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Influence of particle size, antigen load, dose and additional adjuvant on the immune response from antigen loaded PLA microparticles

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Abstract

Polylactide (PLA) polymer particles entrapping tetanus toxoid (TT) were evaluated in terms of particle size, antigen load, dose and additional adjuvant for achieving high and sustained anti-TT antibody titer from single point intramuscular immunization. Admixture of polymer entrapped TT and alum improved the immune response in comparison to particle-based immunization. High and long lasting antibody titer was achieved upon immunization with 2–8 μm size particles. Microparticles within the size range 50–150 μm elicited very low serum antibody response. Immunization with very small particles (<2 μm) and with intermediate size range particles (10–70 μm) elicited comparable antibody response from single point immunization but lower in comparison to that achieved while immunizing with 2–8 μm size particles. Potentiation of antibody response on immunization of admixture of microparticles and alum was also dependent on particle size. These results indicate the need of optimal particle sizes in micron ranges for improved humoral response from single point immunization. Increasing antigen load on polymer particles was found to have positive influence on generation of antibody titers from particle based immunization. Maximum peak antibody titer of ∼300 μg/mL was achieved on day 50 upon immunization with particles having highest load of antigen (94 μg/mg of polymer). Increase in dose of polymer entrapped antigen resulted in concomitant increase in peak antibody titers indicating the importance of antigen stability, particle size and load on generating reproducible immune response. Optimization of particle size, antigen load, dose and use of additional adjuvant resulted in high and sustained anti-TT antibody titers over a period of more than 250 days from single point immunization. Serum anti-TT antibody titers from single point immunization of admixrure of PLA particles and alum was comparable with immunization from two divided doses of alum adsorbed TT.

Keywords: PLA particles; Tetanus toxoid; Particle size; Antigen load; Antibody response

1. Introduction

Polylactide co-glycolide (PLGA) and polylactide (PLA) polymer based particles have been investigated

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as an alternative vaccine delivery system (Hanes et al., 1997; Cleland, 1999; Boehm et al., 2002). They offer many advantages such as single point immunization for multidose vaccines, protection of antigen in vivo, flexibility of rate and profile of antigen release, co-encapsulation of multiple antigens, generation of cytotoxic T cell response and possibility of administration by non-invasive routes (Gupta et al., 1998; Raychaudhuri and Rock, 1998; Shi et al., 2002; Boehm et al., 2002; Jones, 2003; Peyre et al., 2004). Limited adjuvanticity of alum and it's failure to generate immune response against many of the new generation recombinant antigens as well as weak antigens have further necessitated research in the field of microencapsulated antigen. Extensive research has been carried out entrapping TT (Johansen et al., 2000; Raghuvanshi et al., 2002; Peyre et al., 2003), diphtheria toxoid (Boehm et al., 2002; Peyre et al., 2003, 2004) and hepatitis B surface antigen (Singh et al., 1997a; Shi et al., 2002) for developing single dose vaccine.

