Biodegradable Delivery System for a Birth Control Vaccine: Immunogenicity Studies in Rats and Monkeys

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Purpose. The purpose of this study was to develop a single administration delivery system for a model birth control vaccine, in order to reduce the need for multiple injections and enhance immunogenicity.

Methods. The immunogen-loaded microspheres were prepared by solvent evaporation method and characterized for loading levels, size distribution and in vitro release kinetics. The microspheres were immunized intramuscularly in wistar rats and bonnet monkeys, and the antibody response was compared to that obtained with the same total dose of the immunogen on alum given at a monthly interval.

Results. Results indicated that a single injection of the immunogen entrapped in the microspheres generated a response comparable to that obtained by the same immunogen on alum injected at a monthly interval. The antibodies generated by the microspheres in the monkeys also had a good bioneutralization capacity indicating immunogen integrity during the microencapsulation process.

Conclusions. Biodegradable microspheres served as an effective delivery system for a model immunogen used in this study to reduce the need for frequent immunizations and enhance immunogenicity.

KEY WORDS: birth control vaccine; single shot vaccine; biodegradable polymers; vaccine delivery system; antibody response.

INTRODUCTION

The multiple injection schedule of most vaccines often leads to dropouts among subjects to be immunized causing failure of protection. This is all the more true in developing countries where the target population is large and health facilities are inadequate (1). Every extra injection in the immunization schedule leads to additional dropouts. Thus the reduction in the number of injections for any vaccine offers better efficacy and compliance with respect to mass immunization. A birth control vaccine based on β-hCG, currently undergoing clinical trials in India (2–4) also requires 3–4 injections to be administered to a woman in its primary schedule, very similar to the expanded programme of immunization (EPI) schedule for diphtheria, tetanus and pertussis. The development of a single shot vaccine that could generate a comparable antibody response with a single immunization would offer better patient compliance and reduced dropout rates.

Biodegradable microspheres have emerged as promising delivery systems for peptides and proteins. They can also be tailored to release a continuous amount of an immunogen for a prolonged period of time and generate a good immune response (5–8). Vaccine delivery systems based on the microsphere technology have been studied by various groups for the development of single dose vaccines, which would be more immunogenic and require fewer number of injections (9–11). Biocompatible and biodegradable polylactide-co-glycolide polymers have been used extensively in the development of such delivery systems by many groups (7,12–16).

The microspheres can be modified to obtain a desired in vivo release profile by varying the polymer composition and molecular weight. Control of the microsphere size can also help modify the immunogen uptake (9,12) and can thereby influence the immune response. Thus, the biodegradable microspheres can be designed to meet the individual needs of a vaccine and its immunization schedule.

In the present study, we have examined the immunogenic nature of a model birth control vaccine delivered by biodegradable microspheres. The encapsulated immunogen is a hetero species dimer (HSD) of α-ovine luteinizing hormone (α-OLH) and β-human chorionic gonadotropin hormone (β-hCG) linked to diphtheria toxoid (HSD-DT). The main object of the particular study was to examine whether a single injection of three unit doses of the immunogen entrapped in biodegradable microspheres could generate a desirable immune response in two model species. The duration of the antibody response and the bioneutralization capacity of the antibodies was also measured.

MATERIALS AND METHODS

Materials

The immunogen HSD-DT was obtained from the vaccine production unit of the National Institute of Immunology, New Delhi. Polylactide-co-glycolide (PLGA) and poly(D,L-lactide) (PLA) biodegradable polymers were obtained from Birmingham Polymers Inc. Birmingham (Alabama, U.S.A.). All other reagents were obtained from Sigma Chemicals (St. Louis, MO.) and used as received. Aluminum hydroxide gel (Alhydrogel®) was purchased from Superfos (Copenhagen, Denmark). Nunc microtiter plates were used for the enzyme linked immunosorbent assay (ELISA).

