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Prolonged Immune Response Evoked by a Single Subcutaneous Injection of Microcapsules Having a Monophasic Antigen Release

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Abstract

A model antigen, bovine serum albumin (BSA), was successfully incorporated into microcapsules fabricated from blends of poly(*d,l*-lactide-co-glycolide) and poly(*d,l*-lactide). The microcapsules possessed diameters ranging from 10 to 100 μm and exhibited a continuous monophasic release of the protein in-vitro for 3 weeks.

They were found to enhance its immunogenicity, thereby potentiating the anti-BSA antibody response following a single subcutaneous injection in mice and rabbits. Enzyme-linked immunosorbent assays demonstrated that the microcapsules provoked high-titre and long-lived immunoglobulin G immune responses over a period of 192 days in mice. When rabbits were immunized by a single subcutaneous injection of BSA-loaded microcapsules, a high level of anti-BSA antibody was still present in the sera obtained at 17 weeks post-injection. The immunization protocol using the BSA-loaded microcapsules was superior to that using BSA dissolved in saline or adsorbed to alum.

These microcapsules providing the controlled release of antigens may be valuable in designing better vaccine formulations.

Controlled-release technology has been considered with a view to increasing the immunogenicity of antigens and reducing the number of immunizations required to induce a high-titre immunoglobulin response. Among various types of biodegradable polymers used for achieving gradual and sustained release of antigens, lactic/glycolic acid polymers are prime candidates due to their excellent biocompatibility and safety in man. The release rates of antigen from microparticles can be manipulated by varying the molecular weight of polymers, the ratio of lactic to glycolic acids, and the concentration of active ingredients. However, in contrast to successful controlled delivery of small molecular weight drugs, it is found that these polymeric systems provide pulsatile-release kinetics of antigens; initial burst release of antigen is followed by trickle delivery with or without induction periods (Hora et al 1990; Cohen et al 1991; Shah et al 1992; O'Hagan et al 1994).

A heterogeneous microparticulate delivery system is also popular for the release of antigen in a multiphasic or pulsatile manner. Chang (1976) suggested that microcapsules prepared from different polymers would be degraded at various times so as to release entrapped antigen at different times. Eldridge et al (1991) reported a combination of two microspheric populations that had different susceptibility to phagocytosis by macrophages due to their particle size. They proposed that smaller microparticles were phagocytosed and the antigen released immediately whereas larger ones were phagocytosed more slowly, thereby releasing the antigen at a slower rate. In this way discrete primary and booster doses were made available following a single injection. Hazrati et al (1993) also demonstrated that a single shot of the mixture of antigen loaded in microspheres and adsorbed to alum was able to generate immune responses comparable with those generated by a conventional multi-dose immunization regimen.

However, very few studies have been carried out to achieve optimal release profiles for the delivery of antigens, since common multiphasic release characteristics are thought to mimic the effect of conventional booster injections. In the present study we have evaluated the adjuvanticity of model antigen-containing microcapsules to potentiate an immunoglobulin G (IgG) immune response in mice and rabbits. The antigen employed was bovine serum albumin (BSA). In contrast to current immunization approaches which depend upon multiphasic or pulsatile release of antigens, our efforts were directed at achieving prolonged immunity by means of continuous or controlled release of entrapped antigen from microcapsules. The effect of microencapsulated BSA vs alum-adsorbed BSA on immune responses was also investigated.

Materials and Methods

Materials

Birmingham Polymers Incorporated (Birmingham, AL, USA) was the supplier of poly(*d,l*-lactide-co-glycolide) with a lactide to glycolide ratio of 75 : 25 (intrinsic viscosity = 0.48 dL g^{-1} in chloroform at 25°C). This polymer is referred to as PLCG75 : 25. Poly(*d,l*-lactide) (molecular weight (MW) = 2000) was purchased from Boehringer Ingelheim (Ingelheim, Germany) and written

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as PLA2000 in the text. Polyvinyl alcohol (PVA; 88% hydrolysed, MW = 25 000) and sodium carboxymethylcellulose (MW = 80 000) were purchased from Polysciences Inc. (Warrington, PA, USA). Bovine serum albumin (BSA, A7030), alkaline phosphatase conjugated to anti-mice (A3562) or anti-rabbit IgG (A3687), *p*-nitrophenyl phosphate (pNPP, Sigma Fast) and Tween 20 were obtained from Sigma Chemical Co. (St Louis, MO, USA). Pierce (Rockford, IL, USA) was the supplier of Imject alum and bicinchoninic acid solutions.

