Pharmaceutical and Immunological Evaluation of a Single-Shot Hepatitis B Vaccine Formulated With PLGA Microspheres

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ABSTRACT: A single-shot Hepatitis B vaccine formulation using poly(*d*,*l*)-lactide-co-glycolide acid (PLGA) microspheres as a delivery system was examined using a variety of biophysical and biochemical techniques as well as immunological evaluation in C3H mice. PLGA microsphere encapsulation of the Hepatitis B surface antigen (HBsAg), a lipoprotein particle, resulted in good recoveries of protein mass, protein particle conformational integrity, and *in vitro* antigenicity. Some partial delipidation of the HBsAg, however, was observed. The loading and encapsulation efficiency of HBsAg into the PLGA microspheres were measured along with the morphology and size distribution of the vaccine-loaded PLGA microspheres. The *in vitro* release kinetics of HBsAg from the PLGA microspheres was evaluated and found to be affected by experimental conditions such as stirring rate. HBsAg showed enhanced storage stability at 37°C in the slightly acidic pH range reported to be found inside PLGA microspheres; thus, the antigen is relatively stable under conditions of temperature and pH that may mimic *in vivo* conditions. The immunogenicity of the microsphere formulations of HBsAg was compared with conventional aluminum adjuvant formulated HBsAg vaccine in C3H mice. Comparisons were made between aluminum formulations (one and two injections), PLGA microsphere formulations (single injection), and a mixture of aluminum and PLGA microsphere formulations (single injection). The nine-month serum antibody titers indicate that a single injection of a mixture of aluminum and PLGA-formulated HBsAg results in equal or better immune responses than two injections of aluminum-formulated HBsAg vaccine. Based on these *in vitro* and *in vivo* studies, it is concluded that HBsAg can be successfully encapsulated and recovered from the PLGA microspheres and a mixture of aluminum-adjuvanted and PLGA-formulated HBsAg can auto-boost an immune response in manner comparable to multiple injections of an aluminum-formulated vaccine. J Pharm Sci 91:1019–1035, 2002

Keywords: PLGA microspheres; controlled-release vaccination; Hepatitis B surface antigen (HBsAg); single-shot vaccine

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INTRODUCTION

The evaluation of controlled-release formulations for protein-based vaccines has become an active area of vaccine pharmaceutical research.[1–9] Currently, parenteral administration of purified protein-based vaccine antigens involves multiple injections to achieve an optimum immune response in humans. For example, recombinant Hepatitis B surface antigen (HBsAg) adsorbed to aluminum adjuvant is typically administered with multiple injections over many months to obtain a suitable immune response and protection against Hepatitis B viral infection in humans.[10–12] Consequently, for a recombinant vaccine such as HBsAg, the design of an injectable formulation that releases the antigen in a controlled manner over a sufficient time period to induce long-lasting immunity from a single shot would be a promising improvement in terms of immunization coverage and compliance. In fact, based on these considerations, the development of controlled-release vaccine formulations for both current vaccines and for novel vaccine antigens in clinical development has been promoted.[13]

Controlled-release delivery of protein-based vaccine antigens using poly(*d*,*l*)-lactide-co-glycolide acid (PLGA), an FDA-approved polymer, is of particular interest. Various therapeutic protein drugs have been formulated using PLGA microspheres and some have been evaluated in human clinical trials.[14–19] In the case of protein-based vaccine antigens, several reports have now demonstrated that continuous or pulsatile release of protein antigens from PLGA microspheres provides a prolonged immunological response in animal studies, thus avoiding the need of multiple injections.[20–34] Some of these studies also show that protein antigen encapsulation into PLGA polymers may induce a higher immunological response in animals than that achieved with the nonencapsulated vaccine. PLGA microsphere technology may therefore be a promising approach for vaccine formulations, with potential benefits of reducing the number of inoculations as well as enhancing the immune responses. The application of PLGA microspheres as a vaccine delivery system for a specific protein antigen, however, will require specific, detailed investigations because of the diversity of intrinsic stability and biophysical properties of antigenic proteins.[35]

The commercially available Hepatitis B vaccine consists of a purified, recombinant viral lipoprotein particle adsorbed to aluminum adjuvant. The vaccine antigen is HBsAg, which consists of a ~24 kDa viral protein (S protein) that is expressed *in vivo* in yeast, forming a lipoprotein complex of 22 nm.[36–38] These virus-like particles (VLPs) are purified to homogeneity by conventional chromatography methods and then adsorbed to aluminum adjuvant for use as a vaccine.[39–41] Although several previous papers have shown that HBsAg encapsulated in PLGA microspheres can elicit an immune response in animals,[27,42–46] these papers did not emphasize the effect of the formulation conditions on the biophysical and biochemical properties of HBsAg from a pharmaceutical development perspective. Thus, the overall goal of this study was to test the pharmaceutical feasibility of developing a single-shot HBsAg vaccine using PLGA microspheres. In addition, the immunogenicity of the PLGA-formulated vaccine was evaluated in C3H mice and compared with that of the aluminum adjuvant-formulated vaccine.

MATERIALS AND METHODS

Polymer and HBsAg

Poly(*d*,*l*)-lactide-co-glycolide acid (PLGA) with a lactide/glycolide (L/G) ratio of 65/35 (MW 75 000) and PLGA with a L/G ratio of 50/50 (MW 75 000) were purchased from Birmingham Polymers, Inc. In this paper, the corresponding polymers are referenced as PLGA65/35 and PLGA50/50, respectively. Recombinant Hepatitis B surface antigen (HBsAg) S protein was expressed in yeast and purified as outlined previously.[41] The purified HBsAg used for PLGA encapsulation was dialyzed and concentrated to 1–2 mg/mL protein in 5 mM sodium phosphate, 2% sucrose, pH 7.2. Aluminum hydroxyphosphate adjuvant was manufactured by Merck & Company Inc. All other chemicals and reagents were purchased from commercial vendors.

Preparation and Testing of PLGA Microspheres

PLGA microspheres were prepared using a double emulsion method at room temperature, which requires five major steps: primary emulsion, second emulsion, hardening, washing/filtration, and drying. The process for small-scale formulations was performed as follows: PLGA polymer was first dissolved in 8 mL of ethyl acetate (a mixture of 20% *N*-methyl pyrrolidinone and 80%

ethyl acetate was used for a few batches, as indicated later). The dissolved polymer was homogenized with 2 mL of HBsAg stock solution (1–2 mg/mL protein in 5 mM sodium phosphate, 2% sucrose, pH 7.2) at ~10 000 rpm for a few minutes to form a water-in-oil (w/o) emulsion. This primary w/o emulsion solution was poured into 200 mL of 1% polyvinyl alcohol (PVA) aqueous solution while stirring with a magnetic plate stirrer. In this step, a second emulsion of water-in-oil-in-water (w/o/w) forms. After ~30 min of stirring (hardening of microspheres), additional water was added slowly to make a final volume of 4–5-fold more than the first washing. After continuously stirring for 1 h, the microspheres were collected by filtration, then redispersed in 0.5 % PVA solution and incubated with stirring for 2.5 h as the second washing. The main purpose of the washings is to remove ethyl acetate from the PLGA microspheres. The microspheres were then collected by filtration and dried under vacuum at room temperature for ~2 days.

