried microspheres of HBsAg was suspended in microcentrifuge tubes containing 1 ml of PBS (pH 7.4). The samples were incubated at 37°C under gentle shaking (150–200 rpm), and at predetermined time intervals, the vials were removed from the incubator and centrifuged at 4,000 rpm for 10 min at 4°C. The supernatant was collected, and equal amount of PBS buffer was added to replace the volume, and finally the antigen in the supernatant was quantitated by micro BCA. The *in vitro* release studies were conducted for 28 days, and the release profiles are reported as cumulative antigen released against time.

Pulmonary Immunization Studies

Immunization studies were performed according to our previously published method (13). Prior to the experiment, female Sprague-Dawley rats (Charles River Laboratories, Charlotte, NC) weighing between 150–200 g were anesthetized by an intramuscular injection of an anesthetic cocktail containing xylazine (20 mg/ml) and ketamine (100 mg/ml) and divided into five groups (8–10 rats in each group) to receive the following treatments: MS-2, MS-3 or MS-4 formulation, plain HBsAg administered via the pulmonary route, and intramuscular injection of HBsAg. The formulations used in the *in vivo* studies were selected based on the results of the *in vitro* characterization studies described above. The formulations were prepared by dispersing the particles in 100 µl PBS buffer and administered as aerosolized dispersion at a dose of 10 µg HBsAg per animal. All treatment groups received the dose of antigen on days 0 and 14. The formulations were administered as aerosols to the lungs by a Microsprayer™ attached to a syringe (Penn-Century, Inc, PA) as reported by us previously (13,14). Blood samples were collected from the tail prior to administration of antigen, at 1 week after the first dosing, and then every week for three more weeks.

Serum was separated by centrifugation at 6,000 rpm for 10 min and stored at −20°C for further analysis.

Measurement of Serum Antibody Response

Specific antibodies generated to HBsAg were quantitated by using a commercially available ELISA kit, HBsAb (Immuno Diagnostics, Foster City, CA). The microplates for ELISA were precoated with purified heat-inactivated HBsAg. The samples and the standards were added into the microplate and incubated for 60 min at 37°C. Following incubation, microplates were washed, the enzyme conjugate was added into the wells and again incubated for 60 min at 37°C. The enzymatic reaction was stopped by adding sulfuric acid, and the absorbance was measured in a microplate reader at 450 nm. The kit was standardized in our laboratory to further confirm the quantitation using the standard samples provided in the kit. For analysis, the samples were diluted up to 50 times with the sample diluent provided in the kit.

Particle Uptake by Rat Alveolar Macrophages

Alveolar macrophages were obtained from male Sprague-Dawley rats (Charles River Laboratories, Charlotte, NC) weighing 300–350 g by a modification of the bronchoalveolar lavage (BAL) procedure reported earlier (14,18). The lungs were isolated from anesthetized animals, and lavage was performed by instilling 5 ml calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Grand Island, NY) containing 0.5 mM disodium EDTA (Fisher Scientific, Fair Lawn, NJ). The procedure was repeated until a volume of around 30 ml was obtained. The lavage fluid was centrifuged at 400×g for 10 min to obtain pellets of alveolar macrophages. The cell pellet was resuspended in 500 µl 0.1% BSA (w/v) in Hanks balanced salt solution (phenol red free, pH 7.4, Gibco, Grand Island, NY) and counted in a hemocytometer. A density of 4×10^5 cells per ml was added on a cover slip (12 mm in diameter) placed in a 24-well cell plate. The plate was then incubated for 1 h in a humidified chamber at 37°C in 5% CO_2. Following the incubation, media containing the non-adherent cells were aspirated, and the plates were washed with PBS.

To conduct the uptake study, an aliquot of PLGA microspheres dissolved in medium at a concentration of 1 mg/ml was added into each well and incubated for 1 h in a humidified chamber at 37°C in 5% CO_2. The cells were then washed with PBS and fixed with 3% freshly prepared paraformaldehyde for 15 min at room temperature. The cells were washed again with PBS to remove the paraformaldehyde and permeabilized with 0.2% Triton X-100 for 40 min at room temperature. Following permeabilization, tetramethylrhodamine isothiocyanate-phalloidin (TRITC-phalloidin) was added onto the cover slip to stain the actin cytoskeleton microfilament network of the macrophages and incubated at room temperature in the dark for 90 min. After the incubation period, the cells were washed three times with PBS. Nuclear staining was performed with DRAQ5; cells were incubated with the stain for 10 min according to the manufacturer's protocol followed by three to four washes. The glass cover slips were then carefully removed and placed on a glass slide by using a drop of antifade solution and sealed. Uptake of the fluorescent microspheres by the alveolar macrophages was viewed under a Leica Confocal Microscope (TCS SL; Leica Microsystems, Heidelberg, GmbH) with 63×1.4 oil objective and immersion oil. FITC-BSA was excited with the 488 nm line of the systems argon laser, and TRITC-phalloidin was excited with the 546 nm line of the HeNe laser, whereas the nuclear staining with DRAQ5 was visualized upon excitation at 633 nm. Leica Confocal software, version 2.61 Build 1537, was used for image acquisition and analysis.

Safety of PLGA Microspheres

For safety studies, tumor necrosis factor-α (TNF-α) in bronchoalveolar lavage (BAL) fluid and lung wet weights were measured after administration of the formulations. Formulations that elicited maximal increases in the immune response were used in the safety study. The rats were divided into five groups (4–5 rats in each group) to receive the following treatments: (1) formulation MS-2, (2) formulation MS-3, (3) plain HBsAg, (4) saline and (5) 0.1% SDS. The last two treatment groups, saline and SDS, were used as negative and positive controls, respectively; the dose of the antigen for first three treatment groups was 10 µg per animal. The anesthetized animals received the treatment as 100 µl of aerosolized solution via the pulmonary route on day 0 and day 14. On the 14th day, 12 h after administration of the formulations, the animals were re-anesthetized, and the animal weight was recorded before proceeding to the BAL study. The lungs were surgically removed, lung wet weight was recorded, and the BAL study was carried out as described earlier (14). The lavage fluid obtained was centrifuged at 500×g for 10 min. The TNF-α level was determined in the supernatant using a Rat TNF-α ELISA kit (Bio-Source International, Inc., Camarillo, CA). All animal studies were approved by the Texas Tech University Health Sciences Center (TTUHSC) Animal Care and Use Committee and were conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

Statistical Analysis

One-way ANOVA with a Tukey-Kramer or Newman-Keuls post-test was performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San

Diego, CA). The post-hoc tests were only performed if the results of ANOVA were found to be significant. The Tukey-Kramer post-test was performed to compare data on immunogenicity profiles and TNF-α levels, and the Newman-Keuls test was performed to compare lung wet weight data. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Characterization of Microspheres

We first studied the influence of different process parameters—IAP volume, method of homogenization, presence of core-modifying agents, and hydrophilicity of polymers—on the density, porosity, particle size, entrapment efficiency and release properties of the particles (Table I, Figs. 1, 2 and 3).