Preparation of Microspheres

The immunogen-loaded microspheres were prepared by the solvent evaporation technique previously reported by our group (7,10). Briefly, a 1.5% (w/w) loading level of the immunogen in the microspheres was prepared by dissolving the requisite volume of the unadsorbed immunogen and 15 mg of gelatin (stabilizer) in 1 mL of distilled water and then emulsifying at high speed using a overhead stirrer in 10 mL of 10% (w/v) solution of polymer dissolved in methylene chloride. This formed a primary w/o emulsion. The primary emulsion was then added to 100 mL of distilled water containing poly(vinyl alcohol) (1% w/v) and polyvinylpyrrolidone (0.5% w/v), resulting in the formation of a w/o/w emulsion which was stirred for 12 hours at room temperature. The

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methylene chloride was allowed to evaporate overnight and the resulting microspheres were filtered, washed twice with distilled water and dried in a dessicator.

Particle Size, Loading and *in vitro* Release Kinetics

The particle size distribution of the immunogen-loaded microspheres was measured using a stage micrometer. The surface morphology was determined by scanning electron microscopy (35 JEOL SEM) with a 100 A° gold-palladium coating. The amount of vaccine actually entrapped within the biodegradable microspheres was directly determined by dissolving 50 mg of the microspheres in 1 mL of methylene chloride. The immunogen was then extracted from the organic solvent into 1 mL of distilled water for quantitation by radioimmunoassay (RIA). The amounts of entrapped immunogen were also cross-checked indirectly by determining the amount remaining in the external poly(vinyl alcohol) solution after microencapsulation. The difference of theoretical loading and the amount present in the external phase gave the actual loading within the microspheres.

The *in vitro* release rate was studied on a shaking water bath maintained at 37°C. Briefly, 10 mg of the microspheres were placed in a 3 mL glass tube containing 1 mL of phosphate buffered saline (50 mM, pH 7.4). At periodic intervals, one vial was removed and the amount of immunogen released from the microspheres into the supernatant was determined by RIA. The release rate was measured in triplicate. The cumulative percentage of the released vaccine was calculated for each preparation.

Animals and Immunization

Outbred rats (male/female) of stock NII:Wistar, bred randomly, 8–10 weeks old and weighing 170–250 gms, were obtained from the small animal facility of the National Institute of Immunology, New Delhi. The animals were fed commercial pellet diet provided by Hindustan Lever Ltd (Bombay, India) 'ad libidum' and were maintained at 22–25°C with relative humidity of 50–60% and a 14 hr:10 hr (light:darkness) photoperiod. Male and female bonnet monkeys (Macaca radiata) in the age group of 2–3 years were housed at the Primate Research Center of the National Institute of Immunology, New Delhi.

In the first series of experiments, 4 groups of wistar rats having 8 animals per group were used in the study. Two groups were immunized with a single injection of the HSD-DT-loaded microspheres prepared using two different polymers (PLA [lactide monomer-100%] and PLGA [lactide:glycolide monomer ratio of 65:35]). The microspheres dose per animal was 30 μg of HSD-DT (based on 3 injections of a 10 μg unit dose on alum). The microspheres which were suspended in a special vehicle for injection to accord uniform dispersion, were injected intramuscularly. The vehicle comprised of 0.1% methyl cellulose and 0.05% Tween 80 in normal saline. The third group was immunized with placebo microspheres along with the vehicle in the same manner. The fourth group was immunized with three intramuscular injections of 10 μg of HSD-DT adsorbed on alum given at a monthly interval (day 0, 30 and 60). The animals were bled at a periodic interval by retro-orbital plexsus and the serum