Research on controlled release vaccine delivery system has mainly focused on stability and immunogenic potential of the released antigen (Raghuvanshi et al., 1998; Weert van de et al., 2000; Schwendeman, 2002). It is imperative that antigenicity of the entrapped vaccine during polymer particle formulation should be preserved for generation of effective immune response. However, presentation of this immunoreactive antigen and its processing ultimately controls the magnitude and duration of immune response from single point immunization. Microparticle formulation parameters like particle size, load of antigen, presences of additional adjuvants and route of immunization have major influence on the quality and quantity of immune response from single point immunization. Current understanding of immune system and research on antigen entrapped microparticles suggests that these parameters may be very crucial for the presentation of antigen to immune cells and for controlling the overall immune response from these particles (Esparza and Kissel, 1992; Nakaoka et al., 1996; Igartua et al., 1998; Cleland, 1999; Johansen et al., 2000, 2001; Gutierro et al., 2002a,b). Uptake of particles by antigen presenting cells, their transport in to lymph nodes and interaction with tissue play an important role in generation of immune response from particulate vaccine formulation. However, there is no consistency in the literature regarding these above parameters and their influence on immune response from single point immunization. Particle size of the polymeric formulation has been considered as the major parameter for generation of immune response but with varied results (Nakaoka et al., 1996; Coombes et al., 1996; Johansen et al., 2000, 2001; Gutierro et al., 2002b). Same size particles when immunized with different routes are taken up by different antigen presenting cells thus elicit varied immune response (Newman et al., 2002). Even though small size particle (<5 μm) are preferably taken by macrophages (Tabata and Ikada, 1988), there are reports that higher size particles (>20 μm) also generate antibody response (O'Hagan et al., 1993; Hilbert et al., 1999). Antigen load on polymer particles and doses also influence the immune response depending on its route of immunization and uptake by antigen presenting cells (Uchida et al., 1994). Most of the reports on these above parameters have been investigated using oral, intradermal or intraperitonial route of immunization (Uchida et al., 1994; Uchida and Goto, 1994; Tabata et al., 1996; Nakaoka et al., 1996; Gutierro et al., 2002b; Carcaboso et al., 2004a,b). Rarely all these parameters important for improving the immunogenicity of particle entrapped antigen have been studied in relation to each other while using intramuscular route of immunization.

In the present study, polylactide (PLA) entrapped TT was used to evaluate the importance of particle characteristics in terms of size, antigen load, dose and use of additional adjuvant on immune response from single point intramuscular immunization. Combined effects of all these parameters associated with particle formulation were evaluated and optimized taking TT as a model antigen for potentiation of immune response from polymer entrapped antigens.

2. Materials and methods

2.1. Materials

Poly D,L-lactide (PLA, 45 kDa) were purchased from Birmingham Polymer Inc., USA. TT (3000 Lf/mL, protein content 8 mg/mL) was purchased from Serum Institute of India, Pune. Alum was 2% (w/v) Alhydrolgel from Superfos Biosector, Denmark. Rat serum albumin (RSA) [A-6272] and polyvinyl alcohol, MW 30,000 (PVA) was from Sigma

Chemicals, USA. Micro BCA protein assay kit was from Pierce, USA. Polyclonal antibodies to TT and goat anti-TT HRPO conjugate were from Reagent Bank of National Institute of Immunology, New Delhi, India.

2.2. Methods

2.2.1. Preparation of polymer particles

Polylactide polymer particles were prepared using w/o/w multiple emulsion solvent evaporation method (Raghuvanshi et al., 2001). Briefly, primary emulsion between internal aqueous phase containing TT and organic phase (50 mg/mL PLA solution in dichloromethane) was prepared by sonication (20 W, 80% duty cycle, 20 cycles) (Branson, Sonifier 450, USA). Along with TT, emulsifier Rat serum albumin (2.5%, w/v) and lyoprotectant sucrose (10%, w/v) was added to the internal aqueous phase to stabilize antigen during microencapsulation and freeze drying steps. Resulting primary emulsion was added drop wise to external aqueous phase containing 1% (w/v) PVA and 10% (w/v) sucrose solution in MQ water and homogenized (10,000 rpm for 10 min) (Virtis, Cyclone I.Q., USA) to get multiple emulsion. The multiple emulsion thus obtained was stirred overnight at room temperature and resulting particles were collected by centrifugation (20,000 rpm, 20 min), washed thrice with ice-cold MQ water and lyophilized to get free flowing powder. Theoretical TT loading was taken 5%, internal aqueous phase (IAP) to organic phase (OP) ratio was 1:20 and primary emulsion volume to external aqueous phase (EAP) ratio was 1:4 unless otherwise specified. Microparticles with different sizes were prepared by varying energy input and organic phase to external aqueous phase ratio during secondary emulsification stage. The detail formulation parameters are given in Table 1. For preparing microparticles with different antigen loads, formulation parameters used for 2–8 μm size PLA particles entrapping TT were used. Different concentrations of antigens were taken in the same volume of IAP during primary emulsification step to prepare similar sized particle having different antigen load.