Particle Density

The tapped density of first three formulations, MS-1, MS-2 and MS-3, decreased as the IAP volume increased from 160 to 500 µl (Fig. 1A). Further reductions in tapped density were observed when 1% PVA was added to the IAP as a core-modifying agent (MS-4 and MS-6) or when IAP was homogenized by a probe sonifier (MS-5 and MS-7). The reduction in tapped density of the first three formulations (MS-1, MS-2 and MS-3) was possibly due to the formation of internal pores, which made the particles fluffy and lighter. Upon removal of water during drying, interconnected droplets form pores and channels and thereby produce particles of lower density. Compared to the MS-2 formulation, there were reductions in the densities of the MS-4, MS-5, MS-6 and MS-7 formulations though the IAP volume was 250 µl in each case. Both the presence of PVA in the primary emulsion and the use of the probe sonifier may have contributed to the reduction in density of these formulations. PVA is also reported to produce surface pores, which could contribute to reduced density (19–23). Compared to the MS-4 and MS-6 formulations, there was an increase in the tapped density of MS-5 and MS-7 because no PVA was used in the latter two formulations. Apparently, the molecular weight of PLGA was not a factor in determining particle density, as the PLGA used to prepare the MS-4 and MS-5 formulations was nearly ten-fold higher (43.5 kDa) than that used for the MS-6 and MS-7 formulations (4.5 kDa).

Geometric and Aerodynamic Diameters

The particle sizes of all the formulations ranged from ~2 µm to ~12 µm (Fig. 1B). There was a slight increase in

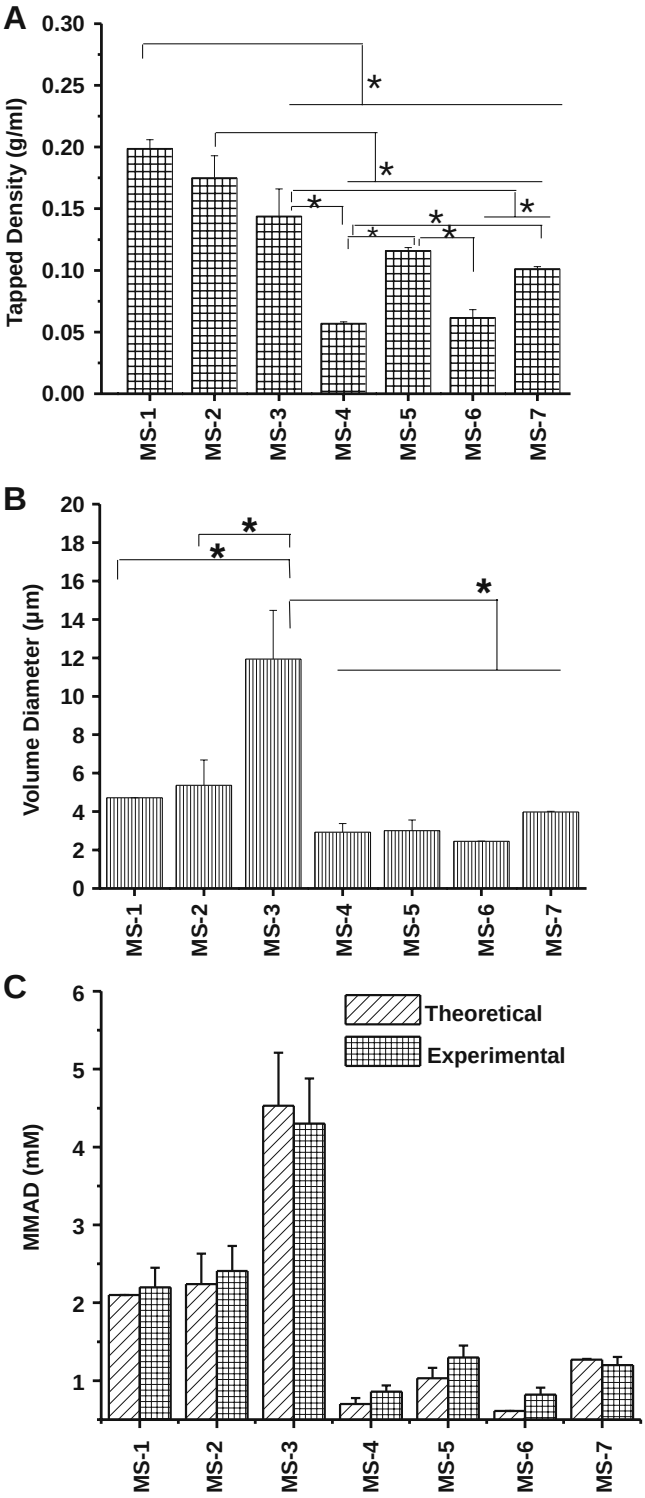

Fig. 1. (**A**) Tapped density, (**B**) particle size of HBsAg-loaded PLGA microspheres and (**C**) theoretical and experimental mass median aerodynamic diameter (MMADt and MMADe). Data represent mean ±SD, $n = 3$. (*Results are significantly different, $p < 0.05$; **Results are significantly different, $p < 0.01$; ***Results are significantly different, $p < 0.001$.)