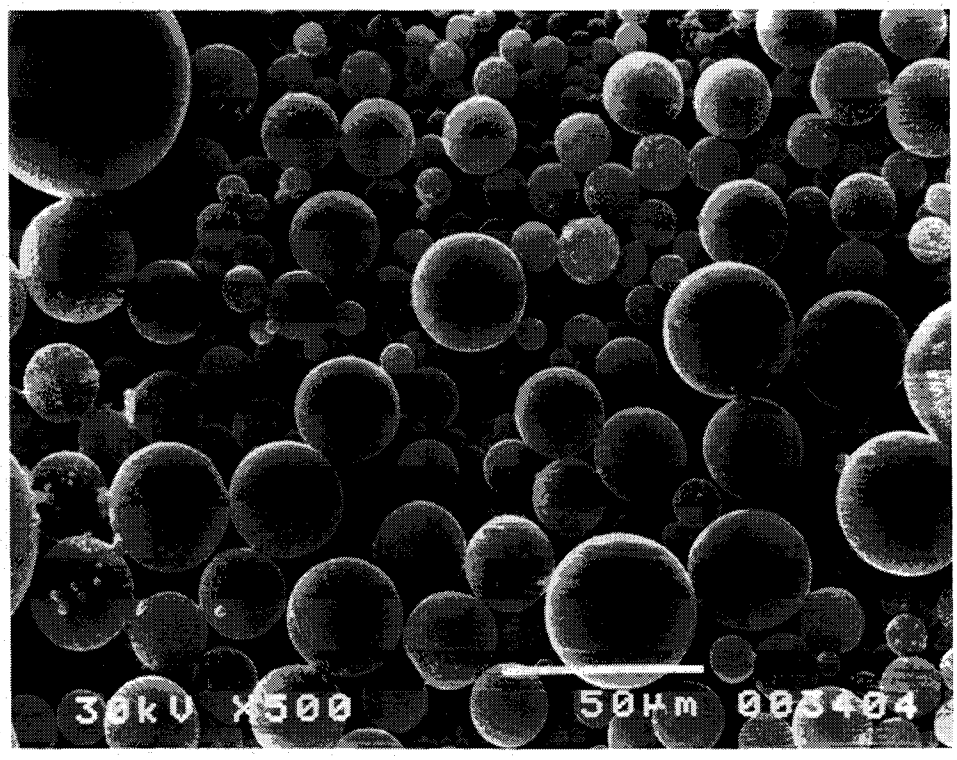

Fig. 1. Scanning electron micrograph of HSD-DT microspheres prepared from PLA Magnification 500×.

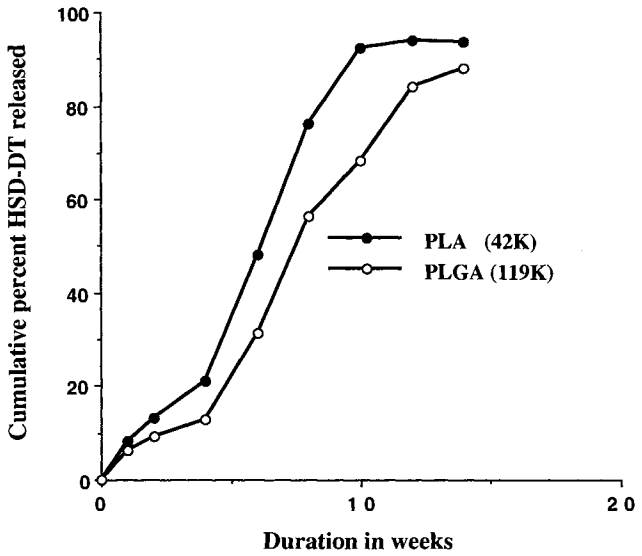

Fig. 2. *In vitro* release of HSD-DT from two different types of polymer microspheres PLA (Mol. wt. of 47,000 daltons) and PLGA (65:35 lactide:glycolide monomer ratio) (119,000 daltons). Average of three samples plotted. Standard deviation too small to represent.

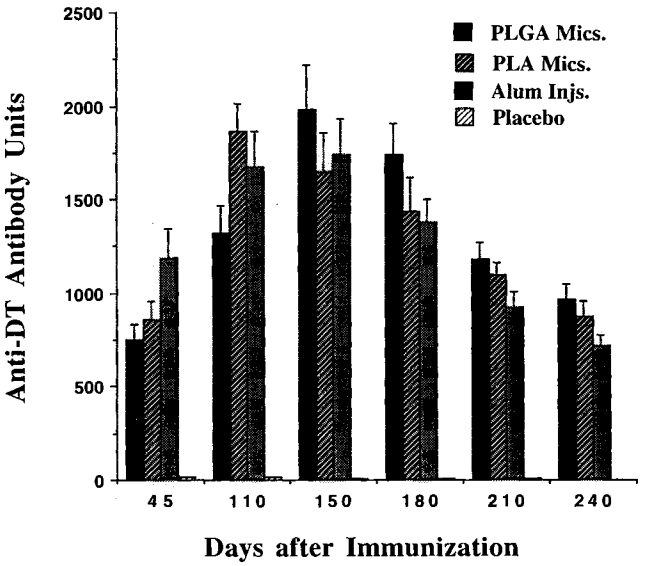

Fig. 4. Anti-DT response in groups of wistar rats receiving HSD-DT vaccine in microspheres as per dosing schedule described in Figure 3. Mean ± S.D.