HBsAg loading was determined by amino acid analysis (see HBsAg Protein Concentration section). The activity of the antigen was determined by measuring the EIA/protein ratio after HBsAg was released from PLGA microspheres by breaking the microspheres through incubation with vigorous stirring with a stir bar followed by EIA assay (see *In Vitro* Antigenicity of HBsAg section) and amino acid analysis. The HBsAg loading and activity data of each formulation are from two samples of duplicate batches. These data are from sized fractions with microsphere size distribution range of 25 to 45 μm. The fractions with size < 25 μm give HBsAg with about the same EIA/protein ratio, but are 40–65% lower in protein loading. An unencapsulated HBsAg control gave an EIA/protein value of 1.0 ± 0.1. Similar EIA/protein ratio values were obtained in repeat measurements after the PLGA microsphere formulation samples were stored dry at 4°C for 3 months.

Other HBsAg Formulations

Aluminum adjuvant formulations of the HBsAg vaccine were prepared by directly adsorbing the HBsAg to aluminum hydroxyphosphate adjuvant. Two doses of HBsAg, 7.5 and 30 μg/mL, were prepared with 450 μg/mL aluminum adjuvant in saline. These formulations correspond to doses of 3 and 12 μg HBsAg, respectively, upon injection of 0.4 mL volume. HBsAg in saline formulation (without aluminum adjuvant) was prepared at a dose of 7.5 μg/mL. This formulation corresponds to the 0.4-mL injection volume of 3 μg HBsAg.

PLGA Microsphere Particle Size

The morphology and size distribution of PLGA microspheres were studied by scanning electron microscopy (SEM) and with a laser scattering particle size distribution analyzer. All samples were dried under vacuum before morphological analysis with an environmental scanning electron microscope (model E-3). The dried samples were also suspended in phosphate-buffered saline (PBS) buffer for particle size distribution analysis with a Horriba laser scattering particle size distribution analyzer (model LA910).

HBsAg Protein Concentration

The concentration of HBsAg in solution was determined by a colorimetric bicinchoninic acid (BCA) assay, ultraviolet (UV) spectroscopy, or amino acid analysis. The protein content in PLGA microspheres was measured primarily by amino acid analysis. The amino acid analysis was carried out by first incubating HBsAg samples (with/without PLGA) in 6 N HCl at 110°C for 20 h. The hydrolyzed samples were then analyzed on a Beckman 6300 (C8 reversed phase) high-performance liquid chromatograph. The total HBsAg concentration was calculated based on known HBsAg amino acid sequence. The S protein of the HBsAg contains 13 tryptophan and 14 cysteine residues. These amino acids were destroyed in the incubation process due to hydrolysis and were not considered in the HBsAg concentration calculation. As a control, a standard mixture of amino acids including nonleucine with known concentrations were also analyzed in parallel to each real sample analysis. All samples were examined in duplicate.

Apparent Density of HBsAg

The apparent density of HBsAg particles was determined by CsCl density gradient analysis. The CsCl density gradients were formed using a Bio-Comp gradient maker. The samples were centrifuged for 5 h at 65 000–80 000 rpm and 20°C in a Beckman model Optima TLX-100 ultracentrifuge using a TLA-100.4 fixed-angle rotor. Fractions of 0.25 mL were collected using a Haake-Buchler fractionator and then weighted for density determination. The presence of the

protein in each fraction was determined by protein fluorescence measurement (excitation at 280 nm and emission at 330 nm).

Circular Dichroism (CD) and Fourier Transform Infrared (FTIR) Spectra of HBsAg

The secondary structure of HBsAg was examined by CD using an Aviv 60 spectropolarimeter and by FTIR using a BioRad FTS 60 spectrometer. The CD spectra were signal-averaged over 3 scans at 20–22°C. The buffer background spectrum collected under the same conditions was subtracted from each sample spectrum. The mean residue ellipticity values were calculated based on HBsAg concentrations determined by BCA measurements. FTIR spectra were scanned using double-pass reflection techniques with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector at ambient temperature with a resolution of 2 cm^{-1}. The samples were buffer exchanged into water, then dried under vacuum on a polished metal plate. A total of 64 scans were signal averaged. The FTIR sample chamber was continuously purged with nitrogen gas. Corrections for residual water vapor and CO_2 were made. Both CD and FTIR results were confirmed by repeated measurements using freshly prepared samples.

In Vitro Antigenicity of HBsAg

The *in vitro* antigenicity of HBsAg was measured with an enzyme immunoassay (EIA) kit ("Auszyme Monoclonal" diagnostic kit) from Abbott Laboratories. Each sample was examined at three different dilutions against a linear fitting to the responses of control standard samples with different dilutions. The *in vitro* antigenicity of HBsAg was evaluated by the ratio of EIA response and protein concentration (EIA/protein).

In Vitro Release Studies

The studies of HBsAg *in vitro* release from PLGA microspheres were performed under two different incubation conditions: (1) with a gentle, slow rocking of the samples, or (2) with a vigorous stirring at 800 rpm using a stir bar. At designated time intervals, a volume of $>70\%$ of the supernatant was collected and the fresh buffer of the same volume was added back to control the pH. *In vitro* release of 100% was claimed if the protein mass of HBsAg released into the supernatant as determined by UV spectrophotometric measurement equaled that of encapsulated HBsAg in PLGA microspheres as determined by amino acid analysis. The correlation coefficient for UV measurement and amino acid analysis was determined to be in the range 0.95–1.00.

Stability of HBsAg Inside the PLGA Microspheres

A stock solution of HBsAg was split into two parts: one part was used for PLGA encapsulation, and half of the other part was stored at 4°C as control and the other half was incubated in solutions of varying pH at 37°C for 1 week, then analyzed for EIA/protein ratio. The 4°C control sample (unencapsulated HBsAg, no 37°C incubation) gave an EIA/protein ratio value of 1.0 ± 0.1. The PLGA microspheres containing HBsAg were first incubated in solutions of varying pH at 37°C for 3 weeks with gentle rocking. The microspheres were then separated from the incubation buffer by filtration. After washing thoroughly, the microspheres were re-suspended in fresh buffer solutions, and then incubated with vigorous stirring at 800 rpm for 2 days, conditions that were shown to physically break apart the microspheres and release the HBsAg. By this method, most of the remaining HBsAg was released after 2 days of incubation. The supernatant containing the released HBsAg was collected, and *in vitro* antigenicity was measured by EIA/protein ratio determination. All data of EIA/protein ratio values are the average of 3–5 measurements.

C3H Mouse Experiments

The *in vivo* immunogenicity of HBsAg vaccines was examined in C3H mice by subcutaneous (sc) injection.[47] It has been reported that PLGA microspheres are easy to handle for sc implantation.[55] A total of 21 groups of 15 six- to eight-week-old C3H mice were injected with 0.4 mL of formulated HBsAg vaccine using a 23-gauge needle. The total injected HBsAg dose range was 3–21 μg. For the higher dose vaccines (~12–21 μg HBsAg), the formulation was composed of 3 μg of HBsAg–aluminum and 9–18 μg of HBsAg encapsulated in PLGA microspheres. Mouse sera were collected at designated time points after initial vaccine injection. A microtiter plate modification of the AUSAB EIA kit (Abbott Labs, N. Chicago, IL) was used to quantify antibodies to HBsAg as described previously.[47] Antibody responses are

reported as geometric titer (GMT) measured in mIU/mL from groups of ~15 mice in each group.