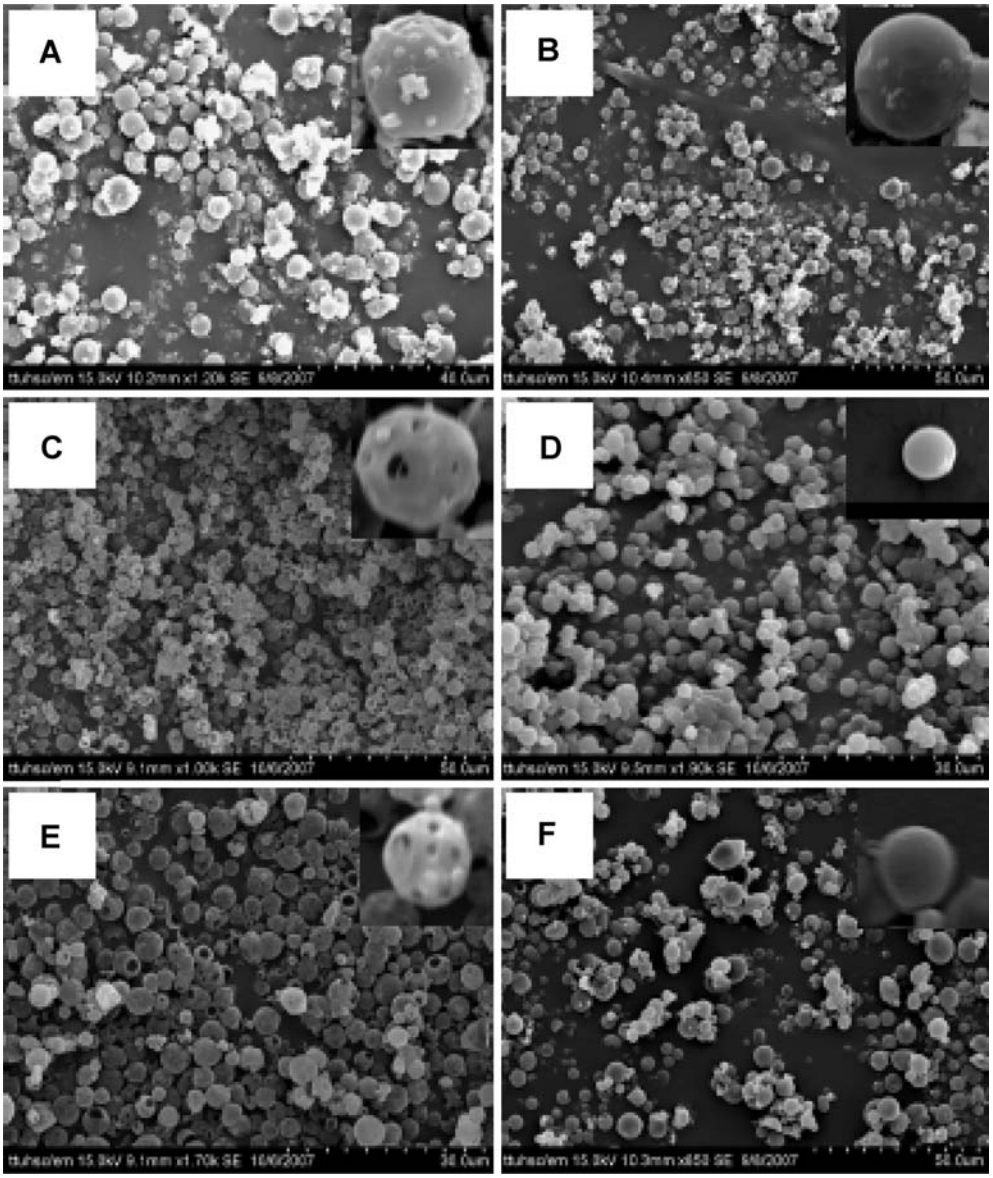

Fig. 2. Surface morphology of HBsAg-loaded porous microspheres; (**A**) MS-2, (**B**) MS-3, (**C**) MS-4, (**D**) MS-5, (**E**) MS-6 and (**F**) MS-7 under the scanning electron microscope. Refer to Table I for composition of the different formulations.

particle size when the IAP volume was increased from 160 µl to 250 µl; however, a significant increase in particle size was observed only when the IAP volume was increased to 500 µl (Fig. 1B). These data are consistent with results of a previous study suggesting that, with an increase in IAP volume, the particle size increases because of the formation of larger aggregates or droplets in the primary emulsion (24). Although geometric particle size was between ~2–12 µ, the theoretical mass median aerodynamic diameters ($MMAD_t$) of all particles were between 1 and 5 µ, suggesting that particles are respirable. To further confirm the results obtained by calculating $MMAD_t$, experimental mass median aerodynamic diameter ($MMAD_e$) was also determined using an eight-stage Marc-II Anderson Cascade Impactor, a widely used technique for simulating particle deposition in lungs. The theoretical and experimental MMAD data presented in Fig. 1C show a pattern similar to that observed for the geometric diameter. Further, MMADe data obtained are in congruence with the $MMAD_t$ data obtained from tapped density measurements. As can be seen in Fig. 1C, $MMAD_e$ increased with increasing IAP volume (MS-3>MS-2 and MS-1) but was still in the respirable range of 1–5 µ. Also, both $MMAD_e$ and $MMAD_t$ decreased when the same formulations were prepared with probe sonication.

Particle Morphology

The morphology of the particles was also influenced by different process parameters. In agreement with the particle size data presented in Fig. 1B, an increase in particle size was observed with increasing IAP volume (Fig. 2A and B).

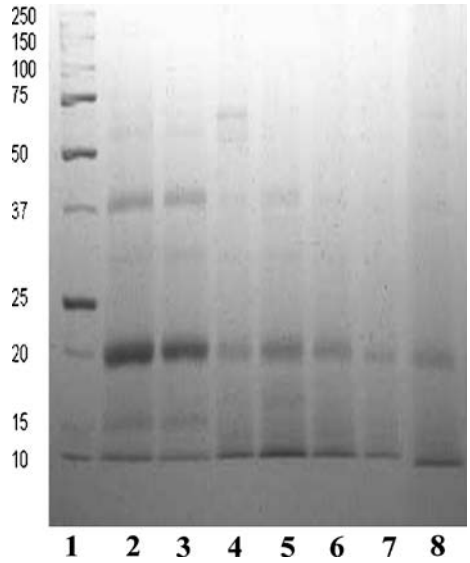

Fig. 3. SDS-PAGE analysis for antigen extracted from the HBsAg-loaded PLGA microspheres (1) molecular weight marker, (2) HBsAg protein, (3) concentrated HBsAg, (4) HBsAg from MS-2, (5) HBsAg from MS-3, (6) HBsAg from MS-4, (7) HBsAg from MS-5, (8) HBsAg from MS-6.