was subjected to antibody titration by RIA (anti-hCG) and ELISA (anti-DT). Serum IgG titers were estimated in all groups.

Two groups of 4 monkeys each (2 males and 2 females) were used for the primate study. The first group was immunized with a single intramuscular injection of 900 μg of HSD-DT (equivalent to three human doses of 300 μg each) loaded in PLA and PLGA microspheres in a 1:1 ratio of these two microsphere types, which were suspended in the vehicle for injection. The second group received three separate intramuscular injections of 300 μg of HSD-DT adsorbed on alum every month. Both groups were re-immunized at day 225 with a single injection of 300 μg each on microspheres (PLA and PLGA in a 1:1 ratio of two types of microspheres) and alum, respectively. Blood was obtained at periodic interval by forearm vein bleeding and the serum was subjected to antibody titration. The bioneutralization capacity of the antibodies was also determined by a receptor binding assay.

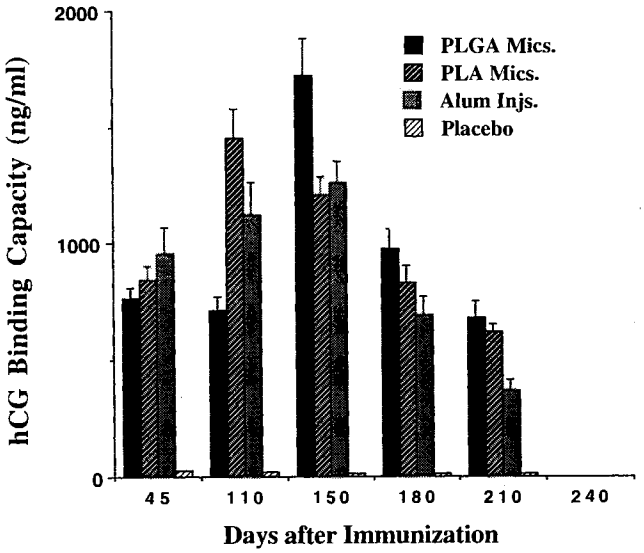

Fig. 3. Anti-hCG response in Wistar rats as measured by hCG binding capacity, expressed as ng/ml. Two groups were immunised with a single injection of PLGA and PLA microspheres containing 30 μg of entrapped HSD-DT, respectively. The third group received three injections of 10 μg of HSD-DT adsorbed on alum at a monthly interval. The fourth group was injected with blank microspheres. Mean ± S.D.

Anti-hCG and Anti-DT Antibody Estimation

Anti-hCG antibodies were assayed using a sensitive RIA method (3–4). The neutralization capacity of the anti-hCG antibodies was determined by a receptor binding assay (4). Anti-DT antibodies were estimated using an enzyme linked immunosorbent assay (ELISA) as previously reported (7). The antibody units of DT were calculated for each serum sample. All samples were also subjected to anti-hCG titration by RIA. An unpaired student's *t*-test was used to assess statistical significance of the mean values at various time points. Results were considered statistically significant if $p < 0.05$.

RESULTS

Microsphere Size, Loading and in Vitro Release Kinetics

The immunogen-loaded microspheres prepared in this study were in the size range of 5–90 μms as determined by optical microscopy. The SEM analysis exhibited the smooth and non-porous structure of the microspheres as shown in Figure 1. The loading efficiency in all of the HSD-DT preparations was over 80%. The mean loading efficiency at a 1.5% (w/w) initial vaccine concentration was measured as 89% for the HSD-DT microspheres.