Preparation of microcapsules

An aqueous solution of BSA (15 mg BSA was dissolved in 300 μL H_2O) was mixed with a polymeric solution (total 0·5–0·6 g polymers was dissolved in 7 mL methylene chloride). A primary water-in-oil (w/o) emulsion was produced, homogenizing the mixture at 15 000 rev min^{-1} for 1·5 min using a Polytron homogenizer (Kinematica GmbH, Switzerland). The emulsion was then poured into 1-L beaker containing 300 mL 4% aqueous polyvinyl alcohol solution to make a w/o/w emulsion, which was stirred at 470 rev min^{-1} using a plate stirrer (Model 400HPS/VWR Scientific; bar size 9·5 mm × 51 mm). After 30 min, an additional 700 mL water was slowly added to the emulsion over 30 min using a peristaltic pump. Microcapsules were then collected by filtration and dried under vacuum for 2 days. The actual loading of BSA in microcapsules was determined using [^{14}C]methylated BSA. To do so, 1 M NaOH was used to digest completely the radiolabelled BSA-containing microcapsules. After adjusting the pH of the solution to 7, its radioactivity was measured using a liquid scintillation counter to calculate the actual loading of BSA in microcapsules.

Scanning electron microscopic analysis

After placing microcapsules on aluminium mounts, they were coated with gold/palladium in a sputter-coating apparatus under an argon atmosphere. To observe the internal structure of microcapsules, they were embedded in epoxy resin and cross-sectioned before mounting. The samples were examined with an Amray 1400 scanning electron microscope operated at 10 kV.

In-vitro BSA release study

Microcapsules (65 mg) were placed into vials in 4 mL 10 mM phosphate-buffered saline (PBS, pH 7·4; 120 mM NaCl and 2·7 mM KCl) containing 0·02% polyvinyl alcohol and 0·02% sodium azide. The vials were kept at 37° C in a shaking water bath. At selected time intervals, microcapsule suspensions were centrifuged at 796 *g* using a CU-5000 Centrifuge (Damon/IEC Division). A portion (0·5 mL) of the supernatant was withdrawn and the suspensions were refilled with fresh PBS. The amount of BSA released from microcapsules was quantitated using a modified bicinchoninic acid (BCA) protein assay. Briefly, BCA working reagent was prepared by combining 50 parts of BCA stock solution and 1 part of 4% $CuSO_4 \cdot 5H_2O$. Forty microlitres of either standard or unknown samples was pipetted into microtitre plate wells to which was added 200 μL BCA working reagent. The microtitre plate was covered with a lid and incubated in a shaking water bath at room temperature for 2 h. Absorbance of the water-soluble purple product was measured at 550 nm using a microplate reader (Model 450/Bio-Rad Lab).

Alum preparation

Pierce's Imject alum was used as a preformulated immunogen-grade alum to effectively adsorb BSA, acting as an adjuvant for injection. Ten millilitres of Imject alum (45 mg mL^{-1}) was added dropwise with stirring to 10 mL BSA solution (5 mg mL^{-1}). The stirring was continued for 30 min at room temperature after alum addition was complete. The BSA-alum mixture was used to immunize animals.

Animal experimental protocol

Balb/c female mice and New Zealand White female rabbits were used and treated as approved by the Institutional Animal Care and Use Committee (IACUC) of Rutgers University.