Particles with rough surfaces and small aggregates were observed when the IAP volume was 250 µl. As the IAP volume was increased, the surface of the particles became smoother and thinner, perhaps because of the formation of internal pores (Fig. 2C). Although no surface pores were observed in the MS-2, MS-3, MS-5 and MS-7 (Fig. 2A, B, D and F) formulations, the MS-4 and M-6 formulations showed particles with surface pores. The presence of surface pores in the MS-4 and MS-6 formulations (Fig. 2C and E) was due to incorporation of PVA in the IAP. PVA, a hydrophilic agent, perhaps facilitates rapid evaporation of the aqueous phase, leaving hollow spaces on the surface. Alternatively, PVA may remain at the oil-water interface of the primary emulsion and later partition into the external phase because of its increased hydrophilicity. Such partitioning may contribute to the increased numbers of pores on the particle surface. Particles of the MS-4 and MS-6 formulations were more evenly distributed because PVA produced a more homogenous dispersion of the primary emulsion droplets compared to formulations without PVA.

Antigen Loading and Encapsulation Efficiency of PLGA Microspheres

A high payload of antigens in PLGA microspheres is required to elicit an amplified immune response in a controlled fashion. This experiment was performed to prepare a set of formulations with a maximal antigen load. Previous studies have shown that an increase in IAP volume is accompanied by a reduction in entrapment efficiency because of increased porosity (15). Encapsulated drugs are believed to leach out through these pores during the washing process of microsphere preparation. To offset this limitation, we used increasing amounts of antigen with increasing IAP volume to achieve optimal antigen loading (Table I). The entrapment efficiency (MS-2 formulation) was increased by twofold when antigen in the IAP was increased from 1.2 mg/ml to 1.875 mg/ml; the entrapment efficiency was about 98% (MS-3 formulation) when the antigen concentration was increased to 3.75 mg/ml, suggesting saturation of the particles with loaded antigen. Because there were no dramatic differences between antigen-loading in formulations prepared with 1.875 mg/ml antigen (MS-2) *versus* 3.75 mg/ml (MS-3), we used 1.875 mg/ml to prepare the rest of the formulations in 250 µl of IAP. The entrapment efficiency was increased from 88.45% to 99.03% when PVA was added to the IAP.

Protein Integrity in PLGA Microspheres

It has been reported that the structural integrity of the antigen is essential for the antigen to generate an immune response. The integrity of HBsAg following its extraction from PLGA microspheres (MS-2, MS-3, MS-4 and MS-5) was examined by SDS-PAGE (Fig. 3). Inactivation of the antigen due to processing of the microspheres will be revealed by a shift in the band toward a higher or lower M_R structure as compared to the standard antigen (17,25) . The antigen extracted from all four formulations of PLGA microspheres appeared as bands co-migrating with the standard, plain HBsAg (original and concentrated antigens) used in the preparation of the microspheres, suggesting that the structural integrity of the antigen formulated in microspheres was not adversely affected by the processing parameters. The differences in intensity of the bands observed for each of the formulations were because different amounts of antigen were loaded in the gel.

In Vitro Release of HBsAg from PLGA Microspheres

The *in vitro* release profiles of all formulations except MS-1 were studied; the MS-1 formulation was excluded from the study because of its very low entrapment efficiency. All formulations tested showed a nearly biphasic release pattern: an initial burst release followed by continuous release (Fig. 4A). About 5–7% of the entrapped antigen was released from MS-2, MS-3 and MS-5 formulations during the initial burst phase, whereas 10% underwent burst release from MS-4 (Fig. 4B). The higher burst release of MS-4 is due to the presence of surface pores, as observed in Fig. 2C. The MS-6 and MS-7 formulations showed a significantly higher burst release of around 20–30%,

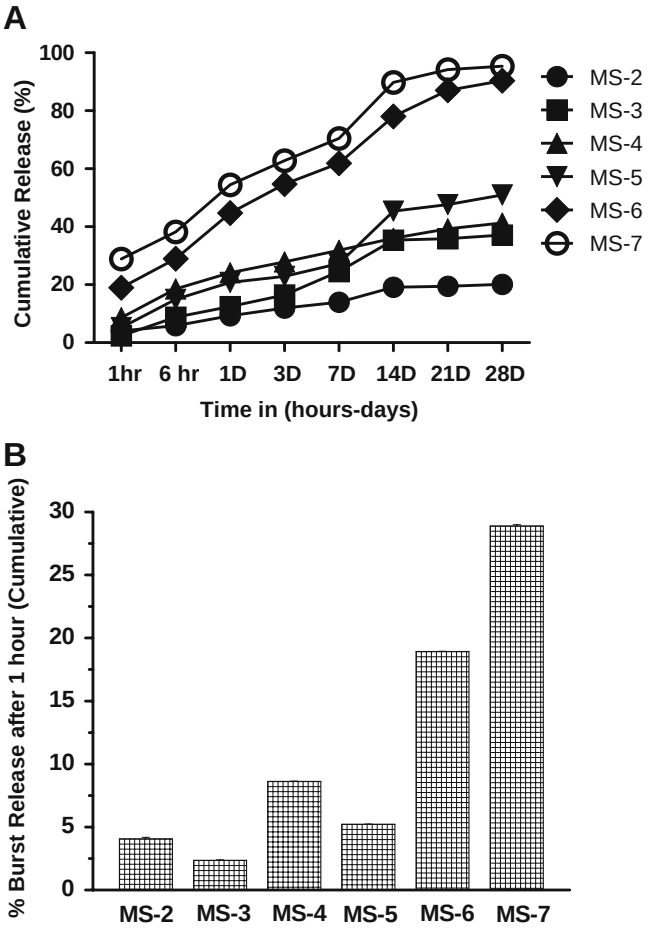

Fig. 4. *In vitro* release profiles (**A**) and percent burst release (**B**) of HBsAg-loaded PLGA microspheres. Data represent mean ± SD ($n = 3$).