The cumulative percent of HSD-DT released (mean of three values) with respect to time is depicted in Figure 2. The profile indicates a variable lag phase for the two microsphere

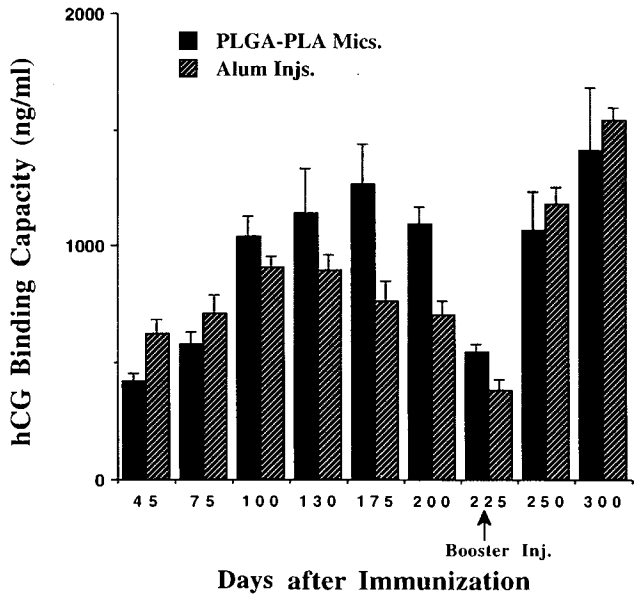

Fig. 5. Anti-hCG response in four bonnet monkeys (Macaca radiata) as measured by hCG binding capacity in ng/ml. The monkeys were immunized with three monthly injections of 300 μg of HSD-DT adsorbed on alum followed by a booster of 300 μg at day 225. A group of four bonnet monkeys were also immunized with a single injection of 900 μg of HSD-DT loaded in PLA-PLGA (1:1 ratio of two polymer microsphere mixture) followed by a booster of 300 μg of HSD-DT in microspheres (PLA:PLGA 1:1 mixture) at day 225. Mean ± S.D.

types. The burst or immediate release was also less than 10% of the total loading in both cases. Total release of HSD-DT from PLA microspheres occurred in 10 weeks, as compared to 14 weeks for PLGA microspheres.

In Vivo Studies

Rats. The anti-hCG response in groups of wistar rats immunized with a single injection of vaccine loaded microspheres, compared to the group receiving three injections of the vaccine on alum, is shown in Figure 3. The peak antibody response was observed at day 150 for both the microsphere and alum-adsorbed vaccine groups. The circulating antibodies were diminished by day 210 and were not detectable by day 240. Figure 4 depicts the carrier (DT) response in the same groups following immunization with HSD-DT in microspheres and on alum. The peak anti-DT titers were observed on day 110 for the alum-adsorbed formulation and day 150 for microsphere formulation. At day 240, the anti-DT titers were still fairly high in comparison to anti-hCG response.

Monkeys. Figure 5 depicts the anti-hCG response in the two groups as measured by the hCG binding capacity assay (RIA) represented in ng/ml. It can be seen that the maximum response in the alum group occurred at day 100 before the booster and rose again 75 days after the booster. The peak average anti-hCG titers were around 1000 ng/ml before the booster for this group. On the other hand it was observed that in the group receiving the microsphere formulation, the peak anti-hCG response occurred at day 175 and the titer values were about 1200 ng/ml. The titers were still rising following a booster in microspheres. The differences in response between the two groups at most time points were not statistically significant ($p > 0.05$ at most time points).

Figure 6 illustrates the binding and bioneutralization capacities of the antibodies generated in monkeys by the HSD-DT microsphere injection. The antibodies generated by the vaccine loaded microspheres has a strong bioneutralization capacity as determined by the receptor binding assay.