RESULTS AND DISCUSSION

Physical Characterization of PLGA Microspheres Containing HBsAg

PLGA microsphere preparation by the "double emulsion" method requires the PLGA polymer to be initially dissolved in an organic solvent, mixed and homogenized with an aqueous protein solution to form a w/o emulsion, subsequently mixed in a second solvent to form a w/o/w or double emulsion, followed by collection and drying of the protein-containing microspheres[48–52] (see Materials and Method section). The PLGA microspheres prepared by the double emulsion method in this work were basically spherical with a smooth surface as indicated by morphology examination using SEM (Figure 1A). Some of the microspheres had a donut or flattened ball-like shape. In addition, some of the microspheres possessed a hollow structure, as indicated by broken microspheres (not shown). The difference between sphere, donut-like, and flattened ball-like microsphere structures may be related to the hardening and drying processes. Compared with the vacuum drying used in this work, freeze-drying or lyophilization may be helpful for overcoming the microsphere flattening problem if required.[53–57] PLGA microspheres with relatively hollow structures are typically seen when manufactured by a double emulsion method,[50,58,59] whereas PLGA microspheres with microcave structures are often observed when manufactured by a spray-drying method.[50,59,60]

The actual size of the individual microspheres was examined by SEM, sieving, and laser light scattering experiments. The SEM results indicate that the particle diameter of the majority of microspheres is in the range 25–50 μm, and a few microspheres approach the size limits of 5 and 150 μm (not shown). The particle size distributions of the microspheres as estimated from yields during size fractionation were also determined. The fractionation was carried out by passing the wet microspheres through a series of sieves with varying size cutoffs. The majority of the microspheres were < 90 μm in diameter before drying (Figure 1B). The particle size distributions of the fractionated microspheres were also determined by laser light scattering after the dried microspheres were re-suspended in aqueous solution.

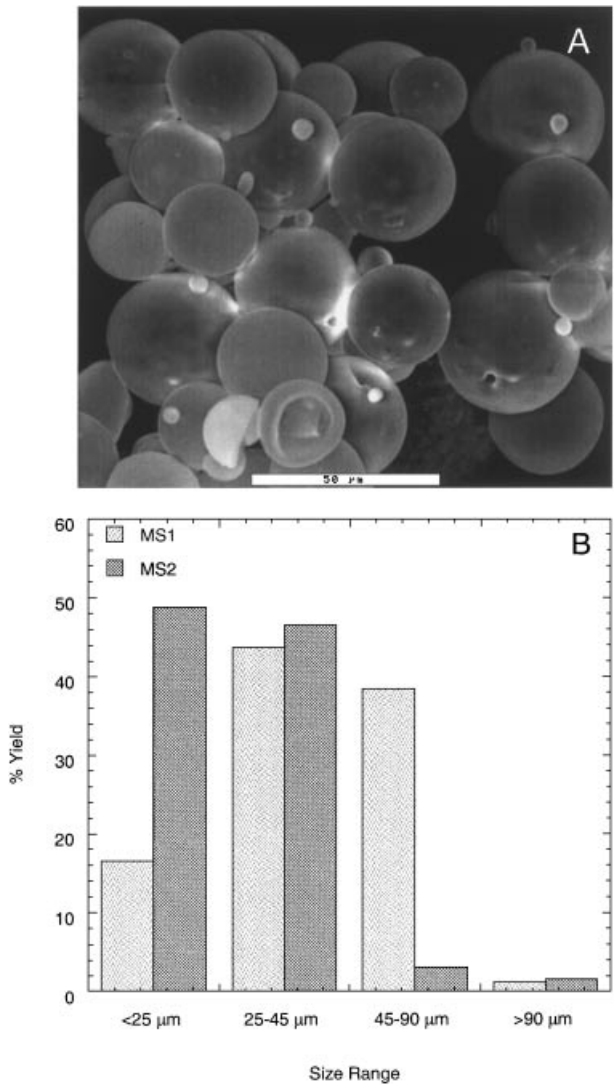

Figure 1. PLGA microspheres containing HBsAg. (A) Scanning electron micrographs (SEM) of PLGA microspheres. (B) The size distribution profiles of PLGA microspheres containing HBsAg. The PLGA microsphere were sized by passing the wet microspheres through a series sieves with different size holes. The yield of each fraction was determined by mass after the microspheres were dried. See Table 1 for description of PLGA formulations MS1 and MS2.

The data indicate that the fractionated samples contained significant amounts of larger particles (centered around 100 μm); thus, the microspheres probably formed aggregates during the drying or re-suspension process, with smaller microspheres forming more aggregates than larger microspheres (not shown).

The apparent bulk density of the PLGA microspheres, defined as the bulk mass of the dried microspheres divided by the bulk volume (space)

Table 1. HBsAg–PLGA Microsphere Vaccine Formulation Preparations Used for *In Vitro* Characterization and *In Vivo* C3H Mice Studies[a]

Lot	PLGA Polymer (Lactide/Glycolide Ratio)	Total PLGA (g)	HBsAg Loading (%)	Microsphere Density (g/mL)	EIA/Protein Ratio
MS1	65/35	5	0.6, 0.6	0.20, 0.36	0.9, 0.9
MS2	65/35	3	1.1, 1.2	0.12, 0.17	0.9, 1.0
MS3	50/50	3	0.8, 0.9	0.19, 0.27	0.0, 0.1
MS4	65/35	5	0.0, 0.0	Not measured	0.0, 0.0

[a]See preparation and testing of PLGA microspheres in Materials and Methods section for details. MS3 was prepared using *N*-methyl pyrrolidone/ethyl acetate as organic solvent instead of ethyl acetate.

occupied, was also determined. The apparent bulk density values of 25–45 μm-sized microsphere fractions was determined by measuring the sample weight and volume (Table 1). The bulk density of dried PLGA microspheres varies from batch to batch, and even varies somewhat between duplicate batches manufactured by the same formulation process (Table 1). The variation in sample density is most likely caused by the difference in the extent of microsphere aggregation in the drying process, as shown by SEM analysis (not shown). It is interesting that although there are batch-to-batch variations, the bulk density values of the PLGA microspheres prepared in this work are close to the published value of 0.27 g/mL (as determined by the same methodology with a batch of PLGA50/50 microspheres).[61]

Loading and Encapsulation Efficiency of HBsAg into PLGA Microspheres

The amount of HBsAg encapsulated in PLGA microspheres was determined either by dissolving the PLGA microspheres in 6 M HCl followed by amino acid analysis or by physically breaking the PLGA microspheres in an aqueous solution. After release from the microspheres by the latter method, the amount of HBsAg released into solution could be determined by either amino acid analysis after hydrolysis in 6 M HCl or UV analysis; both methods gave comparable results (not shown). The loading of HBsAg in PLGA microspheres is presented as the amount of HBsAg compared with the total mass of the microspheres (Table 1). The effect of HBsAg concentration on HBsAg loading was basically negligible because the maximum amount of HBsAg used in the formulation was no more than 0.1% of PLGA used. The encapsulation efficiency of HBsAg is defined as the mass percentage of HBsAg encapsulated into PLGA microspheres out of the total amount of HBsAg used in the formulation process.[22] The HBsAg encapsulation efficiency was tested by varying the total amount of PLGA polymer used in the formulation process. As the total amount of polymer increased from 1 to 5 g, the encapsulation efficiency increased from ~33% up to ~100% under a fixed formulation condition. A similar correlation of protein encapsulation efficiency with PLGA concentration has been reported.[53] A mass balance of HBsAg through the PLGA formulation process was determined by measuring the amount of antigen incorporated into PLGA microspheres and the amount of antigen remaining in the aqueous phase after formulation (not incorporated). A range of ~87–98% mass balance was observed; thus most of the HBsAg could be accounted for in the formulation process.