perhaps because these microspheres were prepared with a lower molecular weight PLGA, which agrees with earlier reports that suggest that lower molecular weight PLGA has a higher degradation rate because it is more hydrophilic and therefore imbibes more water compared to its larger molecular weight counterpart (26). The MS-6 and MS-7 formulations also showed the greatest increase in total cumulative release. Similarly, because of the presence of surface pores, MS-4 showed a higher cumulative release compared to the MS-2 and MS-3 formulations (Fig. 4). The cumulative release of the MS-2 formulation was slightly lower than that produced by the MS-3 formulation, perhaps because MS-2 had fewer internal pores compared to MS-3. Also, a lower antigen amount could be one of the reasons for the lower total release of antigen from the MS-2 formulation. It is worth noting that the release profiles of the MS-2, MS-3 and MS-5 formulations were slightly different compared to those of the other formulations. The former formulations showed a sharp increase in the release of antigen on day 7 to day 14, followed by continuous release.

In Vivo Immunization Studies

In vivo immunization studies were carried out in female Sprague Dawley rats. A dose of 10 µg per animal was administered to each animal. Based on porosity, particle size and *in vitro* release profiles, three formulations (MS-2, MS-3 and MS-4) were selected for the immunogenicity study. As stated in the Methods section, we used plain HBsAg administered intramuscularly (IM) as a positive control and plain HBsAg administered by the pulmonary route as a negative control. When plain HBsAg was administered by IM injection, a gradual increase in the immune response was observed for 21 days followed by a decline in antibody levels on day 28 (Fig. 5). Plain HBsAg administered via the pulmonary route showed an antibody profile similar to that elicited by IM vaccination, but with a reduced magnitude of immune response (Fig. 5). Unlike plain IM or pulmonary HBsAg administration, microspherically administered antigen showed a continual increase in immune response for 28 days. Of the formulations tested, MS-2 elicited the greatest increase in immune response ($p < 0.01$, when compared to plain HBsAg administered by the pulmonary route; $p < 0.05$, when compared to MS-3 formulation or IM administration of antigen).

Particle Uptake by Rat Alveolar Macrophages

These experiments were performed to further test our hypothesis that size of the PLGA microparticles plays a pivotal role in influencing the extent of the immune response. In order to visualize size-dependent uptake of microparticles by macrophages, the cells were incubated

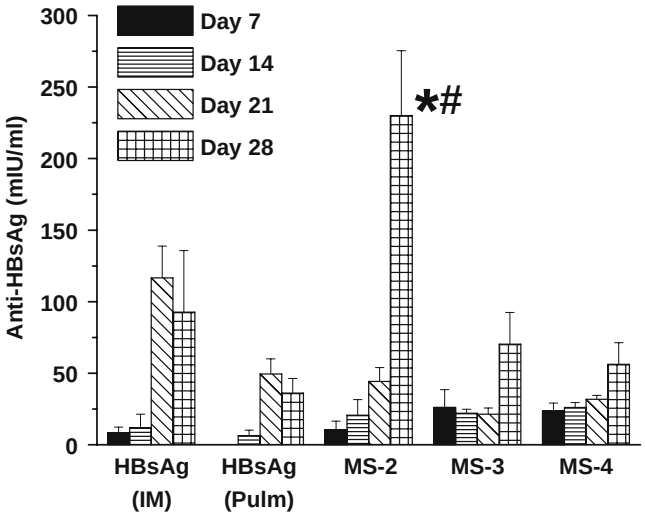

Fig. 5. Immune response profiles after dosing of formulations on day 0 and 14. Data represent mean ± SEM, $n = 5–8$, (*Results are significantly different compared to plain HBsAg by the pulmonary route, $p < 0.01$; # Results are significantly different compared to MS-3, MS-4 and IM plain HBsAg, $p < 0.05$.)

with FITC-BSA-based fluorescent PLGA microparticles of two different sizes: MS-8, 10–15 µm; MS-9, 4–5 µm. These two size ranges were selected on the basis of immune-response studies conducted earlier (Fig. 5). The MS-8 formulation visualized under the confocal microscopy is shown in Fig. 6A. MS-8 (>10 µm) fluorescent microparticles showed no physical uptake by macrophages (Fig. 6B and C) after 1 h of incubation. However, cells showed green fluorescence of BSA-FITC (Fig. 6B, left panel). This fluorescence was because of the release of surface-associated