Animal immunization

Nine Balb/c female mice (Harlan Sprague Dawley Inc., IN, USA), 21–23 g, were injected subcutaneoulsy with 500 μg BSA encapsulated into microcapsules (200 μL vehicle/mouse). BSA-containing microcapsules were suspended in an aqueous solution containing sodium carboxymethylcellulose (0·08%) and Tween 20 (0·02%) just before administration. Five milligrams of BSA, either adsorbed to alum or encapsulated into microcapsules, was used to immunize New Zealand White female rabbits (2·5–2·7 kg, HRP Co, PA, USA) with a subcutaneous injection (1 mL injection vehicle/rabbit, 3 per group). In the mice, one of the lower veins on the underside of the tail was nicked at appropriate time intervals so that blood samples could be taken. For rabbits, a blood collection needle (Vacutainer, 21G1 or 22G1) was inserted into a marginal ear to collect blood. The microcentrifuge tube containing blood was incubated at 37°C for 1 h and was flicked several times to dislodge the blood clot. After storing the tube at 4°C for 2 h, it was centrifuged to collect serum. The serum was frozen at −20°C until assayed for the levels of anti-BSA antibody, Pre-immune blood samples of animals were also obtained following the same procedure and the sera prepared were used as a control for the ELISA experiment.

Enzyme-linked immunosorbent assay (ELISA)

Anti-BSA antibody was detected by a solid-phase-immunoassay. Briefly, flat-well microtitre plates were coated with 100 μL BSA (30 μg mL^{-1} in 50 mM carbonate-bicarbonate buffer, pH 9·6), and refrigerated overnight. Plates were then washed with 50 mM Tris-buffered saline (TBS, pH 8·0; 138 mM NaCl and 2·7 mM KCl) containing 0·05% Tween 20. To avoid nonspecific antibody binding, 100 μL 1% gelatin in TBS was added to each well. Plates were incubated at 37°C for 1 h and washed three times with TBS. The sera obtained from mice or rabbits were diluted in TBS, and 100 μL of the diluted samples was loaded into each well of the microtitre plates. After incubation at room temperature for 2 h, they were washed as before. Then, each well was dispensed with 100 μL anti-mice IgG alkaline phosphatase conjugate (diluted to 1 : 1500 in TBS) or anti-rabbit IgG alkaline phosphatase conjugate (diluted to

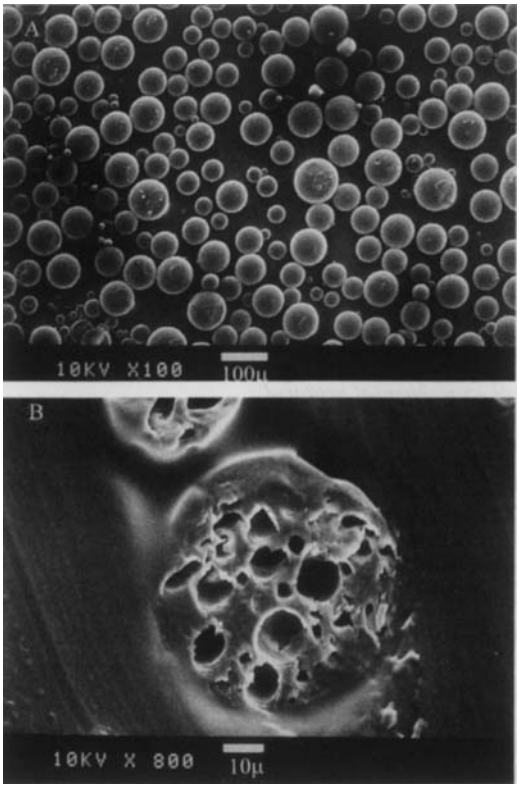

FIG. 1. Scanning electron micrographs of PLCG/PLA2000 (0·5/0·1, g/g) microcapsules. A and B illustrate their surface and internal structures.