Effect of Incubation and Formulation Variables on *In Vitro* Release of HBsAg From PLGA Microspheres

The *in vitro* release of HBsAg from the PLGA microspheres was examined under a wide range of experimental conditions of pH and buffer composition. Because the hydrolysis of PLGA releases lactic and glycolic acids, which may in turn lower the pH of the solution[62,63] as well as the inside of the microspheres,[64] the pH of the incubation solution was controlled by addition of fresh buffer after each sample collection. Under relatively mild incubation conditions of gentle rocking at 37°C, HBsAg release profiles showed delayed release from the PLGA microspheres (Figure 2) under a variety of conditions of pH and salt concentrations. Although alkaline[29,62,65] and acidic pH values[56] promote PLGA polymer

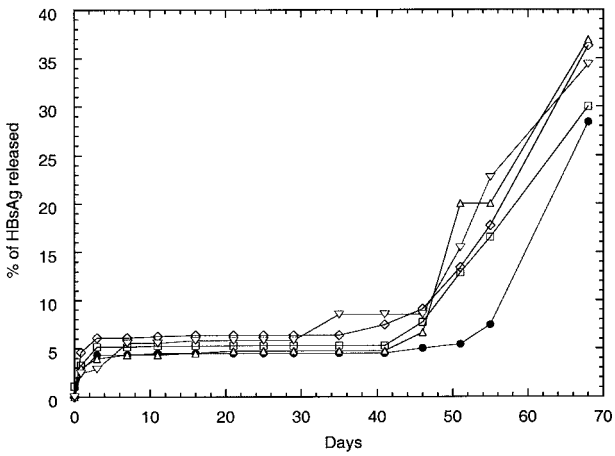

Figure 2. *In vitro* release profile of HBsAg from PLGA microspheres. Percentage of HBsAg protein released into the supernatant was measured by UV spectroscopy and compared with the total HBsAg protein encapsulated into the PLGA microspheres (PLGA65/35). Delayed release of HBsAg was observed by gentle rocking of the samples incubated at 37°C. Key: (open symbols) incubation buffers containing 10 mM NaCl, 0.01% polysorbate 20, and 5 mM phosphate buffer with pH range 5.0–8.5: (filled symbols) incubation buffers containing 200 mM NaCl and 5 mM phosphate at pH 8.5.

hydrolysis, the HBsAg release rate was not significantly affected by solution pH in the range 5.0–8.5. A small delay in HBsAg release was observed when the NaCl concentration was increased from 10 to 200 mM with gentle rocking (Figure 2). This delay may be caused by the balance of osmolality inside and outside of microspheres.[56,64] The effect of temperature during incubation was not examined in detail because most experiments were performed at 37°C to mimic *in vivo* conditions. The release profile observed for HBsAg in Figure 2 is similar to the multiphase release process thought to be required for successful vaccination: that is, initial antigen diffusion, polymer erosion, and final antigen diffusion.[49,66]

The effect of PLGA composition and formulation processing conditions on the *in vitro* release profile of HBsAg was also evaluated. The degradation half-life of a PLGA polymer has been shown to be related to its monomer composition and will approach the minimum when its glycolide/lactide ratio reaches 50/50 and will increase with both increasing and decreasing of glycolide/lactide ratios.[49,67–70] However, the degradation rate of PLGA with lactide/glycolide increased to 75/25 has been reported to be faster than that of PLGA50/50 under certain conditions,[55] suggesting that the PLGA polymer microsphere erosion and the protein antigen release may be complex functions of several variables and not solely controlled by PLGA degradation. It has been reported, for example, that the degradation rate of microspheres is related to the molecular weight of the PLGA polymer.[71] *In vitro* HBsAg release experiments with PLGA65/35 and PLGA50/50 microspheres were performed at 22°C with vigorous stirring (as described later), and results indicate that microspheres formulated with PLGA50/50 had the slowest release rate of HBsAg (not shown). However, the experimental condition of physical stirring the sample may have significantly affected the *in vitro* antigen release profiles.

To determine if the presence of vigorous stirring during the *in vitro* release incubation alters HBsAg release kinetics predominantly by the process of physically breaking the PLGA microspheres, a series of *in vitro* release studies were performed. SEM imaging in Figure 3A shows that almost 100% of the PLGA microspheres were broken when exposed to vigorous stirring for 1 week. As a comparison, PLGA microspheres of the same batch exposed to gentle rocking for 1 week showed no detectable damage as determined by SEM imaging with morphology similar to that shown in Figure 1A. A comparison of the *in vitro* release profiles of HBsAg from these PLGA microspheres in the presence and absence of vigorous stirring is shown in Figure 3B. It can be seen that the rapid release of HBsAg (Figure 3B) is not caused by PLGA hydrolysis but by physically damaging (breaking apart) the microspheres.

For different formulations of PLGA microspheres containing HBsAg, antigen release is most likely affected by multiple factors including polymer composition, polymer amount, and formulation processing conditions. For example, in a separate experiment to further explore the effect of PLGA amounts on HBsAg release rates, samples were formulated with less (0.5 g) and more PLGA (5 g) and were incubated with gentle rocking at 37°C. The formulation with less PLGA released almost 100% of HBsAg in the burst phase within 24 h, whereas the formulations with more PLGA displayed a slow and controlled-release pattern (not shown). In this work, an effect of apparent bulk density of PLGA microspheres on the initial release of HBsAg may have been observed. For example, under identical *in vitro* release conditions with gentle rocking at 37°C,