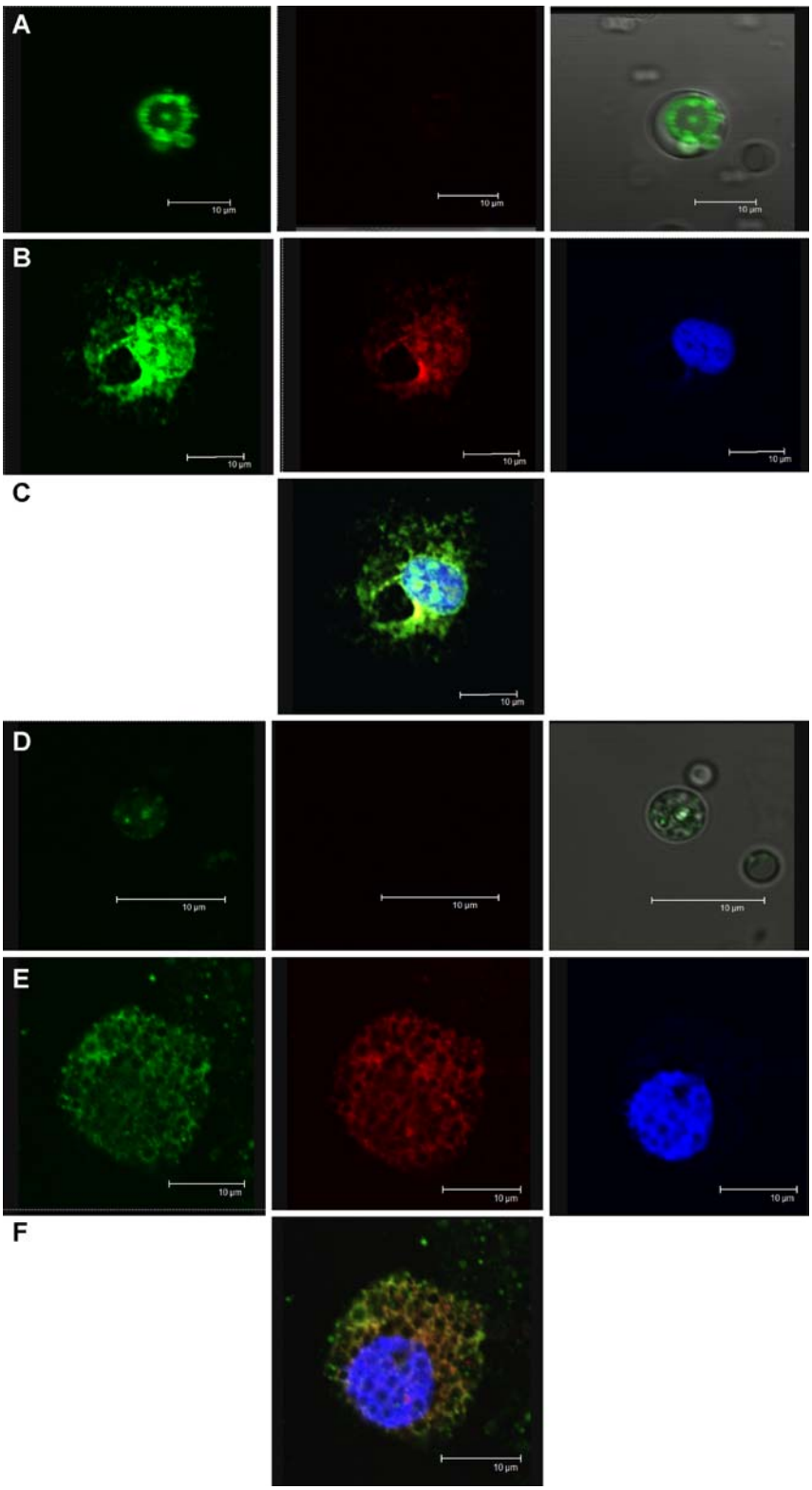

Fig. 6. CLSM images of (**A**) BSA-FITC PLGA microparticles of ~10–15 μm size, (**B**) no physical uptake of microparticles at end of 1 h incubation. The left panel shows slight green fluorescence because of the release of the surface-associated BSA-FITC during the incubation period. The actin cytoskeleton was visualized by labeling with rhodamine-phalloidine (red; middle panel), the microspheres by labeling with FITC (green) and far red fluorescent DNA dye, DRAQ5, was used as a nuclear stain (right panel), (**C**) shows the overlay of particle uptake studies, (**D**) BSA-FITC microparticles of ~4–5 μm. (**E**) The left panel shows enhanced uptake of BSA-FITC (green) microparticles of ~4–5 μm at the end of 1 h incubation. The actin cytoskeleton was visualized by labeling with rhodamine-phalloidine (red, middle panel), far red fluorescent DNA dye DRAQ5 was used as a nuclear stain (right panel) and (**F**) shows the overlay of particle uptake studies.

BSA-FITC during incubation of rat alveolar macrophages with BSA-FITC-encapsulated particles that resemble the burst release profiles of HBsAg-entrapped particle shown in Fig. 4B. Fig. 6D shows the confocal microscopy images of MS-9 (4–5 μm) microparticles. Compared to MS-8 formulation, MS-9 particles clearly showed enhanced phagocytosis by rat alveolar macrophages after 1 h of incubation (Fig. 6E and F). These differences in the uptake of microparticles of two different sizes agree with the immunogenicity data presented in Fig. 5 that show an enhanced immune response with the MS-2 (~5 μm) formulation compared to the MS-3 formulation (~12 μm).

Safety Studies of PLGA Microspheres

The safety of the formulations was studied by recording lung wet weight and measuring tumor necrosis factor-alpha (TNF-α) levels in BAL fluid. As can be seen in Fig. 7A, none of the test formulations (plain HBsAg, MS-2 and MS-3) produced any significant changes in the lung wet weight after the two doses on days 0 and 14; only the positive control, SDS, caused a slight increase in the lung wet weight after administration by the pulmonary route ($p<0.05$). These absences of change in lung weights upon instillation of the formulations suggest that the test formulations did not produce any edema or increase in fluid volume in the lungs. An increase in lung weights would be an indication of lung injury due to the accumulation of extracellular fluid by the respiratory epithelium (14,27,28).

We also measured TNF-α levels in BAL fluid as a marker of inflammation. As shown in Fig. 7B, the positive control, SDS, produced a significant increase in TNF-α levels at the end of 12 h ($p<0.001$). However, when plain HBsAg was administered by the pulmonary route, there was no significant increase in TNF-α level compared to that of the saline control ($p>0.05$). Similarly, the optimized formulations, MS-2 and MS-3, generated no significant changes in TNF-α levels ($p>0.05$). It is important to point out that inflammatory mediators that play important roles in pulmonary host defense include cytokines like TNF-α, IL-12, IL-10. Any form of injury to the respiratory tract will lead to infiltration of cytokines into the circulation or at the site of injury (9). Once the respiratory epithelium encounters a potential irritant, the Toll-like receptor mediates the production of TNF-α and IL-1β (29). That our formulations produced no such increase in TNF-α suggests that their administration via the lungs was relatively safe.