1 : 1000 in TBS). The microtitre plates were incubated at room temperature for 2 h followed by extensive washings with TBS. Then, 200 μL *p*-nitrophenyl phosphate substrate solution (Sigma Fast) was added to each well and incubated again at room temperature for 15 min. The absorbance at 405 nm of the coloured enzymatic product was measured with a microplate reader (Model 450/Bio-Rad Lab).

Results and Discussion

It was possible to prepare microcapsules with different sizes by varying the concentration of polyvinyl alcohol in the external aqueous phase, the concentration of polymers in methylene chloride, shear force, and types of mixer to make the secondary w/o/w emulsion. More than 95% of microcapsules, prepared following the experimental conditions provided in this report, possessed diameters ranging from 10 to 100 μm as shown in Fig. 1. The surface morphology of all microcapsules was smooth and spherical, but the internal structures possessed multiple cavities. It has been previously reported that when microspheres are administered subcutaneously, depending on their size and hydrophobicity, they can either remain in the subcutaneous tissue or be phagocytosed by various macrophage populations (Tabata & Ikada 1988). The microcapsules reported in this study remain localized at the site of injection since they are too big to be engulfed and migrated into draining lymph nodes by macrophages.

In-vitro release profiles of a model antigen BSA from typical microcapsules are illustrated in Fig. 2. The results are plotted as cumulative percentage release of BSA against time in days (Fig. 2A). The microcapsules prepared from 0·5 g PLCG75 : 25 and 0·1 g of PLA2000 provided a near linear release of BSA upon incubation in PBS at 37 °C. When the amount of PLCG75 : 25 was decreased from 0·5 to 0·4 with PLA2000 fixed at 0·1 g, the cumulative release of BSA as a function of time increased. Both release characteristics can be described by first-order kinetics (Fig. 2B); ($r^2 > 0·99$). These continuous-release profiles are in contrast to common multiphasic and pulsatile release patterns of antigens from lactic/glycolic acid polymer systems, and are devoid of a typical initial burst effect often reported in current literature. Our preliminary experiments dealing with the optimization of microcapsule formulations showed that the duration and release characteristics of proteins could be further controlled by manipulating polymer composite, the loading degree of protein and the shear force to make the primary water-in-oil emulsion (Sah et al 1994).

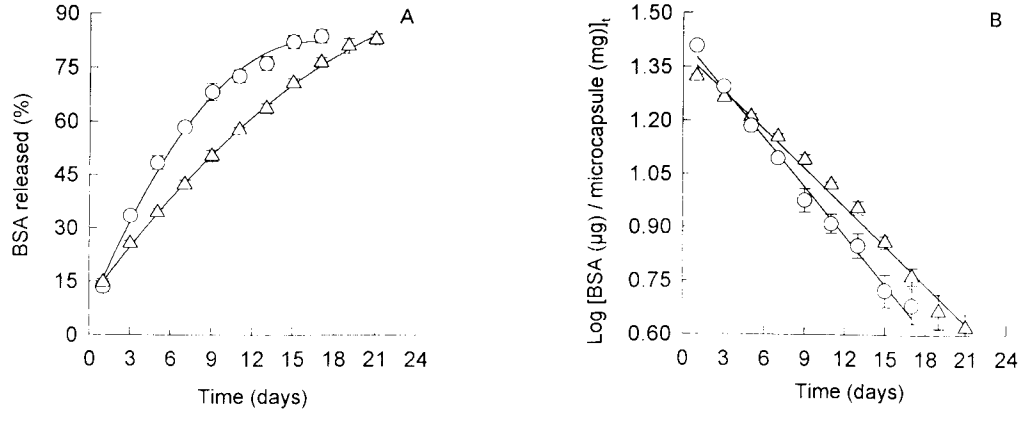

FIG. 2. In-vitro release profiles of BSA from microcapsules prepared with different PLCG75 : 25/PLA2000 ratios (g/g): △ 0·5/0·1 containing 24·6 μg BSA mg^{-1}; ○ 0·4/0·1 containing 29·5 μg BSA mg^{-1}. A. The results are plotted as cumulative percentage release of BSA against time. B. y-axis is the log value of BSA remaining (μg mg^{-1}) at time t. Values are mean ±s.d. (n = 3).