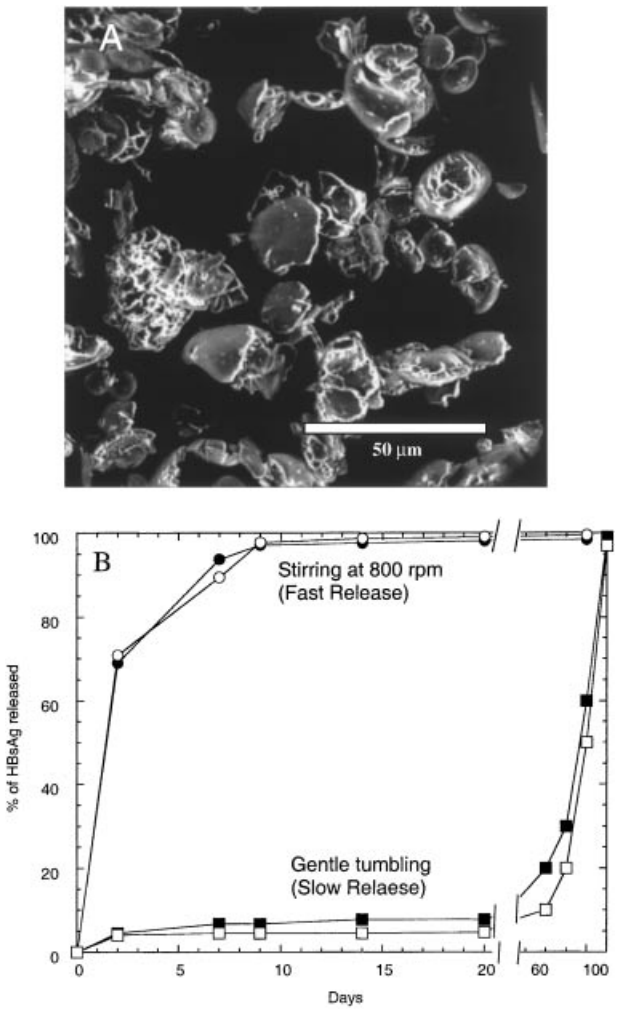

Figure 3. Effect of stirring on the kinetics of HBsAg *in vitro* release from PLGA microspheres. (A) Scanning electronic micrographs (SEM) of PLGA microspheres after incubation for one week with vigorous stirring at 800 rpm using a stir bar. (B) HBsAg *in vitro* release profiles: Percentage of HBsAg protein released into the supernatant was measured by UV spectroscopy and compared with the total HBsAg protein encapsulated into the PLGA microspheres. The top two curves show release of HBsAg from microspheres with vigorous stirring at 800 rpm using a stir bar at 37°C, and the bottom two curves show release of HBsAg with a gentle tumbling of the samples at 37°C. All samples were in an incubation buffer of 10 mM NaCl, 0.01% polysorbate20, and 5 mM phosphate at pH 7.

lower density microspheres show an initial HBsAg release kinetics faster than that of higher density microspheres (not shown). The difference in the apparent bulk density of PLGA microspheres (Table 1) is attributed to the variation in the degree of microsphere aggregation formation, as indicated by SEM analysis (not shown), most likely caused by surface contacts among the wet microspheres during the vacuum drying process. It has been reported that the condition of the PLGA microsphere drying process has a significant impact on burst antigen release.[53] However, others have found no good correlation between the drying conditions and the initial release of protein.[72] The inconsistency in the aforementioned observations may be attributed to the randomness in drying process induced microsphere aggregation. To further reveal the correlation between antigen release rate and PLGA polymer degradation rate, more detailed studies will need to be carried out employing multiple formulation processes.[61,73,74] Nonetheless, a well-defined *in vitro* release test is critical to ensure consistency of manufacturing and is a typical component of quality control assays for proteins in PLGA microspheres.[75]

Effect of PLGA Microsphere Encapsulation and Storage on the Biophysical and Biochemical Properties of HBsAg

Many proteins will denature under conditions of PLGA manufacturing, such as exposure to organic solvents and/or physical stress.[35,76–80] For example, it has been reported previously that methylene chloride or ethyl acetate may have a detrimental effect on a model protein (bovine serum albumin) if not properly stabilized, yet these two solvents are the most suitable solvents for preparing PLGA microspheres via the double emulsion technology.[54,81–83] To examine the effect of organic solvent exposure on the structure or lipid content of HBsAg in more detail, however, a more concentrated protein preparation is required. Lyophilized HBsAg in which either ethyl acetate, methylene chloride, or a chloroform/methanol mixture (known to extract lipids effectively) was mixed with lyophilized HBsAg formulated in the presence of a mixture of mannitol and sorbitol (3/2%), was thus examined. In each case, extraction of ~40% (by ethyl acetate or methylene chloride) to 80% (by chloroform/methanol) of the lipids from HBsAg particles was observed. In addition, methylene chloride and chloroform/methanol caused about a 30–80% decrease in the *in vitro* antigenicity of HBsAg, whereas ethyl acetate did not show a significant effect on the *in vitro* antigenicity of HBsAg as determined by EIA (not shown).

It has been reported that sugars such as mannitol and trehalose protect human growth

hormone and interferon-γ from organic solvent-induced damage by preventing direct protein–organic solvent interaction through preferential hydration.[84] In this study, the stabilizing effect of different sugars on HBsAg in the homogenization process of HBsAg solutions was examined in a small-scale laboratory study (no PLGA present). All four tested sugars, sucrose, trehalose, lactose, and mannitol, provided significant protection to HBsAg against loss of EIA activity. For example, the presence of 2% sucrose resulted in ~65% HBsAg *in vitro* antigenicity remaining, whereas the absence of sucrose resulted in < 20% *in vitro* antigenicity being retained. Based on these results, all PLGA microspheres containing HBsAg discussed in this study contain sucrose (see Materials and Methods section). The *in vitro* antigenicity values of HBsAg encapsulated in PLGA microspheres shown in Table 1, as determined by EIA/protein measurement after release, suggest no significant antigenicity loss induced by the encapsulation and subsequent release of HBsAg from the PLGA microspheres (the EIA/protein value of encapsulated/released HBsAg is similar to that of unencapsulated HBsAg, see Table 1).

To better understand the effect of PLGA microsphere encapsulation on the integrity of HBsAg, the conformational integrity and relative lipid content of HBsAg were analyzed. The effect of PLGA formulation on the secondary structure of the S protein comprising the HBsAg was characterized using CD and FTIR (Figure 4). The encapsulated HBsAg was released from the microspheres after incubation *in vitro* at 37°C for 2 days with stirring. The CD spectra suggest no change in secondary structure of the S protein in HBsAg induced by PLGA microsphere encapsulation (Figure 4A). In both cases, a typical α-helical conformation of the S protein is observed.[37] The FTIR spectra of the same samples (Figure 4B) confirms the results of the CD measurements by showing the similar amide I bands at ~1656 cm^{-1}. A small difference in peak intensity at 1740 cm^{-1} (lipid carbonyl band) was observed, as shown in Figure 4B, suggesting a lower lipid content in HBsAg particles released from PLGA microspheres. These observations suggest a possible delipidation of HBsAg particles, induced by the PLGA formulation process. A CsCl density gradient analysis was carried out for both control (unencapsulated) and encapsulated/released HBsAg samples. The control HBsAg sample yields an apparent density of 1.20 g/mL that is consistent with reported values.[85,86] The apparent density increased to at least 1.26 g/mL when HBsAg was recovered from the PLGA microspheres (not shown). This density increase is consistent with the partial delipidation of HBsAg particles. The density of a globular protein containing no lipid is normally around 1.33–1.42 g/mL.[87]