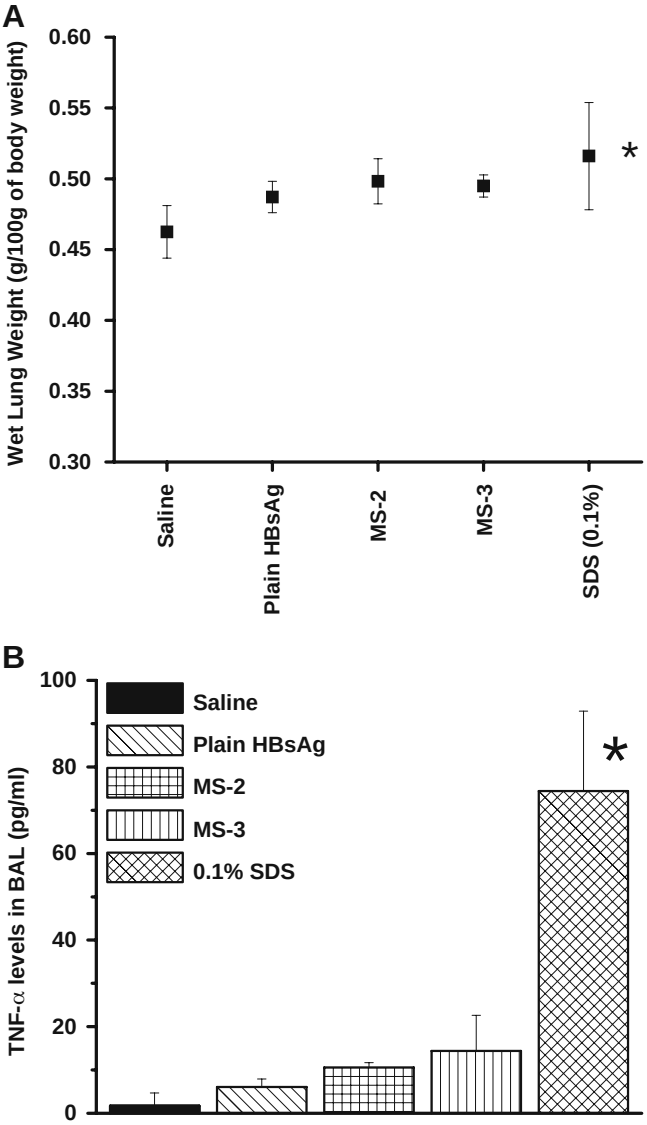

Fig. 7. (**A**) Corrected wet lung weights at 12 h after pulmonary administration of different PLGA microspheres on day 0 and day 14. Data represent mean ± SD, $n=3–5$ (*Results are statistically different from that obtained with saline treated group, $p<0.05$), (**B**) Tumor necrosis factor-α (TNF- α) in BAL fluid 12 h after pulmonary administration on days 0 and day 14. Data represent mean ± SD, $n=3–5$. (*Results are significantly different from that obtained with other treatment groups, $p<0.001$.)

DISCUSSION

In this study, we investigated the influence of size of HbsAg-entrapped PLGA particles on generation of immune response upon pulmonary administration. To this end, we characterized the particles for size, aerodynamic diameter, antigen content and protein integrity. The data on physical characterization, presented in Figs. 1 and 2, reveals that the particle density, size and morphology of PLGA microspheres of HBsAg are significantly influenced by IAP volume, use of surfactant, and method of homogenization. Tapped density, which provides information on the flowability, porosity, size distribution, and inter- and intra-particulate forces, was reduced with the increase in IAP

volume because of the formation of larger particles, as was observed by others (24). The data also suggest that the molecular weight of PLGA did not influence particle density. Further, both theoretical and experimental aerodynamic profiles suggest that the particles were respirable. In fact, aerodynamic diameter of inhaled particles is required to be maintained between 1 and 5 μ because particles within this size range are better deposited in the respiratory tract; outside this size range, particles are either exhaled or deposited in the upper respiratory tract. The particle size parameter used in pulmonary formulations is aerodynamic diameter presented in Fig. 1C rather than actual geometric diameter observed in Fig. 1B. In a seminal paper, Edwards *et al.* have shown that particles with mass densities <0.4 g/cm^3 but geometric diameter >1–5 μ could be used in inhalable formulations (30–32). This assumption was based on the fact that the deposition of particles in the respiratory tract depends on the settling velocity of the particles in the respiratory tract rather than the actual geometric diameter of the particles. However, in the case of vaccine delivery, geometric diameter or actual particle size also plays a role in generating a robust immune response. An aerodynamically favorable particle with a large geometric diameter may be too big to be phagocytosed by macrophages. So particles for pulmonary vaccine delivery should be both inhalable via the respiratory route and uptakable by alveolar macrophage. The actual particle size and aerodynamic diameter suggest that these particles will be optimal both for inhalation and vaccination.

Similar to particle characteristics, the amount of antigen loading was also influenced by the presence of surfactant, amount of antigen, molecular weight of PLGA and method of homogenization. The antigen loading was increased upon incorporation of PVA in the IAP because PVA acts as an emulsifying agent and helps to stabilize the primary emulsion, as observed by Yang *et al.* (23). However, there was a reduction in the extent of antigen loading when lower molecular weight PLGA was used to prepare the microspheres. Because of the lower molecular weight and increased hydrophilicity of the PLGA used, the MS-6 and MS-7 formulations (Table I) possibly were not able to hold as much drug as the MS-4 formulation (26). The formulations that were prepared by probe sonifier showed a lower loading efficiency as compared to the formulations prepared by homogenizer alone, perhaps because the sonifier causes ruptures in the particles that lead to a loss of drugs.

In vitro release study showed two types of release patterns: continuous and discontinuous release. Discontinuous release (33) is a sharp increase in release followed by continuous release, exhibited by MS-2, MS-3 and MS-4 formulations, (Fig. 3) because these particles did not have any surface pores (Fig. 4). However, the extent to which generation of an enhanced immune response is dependent on continuous or discontinuous release of antigen from microspheric formulations is poorly understood. While it is widely believed that continuous release of antigen *in vitro* usually translates into a high immune response *in vivo*, there are also reports that suggest that discontinuous release generates a better immune response (33). It is also possible that a fraction of antigen encapsulated in the particles underwent degradation during the 28-day study period, and this degradation may contribute to the differences in the *in vitro* release profiles. But the integrity and *in vivo* immunogenicity study data demonstrate that such degradations, if any, will have little or no impact on the overall biological activity of the antigen. Nevertheless, based on the *in vitro* release profile, we assume that four of the formulations tested (MS-2, MS-3, MS-4 and MS-5) are likely to produce continuous release of antigens after delivery *in vivo*. Based on the fact that MS-6 and MS-7 showed the highest burst release of the antigen, it is reasonable to argue that these particles are not likely to produce an enhancement in immune response.