DISCUSSION

This study illustrates an attempt towards the development of a "single-shot delivery system" for vaccines currently requiring multiple injections. Our earlier work with other vaccines (7,10,11) and reports from other research groups (5,6,9) indicated the feasibility of such a delivery system for vaccines with biodegradable microspheres

The solvent evaporation method used in the preparation of the microspheres in this study resulted in smooth and free flowing vaccine loaded microparticles. The loading efficiency was over 80% at the 1.5% (w/w) theoretical loading level. The SEM analysis exhibited smooth and non-porous topography of these microspheres. The size of the microspheres ranged from 5 to 90 μms. The percentage of microspheres less than 10 μm (the range that is said to be phagocytosed by the macrophages) varied from 20 to 30% in the total formulation. This size-range probably engendered a priming and initial immunogenic response, followed by controlled release from larger microparticles (70 to 80%). It is reported (12,14,17) that the small sized microspheres (<10 μms) are first engulfed by the macrophages (antigen presenting cells) and the immunogen entrapped in them processed rapidly within the macrophages leading to the initial immune response. The larger microspheres (>10 μms) on the other hand tend to release the entrapped immunogen extracellularly but within close proximity of the macrophages and the immune response from these is delayed but more sustained.

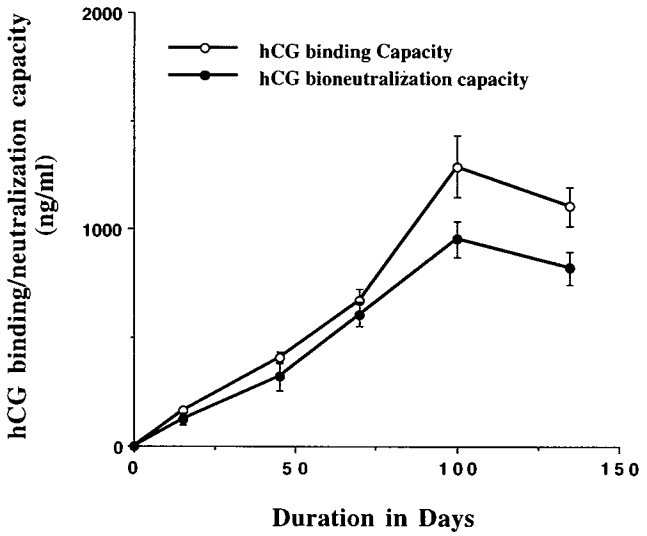

Fig. 6. The binding and bioneutralization capacities of anti-hCG antibodies generated in bonnet monkeys following immunization with HSD-DT entrapped microspheres. Mean ± S.D.

The cumulative response observed in this study is expected to arise from a combination of the two different size groups which present the antigen by different mechanisms. Earlier results (data not shown) with a single size range of microspheres suggested that the optimum response was observed with a polydispersed formulation rather than with a monodispersed formulation.

The in vitro release of the vaccine from the microspheres indicated that the molecular weights of polymers used (molecular weights 47 and 119 Kd) could sustain the release only upto a maximum period of 14 weeks in vitro. Increasing the polymer concentration beyond 10% to further retard release rates did not yield uniform and smooth microspheres due to increased viscosity of the polymer solution (data not shown). The duration of maximum release in vitro did not correlate with peak antibody response in vivo. This suggests that the immunogen release is much more retarded under in vivo conditions.

The in vivo response in rats demonstrated the ability of a single injection of the microspheres to elicit an antibody response comparable to that obtained with three divided doses of the same quantity of vaccine on alum administered at a monthly interval. Both polymer preparations were equally immunogenic and exhibited strong anti-hCG responses. The molecular weight of the polymer selected did not enable the microspheres to sustain the anti-hCG response beyond 12 months.

The study in primates at human dose level also reinforced the findings. The animals had to be boosted at day 225 to sustain the level of anti-hCG response over the minimum protective limit required for prevention of conception in humans (50 ng/ml as established by earlier studies) (4). The antibodies had a good bioneutralization capacity, which is a critical aspect in obtaining effective contraception with a birth control vaccine. This result also suggests the antigen integrity during the process of microencapsulation and lack of destruction of relevant epitopes by the entrapment process.

Keeping other parameters, such as size, polymer concentration, loading levels and monomer ratios constant, selection of a higher polymer molecular weight, is expected to retard the immunogen release even further and maintain the antibody levels above minimum protective limits for a year and beyond, thus warranting only a single injection of the birth control vaccine for a yearly protection from conception.

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