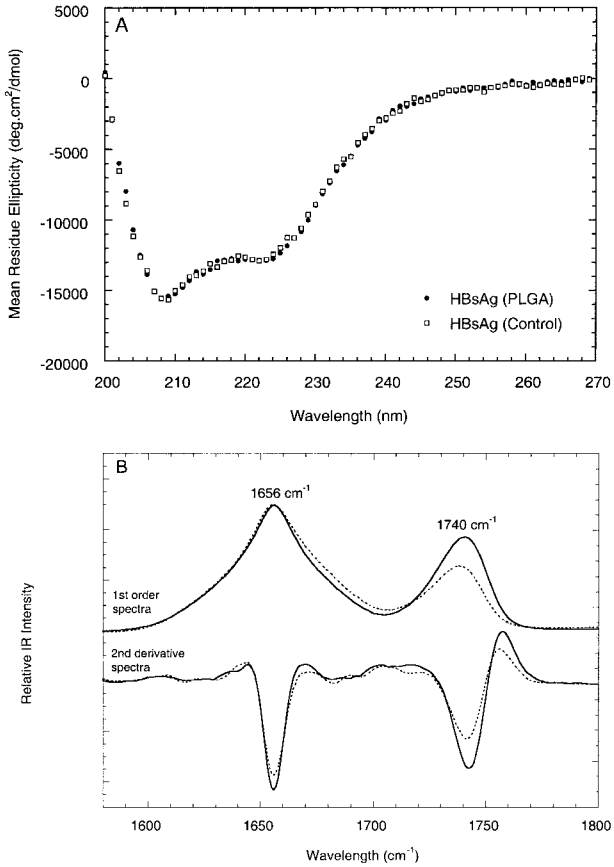

Figure 4. Circular dichroism (CD) and Fourier transform infrared (FTIR) spectra of HBsAg samples before and after the encapsulation into and release from PLGA microspheres. (A) CD spectra. Key: (filled circles) untreated HBsAg; (open circles) HBsAg released from PLGA microspheres. (B) FTIR spectra and the second derivatives of the original spectra. Key: (solid lines) untreated HBsAg; (dashed lines) HBsAg released from PLGA microspheres. The HBsAg released from PLGA microspheres were obtained as follows: the HBsAg–PLGA microspheres were incubated in solution with vigorous stirring at 800 rpm for 24 h, and the supernatant containing released HBsAg was collected for analysis.

For both *in vivo* and *in vitro* studies with PLGA encapsulated HBsAg, it is important to determine if the intrinsic stability of the protein is maintained under the experimental conditions within

the microspheres (temperature and pH) during storage. Therefore, the effects of pH and temperature on the intrinsic stability of HBsAg (no PLGA) were studied *in vitro* to see if the protein has sufficient intrinsic stability under the conditions of *in vivo* experiments. The results shown in Table 2 (HBsAg in solution at 37°C for 1 week) indicate that HBsAg gradually loses *in vitro* antigenicity in aqueous solution at 37°C at a pH value >6. The loss of EIA activity was observed without any detectable changes in protein secondary structure as determined by CD and protein particle aggregation as determined by DLS (not shown).

The stability of HBsAg inside the PLGA microenvironment was evaluated by incubating encapsulated HBsAg in buffer solution at 37°C and then releasing the HBsAg from the microspheres by vigorous stirring as already described (also see Materials and Methods section). As shown in Table 2, HBsAg remains ~100% active after staying inside of PLGA microenvironment for 3 weeks at 37°C in the pH range 5–6. The pH of PLGA microenvironments is believed to relatively acidic due to the slow acid release from PLGA hydrolysis.[63,64,88] This special pH feature may be particularly favorable to the stability of HBsAg because HBsAg is more stable in a relatively acidic pH environment (Table 2). Similar experiments then performed at higher pH values using the same experimental protocol. The data demonstrate that HBsAg loses ~30–40% of *in vitro* antigenicity after 3 weeks of incubation at pH 8 at 37°C (Table 2).

As a final note, the storage stability of encapsulated HBsAg in PLGA microspheres in the dried state was followed and observed to be stable after 3 months of storage at 4°C (data not shown). The data collection for dry samples stored at 37–40°C was not successful because PLGA microspheres melt into rubber-like structures, thereby blocking rapid release of HBsAg, because this temperature is near the glass transition temperature of the polymer.[51,72,73]

Table 2. Relative *In Vitro* Antigenicity (EIA/Protein) of HBsAg Preparations Incubated at 37°C Both in Solution and Inside PLGA Microspheres[a]

HBsAg Formulation	pH 5	pH 6	pH 7	pH 8
HBsAg in solution at 37°C for 1 week	1.0	1.0	0.9	0.6
HBsAg in PLGA at 37°C for 3 weeks	1.0	1.0	0.9	0.7

[a]See stability of HBsAg inside the PLGA microspheres in Materials and Methods section for details.

In Vivo Immunogenicity of HBsAg–PLGA Formulations in C3H Mice

The physical properties of the four PLGA formulations used in these animal studies are as listed in Table 1. The *in vitro* antigenicity results indicate that two HBsAg–PLGA microsphere formulations (MS1 and MS2 in Table 1) contain active HBsAg and one of the HBsAg–PLGA formulations (MS3 in Table 1) contains partially to completely inactivated HBsAg (as determined by *in vitro* antigenicity). The antigen inactivation is most likely due to over exposure to organic solvents during formulation processing (see Table 1 for details). The mostly inactivated formulation was used, along with a placebo PLGA microsphere formulation (MS4 in Table 1) in the animal experiments (see Table 3). Details of the *in vivo* testing of C3H mouse immune response to HBsAg vaccines are shown in Table 3. The dosing regimens evaluated included two injections with aluminum adjuvant-formulated HBsAg; a single injection with PLGA microsphere-formulated HBsAg; and a single injection with the mixture of aluminum- and PLGA microsphere-formulated HBsAg.

The results in Figure 5A show that a single injection of 3 μg of aluminum-formulated HBsAg does not result in a significant antibody response in C3H mice, whereas two injections of 3 μg of aluminum-formulated HBsAg boost animals and a good antibody response is observed. It can also be seen that a single dose of large amounts of aluminum-adjuvanted HBsAg (12 μg) will also result in a significant immune response. The original purpose of this high dose of HBsAg was to demonstrate the dose tolerability in C3H mice (12 μg injected sc in this study can be compared with 30 μg injected intramuscularly in CD1 mice and 20 μg injected intraperitoneally in guinea pigs[27,44]). The immune response from a single injection of a high dose of aluminum-formulated HBsAg suggests that the C3H mice animal model can be exposed to sufficient amounts of HBsAg dose to result in an effective prime and boost. In Figure 5B it can be seen that a single injection of 12 μg of PLGA-formulated HBsAg did not result in significant antibody titers and that the overall anti-HBsAg antibody response is basically no different from the response induced by a single injection of 3 μg of aluminum-formulated HBsAg

Table 3. HBsAg Vaccine Formulations and Vaccination Schedule for *In Vivo* Testing of C3H Mice[a]