The *in vivo* immunogenicity data (Fig. 5) reveals that when plain IM HBsAg was used as a positive control, an increase in immune response was followed by decline as observed previously for IM hepatitis B vaccine in mice (34). In our study, the decline in immune response may be because of deviation from the standard immunization schedule. Hepatitis B vaccination normally entails a three-dose regimen, with the third dose acting as a booster. The lack of a booster dose in the present study may be a reason for the decline in the immune response after IM injection. Furthermore, traditional vaccination by the IM route depends on alum as an adjuvant to generate a robust antibody response; alum was not used in the present study. The immune response data (Fig. 5) clearly demonstrate that antigen encapsulated in microspheres was biologically active and was capable of generating antibody specific to HBsAg. These data are also consistent with our recently published study wherein we have shown that inhalable and positively charged PLGA particles of HBsAg elicit both systemic and mucosal immune response. Upon pulmonary administration, an increase in secretory IgA level in three different mucosal fluids—salivary, vaginal and bronchoalveolar lavage fluids—and cytokine levels—IL-2 and IFN-γ—in spleen homogenates was observed (13). If there was any loss of native properties of antigen, there would have been little or no increase in serum antibody levels. This study, along with our previously published study, suggests that both particle size and surface charge play a role in PLGA particle-mediated generation of immune response. In the current study, we have used negatively charged microparticles to investigate size-dependent uptake and thus generation of immune response. In fact, negatively charged PLGA particles of HBsAg used in this study

produced a reduced immune response compared to positively charged particles used in our recently published study (13). Similar charge-dependent differences in immune response were observed by others (8,17). However, immune response produced by negatively charged particles used in the current study was more pronounced than that produced by particles of similar charge used in our published work. This difference in the immune response of two sets of particles of similar charge is because of the fact that particles of this study was smaller in size compared to those used in earlier study (13).

Moreover, the differences in the immune responses between MS-2 and MS-3 formulations are related to the differences in their actual particle size. Although aerodynamic diameters of the particles of three formulations were within the respirable range (Fig. 1C), the differences in immunogenicity were because of the variations in the actual geometric diameter. The uptake of particles by alveolar macrophages depends on the geometric diameter rather than aerodynamic diameter, a parameter that dictates the deposition patterns of aerosolized formulation after pulmonary administration. So immunogenicity data has been discussed in terms of the actual geometric diameter rather than aerodynamic diameter. In fact, many published reports suggest that particle size plays an important role in the immunogenicity of antigens entrapped in particulate carriers: particles smaller than 10 μm in diameter are reported to be far more immunogenic than larger particles (10,35–38). Based on these published reports, it is reasonable to argue that MS-2, with a particle size of ~5 μm, was internalized by macrophages more efficiently than MS-3, with a particle size of ~12 μm. Enhanced immunogenicity could also be the result of a more sustained release of antigen from MS-2 compared to the MS-3 formulation (Fig. 4).

However, despite having a particle size of ~3 μm, the antibody levels produced by MS-4 were significantly lower than those produced by the MS-2 formulation. In other words, MS-4 failed to increase the antibody levels after 21 days. The immunization study for this set of formulations was continued up to 42 days to investigate whether there was an increase in the immune response later on. No further increase was observed after 28 days (data not shown). The reduced immunogenicity of the MS-4 formulation may be related to its increased porosity, as observed in Fig. 2C. Because of the increased porosity, the entire dose of antigen was perhaps released during the first 2 weeks. For this reason, although a booster dose was given on day 14, there was no continual antigen stimulation that is required to achieve a sustained HBsAg level in the germinal centers of lymphoid tissue for generation of a long-term immune response (39). The dissimilar immune responses of MS-2 and MS-4 support the assumption that in addition to having particles with optimal size for internalization by APCs, there are other factors that may influence the generation of high antibody levels by particulate antigen. Continuous processing and presentation of the antigen to the lymphocyte, for example, can also influence the magnitude of the immune response (40–42). Overall, the data presented in Fig. 5 suggest that pulmonary administration of PLGA microparticles elicits an increase in the immune response that is dependent on particle size.

The particle uptake study also confirms the fact the *in vivo* immune response is a function of particle size. A similar size-based differential uptake by macrophages and dendritic cells has been reported in the literature. For example, Kanke *et al.* (43) showed that particles 1–3 μm in diameter were taken up more efficiently by macrophages than particles 12 μm in diameter. However, it is important to point out that particle uptake data were generated using BSA-FITC particles. Although these data have provided important insights into the influence of particle size on the uptake by alveolar macrophages, it is not known whether the propensity or pattern of uptake of HBsAg-entrapped particles will be similar to or different from that of BSA-FITC particles.

Overall, the result of *in vivo* immunization study and *in vitro* particle uptake study demonstrate that particle size plays an important role in generating systemic immune response upon inhalation. Although published studies, including ours (13), suggest that the mucosal route of administration, including nasal, pulmonary and vaginal routes, are a viable non-invasive alternative for delivering HBsAg antigen (44–47), no studies have specifically investigated the role of particle size in generation of immune response upon inhalational delivery. This manuscript is the first to investigate the effect of particle size on the uptake by rat alveolar macrophages and relate the size-dependent uptake to *in-vivo* immune response. This study is also first to show that particle size in the range of 5 μm is optimal for eliciting an enhanced immune response after immunization via the pulmonary route. The presence of a large number of surface pores on the microspheres is not desirable for continual release of antigen. Acute toxicity studies suggest that the formulations may be safe for pulmonary administration. Nevertheless, more detailed studies are required to determine the long-term cytotoxic and histopathological changes that may occur upon vaccination via inhalation. Further, particle uptake study presented in this manuscript was qualitative rather than quantitative. A detailed quantitative study can only ascertain and directly relate generation of immune response to particle size. Further studies are required to elucidate the mechanistic aspects of inhalable HBsAg-entrapped PLGA particles.

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