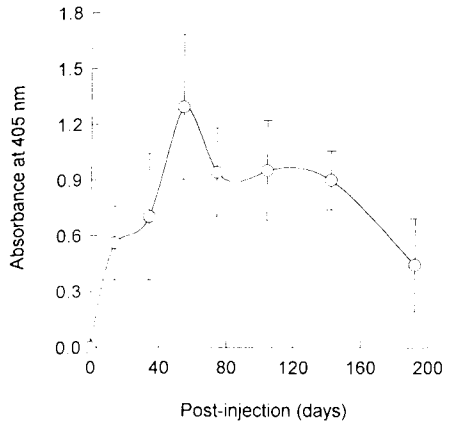

FIG. 3. IgG Immune responses of mice induced by a single subcutaneous injection of 500 μg BSA loaded in PLCG75 : 25/PLA2000 (0·5/0·1, g/g) microcapsules. Before the ELISA experiment, the sera obtained were diluted to 1 : 1600 in Tris-buffered saline. Error bar = s.e. (n = 9).

In-vivo experiments using mice were carried out to investigate the immunogenicity of a model antigen BSA in various dosage forms. Mice were immunized with a single subcutaneous injection of 500 μg BSA encapsulated in PLCG75 : 25/PLA2000 (0·5/0·1 g/g) microcapsules with BSA loading of 2·4 weight%. Before the ELISA experiment, the sera obtained were diluted to 1 : 1600 in Tris buffered saline (TBS). The levels of IgG anti-BSA antibody that appeared in response to such an immunization protocol are expressed as absorbance at 405 nm in Fig. 3. The highest anti-BSA antibody titre was observed at 54 days post-injection, and the strong immunity lasted over 192 days. This indicates that the entrapment of BSA in microcapsules and its controlled release made the protein strongly immunogenic. In contrast, when mice were immunized by a subcutaneous injection of 500 μg BSA in 0·9% NaCl (saline) solution, all blood samples obtained at the same time intervals provided absorbance values of less than 0·1. Spitznagel & Allison (1970) reported that repeated injections of high or low doses of free BSA for several weeks tend to induce tolerance rather than increase the antibody response. However, our microcapsules that exhibited a continuous release of BSA for 3 weeks in-vitro were found to stimulate its immunogenicity in-vivo.

Rabbits were also immunized by 5 mg BSA either adsorbed to alum or encapsulated in the same microcapsules. The blood samples were diluted from 1 : 800 to 1 : 25600 in TBS before the ELISA experiment. Fig. 4 illustrates titration curves of the sera obtained at the time intervals indicated. When rabbits were immunized subcutaneously with BSA adsorbed to alum, antibody titre reached a maximal level at week 2; considering that week 2 is the first time point, it can be speculated that the maximum could occur anytime between weeks 0 and 4. The rapid release of the anitgen may give rise to stronger adjuvant effect of the alum in an early immunization period. However, thereafter the level of anti-BSA antibody quickly declined. For example, the sera diluted to 12800 in TBS provided an absorbance ($\pm$ s.d.) of 1·71 $\pm$ 0·52 at week 2, but the value fell to 0·07 $\pm$ 0·03 at week 15 (Fig. 4A). The poor depot effect may be one reason for the decrease in the level of anti-BSA antibody. It is reported that the repository effect of alum is very low (Beh & Lascelles 1985). By contrast, BSA-loading microcapsules evoked immune responses that lasted much longer as shown in Fig. 4B. A peak antibody titre was observed at week 6, suggesting that maximal immune responses could occur between weeks 2 and 10. The strong immunity still persisted at week 17; at this time the sera diluted to 12800 in TBS displayed an absorbance value of 0·18 $\pm$ 0·09. The long-lived immunity evoked by BSA-loaded microcapsules, compared with that caused by BSA adsorbed to alum, may be attributed to the difference in the release rate of antigen from the adjuvants used. The depot effect seems to be enhanced by a microcapsule delivery system.