Group	Vaccine Formulations and Vaccination Schedules
	Two injections of 3 μg HBsAg–Alum (except for groups 1 and 7)
1	3 μg HBsAg–Alum on day 1
2	3 μg HBsAg–Alum on day 1 and day 28
3	3 μg HBsAg–Alum on day 1 and 3 μg HBsAg in saline on day 28
4	3 μg HBsAg–Alum on day 1 and day 56
5	3 μg HBsAg–Alum on day 1 and 3 μg HBsAg in saline on day 56
6	3 μg HBsAg–Alum + MS4 (PLGA without HBsAg in Table 1) on day 1 and day 28
7	12 μg HBsAg–Alum on day 1
	One injection of 12 μg HBsAg–PLGA
8	12 μg MS3 (inactivated HBsAg–PLGA in Table 1) on day 1
9	12 μg MS1 (activated HBsAg–PLGA in Table 1) on day 1
10	12 μg MS2 (activated HBsAg–PLGA in Table 1) on day 1
	One injection of 3 μg HBsAg–Alum + 9–18 μg HBsAg–PLGA
11	3 μg HBsAg–Alum + 9 μg MS2 on day 1
12	3 μg HBsAg–Alum + 9 μg MS1 on day 1
13	3 μg HBsAg–Alum + 9 μg MS3 on day 1
14	3 μg HBsAg–Alum + 4.5 μg MS1 + 4.5 μg MS2 on day 1
15	3 μg HBsAg–Alum + 3 μg MS1 + 6 μg MS2 on day 1
16	3 μg HBsAg–Alum + 6 μg MS1 + 3 μg MS2 on day 1
17	3 μg HBsAg–Alum + 6 μg MS1 + 6 μg MS2 on day 1
18	3 μg HBsAg–Alum + 6 μg MS1 + 12 μg MS2 on day 1
19	3 μg HBsAg–Alum + 12 μg MS2 on day 1
20[b]	3 μg HBsAg–Alum + 12 μg MS2 on day 1

[a]Each vaccine dose contained 0.4 mL volume and was administrated by subcutaneous injection as described in the Methods section; HBsAg–Alum, aluminum-adjuvanted HBsAg vaccine; MS, PLGA microsphere-formulated HBsAg vaccine (see Table 1) in which vaccine dose in micrograms is the amount of HBsAg.

[b]Group 20 used < 25 μm HBsAg–PLGA microspheres, whereas groups 6, and 8–19 used 25–45 μm HBsAg–PLGA microspheres.

(Figure 5A). In contrast, a single injection of a mixture of 3 μg of aluminum-formulated and 9 μg of PLGA-formulated HBsAg does result in a significant antibody response, as shown in Figure 5B. Thus, when the aluminum– or PLGA–HBsAg formulation was injected individually, both failed to elicit an appreciable immune response, yet when combined, a significant antibody was observed.

The data shown in Figures 6 and 7 indicate that the single injection of a mixture of aluminum-formulated HBsAg combined with PLGA microsphere-formulated HBsAg results in immune response profiles that are the same as or better than the two injections of aluminum-formulated vaccine. The antibody titers over time are examined in Figure 6, and the percentage of mice in each experimental group giving an anti-HBsAg serum antibody response > 10 mIU/mL at 9 months post the initial antigen injection are shown in Figure 7. Almost all single injections of aluminum-adjuvanted HBsAg mixed with PLGA-formulated HBsAg groups (groups 11, 12, 14–20 in Figure 7) have $> 80\%$ mice responding. In contrast, only two of the HBsAg–aluminum alone groups (both with two injections, groups 2 and 3 in Figure 7) have $> 80\%$ mice showing significant antibody response. The one low antibody titer group (group 13 in Figure 7) contains the mostly inactivated HBsAg formulation MS3 (Table 1).

The effect of total HBsAg dose in the PLGA microspheres on C3H mouse immune response was evaluated by measuring the serum antibody titers as a function of total HBsAg dose injected. A single injection of a mixture of aluminum- and PLGA-formulated HBsAg vaccine was performed for all groups in Figure 6 and groups 11–20 in Figure 7. In these experiments, the dose contribution from aluminum-formulated HBsAg is a constant of 3 μg of HBsAg, whereas the dose contribution from PLGA-formulated HBsAg varies from 9 to 18 μg. The results in Figures 6 and 7 show that increasing the total HBsAg dose in the PLGA formulation does not result in any significant increase in antibody titer.

No significant effect of PLGA–HBsAg microsphere size was observed on the anti-HBsAg serum antibody titers. Subcutaneous injection of PLGA–HBsAg microspheres with size ranges of 25 to 45 μm (groups 11–19 in Figure 7 and Table 3)

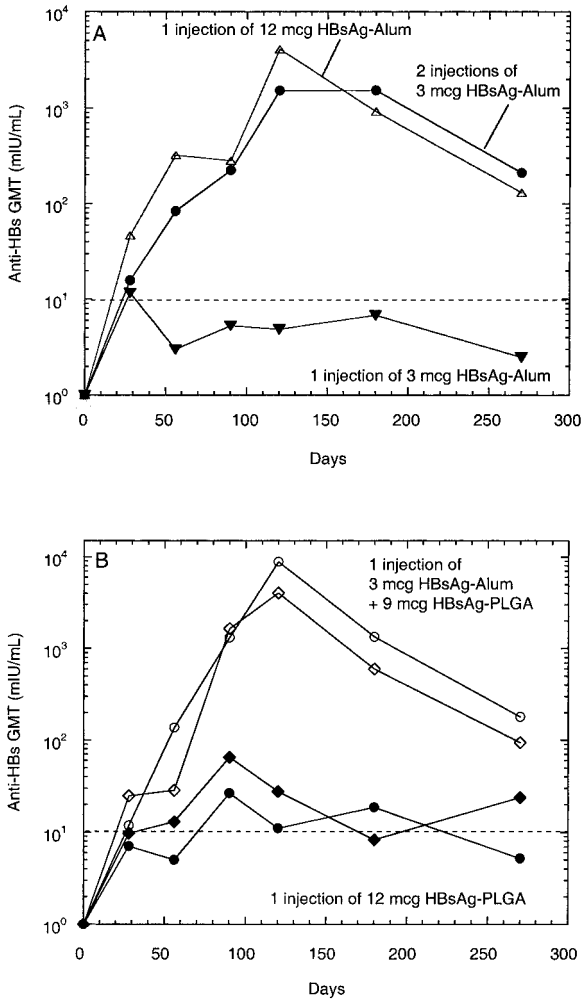

Figure 5. Geometric mean serum antibody titer response of C3H mice to various HBsAg formulations: (A) aluminum-formulated HBsAg vaccines; (B) PLGA microsphere-formulated HBsAg vaccines (filled symbols) and the mixture of aluminum-adjuvanted HBsAg and PLGA microsphere-formulated HBsAg vaccines (open symbols). The total number of doses administered (one or two) are indicated in the figure. "HBsAg–Alum" is aluminum-formulated HBsAg and "HBsAg–PLGA" is PLGA-formulated HBsAg as described in the Methods section. The formulations are as described in detail in Table 3: (A) open triangles (Group 7); filled circles (Group 2); filled triangles (Group 1); (B) open diamonds and circles (Groups 11 and 12); filled diamonds and circles (Groups 9 and 10).

and < 25 μm (group 20 in Figure 7 and Table 3) resulted in GMT profiles with minor differences (Figures 6A and 7). Previous work has suggested that sc injection of microsphere vaccines is less sensitive to the size and the number of microspheres than intraperitoneal injection.[89,90] Previous work with HBsAg encapsulated in

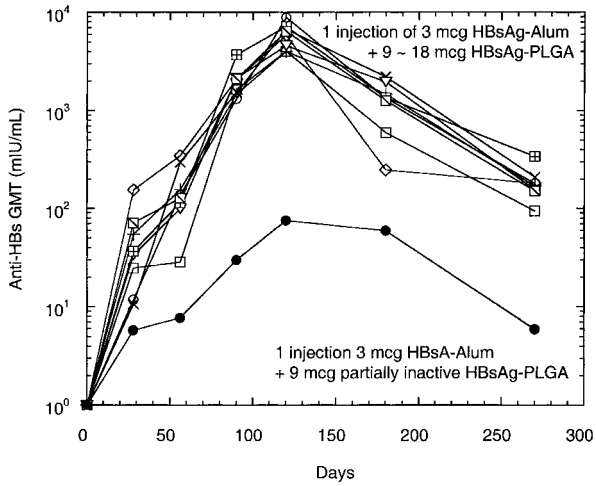

Figure 6. Geometric mean serum antibody titer response in C3H mice to a single injection of a mixture of aluminum- and PLGA microsphere-formulated HBsAg preparations. The total doses of HBsAg injected are indicated in the figure. The HBsAg–PLGA microsphere formulations used for the study were as follows: bottom curve (filled circles) was mostly inactivated (see MS3 in Table 1) and the top curves (8 groups) correspond to active groups 11, 12, and 14–20 as described in Table 3.

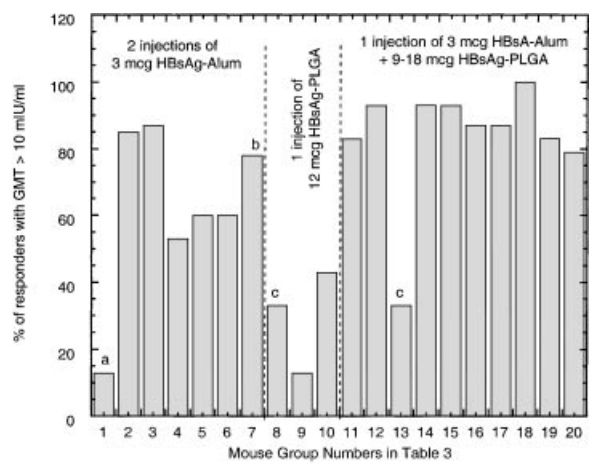

Figure 7. Percentage (%) of C3H mice with geometric mean serum antibody response > 10 mIU/mL at 9 months post initial injection. The percent values were determined based on the number of mice with a response > 10 mIU/mL out of total number (~15 of mice in the group). Formulations, doses, and injection times are indicated in Table 3: (a) 1 injection of 3 μg HBsAg–Alum; (b) 1 injection of 12 μg HBsAg–Alum; and (c) 1 injection of HBsAg–PLGA with mostly inactivated HBsAg.

polyglycolic acid (PLG) polymer microspheres had shown that smaller size microspheres administered intraperitoneally to guinea pigs elicited earlier antibody response, whereas the larger size microspheres provided delayed and longer duration of antibody production.[44] To benefit from the perceived effect of microsphere size on the antigen release profiles, small and large size microspheres have been used in combination to induce the immune response of CD1 mice to HBsAg.[27]

The effects of different HBsAg formulations for a booster dose were also evaluated to better understand the vaccination mechanism of PLGA microsphere-formulated HBsAg vaccine. The anti-HBsAg antibody responses induced by multiple vaccine injections in C3H mice were evaluated with the second injection differing in either vaccine formulation or injection time (groups 2–5 described in Figure 7 and Table 3). The first injections were all done with an aluminum-formulated HBsAg, whereas the second dose administrations were done with either an aluminum-formulated or unformulated (in saline) HBsAg. The second injections were performed at either day 28 or day 56 after the initial injection. The formulation differences of the second doses caused either a small or no significant differences in overall immune response profiles (9 month data of groups 2–5 shown in Figure 7). The nearly equal effectiveness of the aluminum-formulated and non-adjuvant HBsAg used for the second injection suggests that aluminum adjuvant is not needed for boosting the mice. This observation is consistent with the single injection of a mixture of aluminum- and PLGA-formulated HBsAg, which results in equal or better immune responses compared with multiple injections of aluminum-formulated HBsAg vaccine (Figures 5–7). When the second administration was changed from day 28 to day 56, a little lower overall immune response was induced in C3H mice (only 9 month data shown in Figure 7). Although this difference may be directly caused by the variation in availability of effective antigen dose at the assay time, it does suggest that the immune response of C3H mice maybe somewhat sensitive to second administration time, and therefore sensitive to the antigen release profile from the PLGA microsphere-formulated vaccine.[91]

PLGA polymers are frequently referred to as adjuvants when their sustained delivery function is discussed in the literature.[9,33,44,92] Adjuvants are compounds that when used in combination with specific vaccine immunogens, augment or stimulate the immune response.[92–94] In fact, these compounds mediate the vaccine immune enhancement through a variety of possible mechanisms, including nonspecific induction of several cytokines that regulate immune interactions.[92,94] Two groups of 15 mice were evaluated to test the possible adjuvant effect of PLGA microspheres using placebo PLGA microsphere formulations (MS4 in Table 1). The addition of a placebo PLGA formulation to aluminum-formulated HBsAg (Group 6 in Figure 7) resulted in somewhat lower serum antibody response in C3H mice than the same study without PLGA (Figure 5A and Group 2 in Figure 7). In addition, when the placebo PLGA is replaced by inactivated PLGA-formulated HBsAg, a similar low response is observed (Group 13 in Figure 7). An effective adjuvant would be expected to further enhance the immune response of C3H mice to the aluminum-formulated HBsAg vaccine. These results indicate that the PLGA, in this experimental system, does not act as a traditional adjuvant. The PLGA appears to instead delay the release of antigen *in vivo* to provide an auto-boost of the immune response.

CONCLUSIONS

The purpose of this study was to pharmaceutically characterize a HBsAg vaccine formulation with PLGA microspheres. HBsAg has been successfully encapsulated in PLGA microspheres and active antigen has been recovered upon release from the microspheres. The *in vitro* release studies indicate a controlled (delayed) release of HBsAg from PLGA microspheres, but that the *in vitro* release kinetics can be varied by experimental conditions, such as stirring rate. The biophysical and bioanalytical characterization studies indicate that the PLGA microsphere encapsulation process does not significantly affect the conformational integrity of the S protein or the *in vitro* antigenicity of HBsAg; however, partial delipidation of the HBsAg is observed. The *in vitro* storage stability in solution and *in vivo* immunogenicity in animals of these formulations were also examined. HBsAg is stable under slightly acidic conditions at 37°C, which is compatible with the reported pH conditions inside the PLGA microspheres.

A single injection of aluminum-formulated HBsAg (3-μg dose) in C3H mice does not result in any significant antibody titers, but the vaccine

does prime for an antibody response after a second injection. The second injection results in significant boosting from either aluminum-formulated or unformulated (in saline) HBsAg when the second injection occurs 1 or 2 months after the initial injection. A single injection of PLGA formulated vaccine did not result in any significant antibody titers. However, a single injection of a mixture of aluminum- and PLGA-formulated HBsAg results in a good antibody response that mimics multiple injections of aluminum-formulated vaccine.

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