A single challenge using a large antigen dose can evoke the generation of antibody that can be maintained in-vivo, but it is produced in a cyclic fashion not at a constant rate. Follicular dendritic cells (FDC) which can trap and retain antigens are believed to be involved in the regulatory mechanism. A major function of FDC is to trap and retain antigen. It was suggested that they provide B-cells and provide B-cells with antigen that can then be presented to

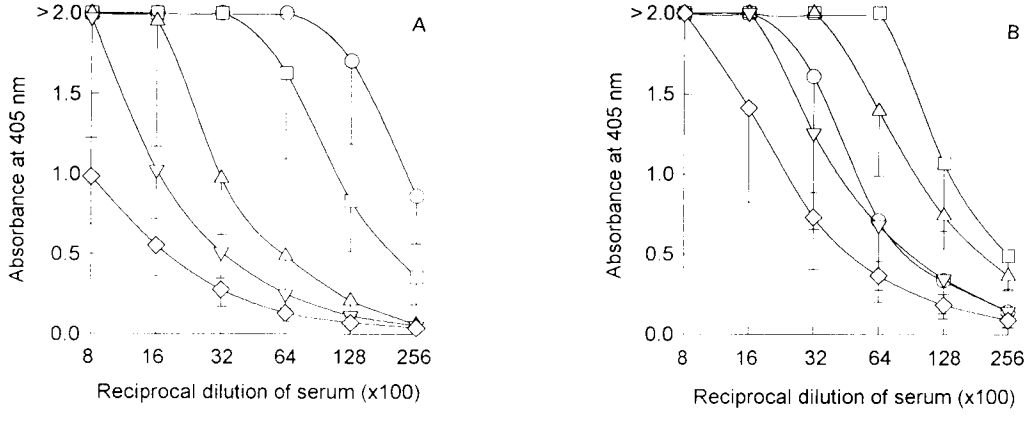

FIG. 4. Comparison of IgG immune responses of rabbits evoked by 5 mg BSA either adsorbed onto alum (A) or loaded in PLCG75 : 25/PLA2000 microcapsules (B). Before the ELISA experiment, the sera obtained were diluted to 6400 in Tris-buffered saline. Error bar indicates standard deviation of absorbance values (n = 3). A: ○ week 2, □ week 4, △ week 8, ▽ week 12, ◇ week 15; B: ○ week 2, □ week 6, △ week 10, ▽ week 14, ◇ week 17.

T-cells to elicit B-cell growth and differentiation factors (Tew et al 1980; Szakal & Tew 1992). Therefore, it is recommended to formulate vaccines in such a way that antigen is presented to the immune systems such as FDC in lymphoid tissues over an extended period of time. However, the results reported in this study suggest that even though directing antigen to FDC or antigen-presenting accessory cells is important to immunostimulatory mechanisms, the release rate of antigens from adjuvants at the site of injection also seems to play a pivotal role in immune responses. Gray & Skarvall (1988) reported that B-cell memory quickly disappears in the absence of antigen since it does not reside with very long-lived resting cells. Therefore, the presence of antigen for the continued stimulation of B-cell memory populations may result in the maintenance of a strong immune response. It can be suggested that microcapsules can carry out such a function by not only preventing the quick diffusion of free antigen through tissues but also providing entrapped antigen to immune accessory cells in a controlled manner for a long time.

In summary PLCG75 : 25/PLA2000 microcapsules that provide continuous release of a model antigen BSA in-vitro provoked a long-lasting immunity in mice and rabbits. The microcapsule system can be utilized to augment the immunogenicity of purified or synthetic protein antigens recently developed by recombinant technology. These antigens are usually poorly immunogenic and require adjuvants to stimulate their immunogenicity. Modification of the release rates of antigen from microcapsules and their combination with other chemical adjuvants such as muramyl dipeptide are likely to further potentiate the immunogenicity of antigen, although the further investigation of these parameters is required.

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