2.2.2. Characterization of particle size and surface morphology

Size distribution of the particles was determined using GALAI-CIS-1 particle size analyzer. Surface morphology was viewed by scanning electron microscope (Jeol JSM 6100, Tokyo) after coating the particle surface with gold–palladium.

2.2.3. Estimation of protein content of polymer particles

To measure the protein content of particles, accurately weighed particles were dissolved in acetonitrile to solubilize the polymer while precipitating the encapsulated protein. The precipitated protein was pelleted by spinning at 5000 rpm for 10 min and was dissolved in 1% SDS solution. The protein content of the solution was determined by micro BCA assay. Protein was resolved in SDS-PAGE gel and the amount of TT in the mixture was determined by densitometry scanning of gel picture. Antigen load was calculated as the percent weight of TT per unit weight of polymer (μg TT/mg of PLA particles).

2.2.4. Estimation of TT released from microparticles by ELISA

To measure the immunoreactivity of TT released during in vitro release studies from the particles, following ELISA protocol was used. One hundred microlitres of anti-TT-antibody (10 μg/mL) solution in PB pH 7.4 was loaded in wells of 96 well, flat bottom,

Table 1
Preparation and characterization of different sized microparticles

Primary emulsion	Secondary emulsion	OP:EAP	Particle size range (μm)	Encapsulation efficiency (%)	TT load (μg/mg)
Son.	Stirring	1:100	50–150	30.5	26.4
Son.	Hom.	1:25	10–70	42.1	36.0
Son.	Hom.*	1:4	2–8	60.4	53.2
Son.	Son.	1:4	<2	56.8	49.3

OP, organic phase: 50 mg/mL polylactide in dichloromethane; EAP, external aqueous phase: 1% (w/v) polyvinyl alcohol and 10% (w/v) sucrose in water; Son., sonication (30 W, 0.2 output control, 40% duty cycle), 1 min; Hom., homogenization: 5000 rpm, 5 min; Hom.*, homogenization: 10,000 rpm, 10 min; Stirring: Thermolyne Cimarac-2 magnetic stirrer at 200 rpm, 10 min.

Nunc immunoplate and incubated at 37 °C for 1 h. Plate was then washed once with PBS-T (0.1% Tween 20 in 50 mM PBS pH 7.4) and blocked by filling the wells with 300 μL 1% BSA solution in PBS-T and incubating for 1 h at 37 °C. After washing it again with PBS-T, dilutions of TT, test solution and standard, in PBS-T were loaded in the wells and incubated for 1 h. Plate was then washed thrice with PBS-T and 100 μL of anti-TT-HRPO conjugate was put in the wells. After 1 h of incubation at 37 °C, the plate was washed thrice with PBS-T and color was developed with the help of *O*-phenyl diamine (OPD)/H_2O_2 in citrate phosphate buffer (pH 4.5). After 20 min the reaction was stopped with 50 μL/well of 5N H_2SO_4 and absorbance was read at 492 nm.

2.2.5. In vivo studies

2.2.5.1. Animals. Immunogenicity of the microparticles containing TT with different stabilizers or their combinations was evaluated in Wistar rats. Six female out bred Wistar rats were taken in each immunization group. Animals were maintained according to the guidelines established by the Institute Animal Ethics Committee of the National Institute of Immunology, New Delhi.

2.2.5.2. Immunization protocol. Particles containing required doses of TT were weighed and suspended in saline just before immunization. Immunization of admixture of particles and alum were carried out by adding 25 μL of alum (Aluminium hydroxide gel, 2%, w/v) per rat. Dose response study for microencapsulated antigen was carried out by dilution of particles suspended in saline but containing 25 μL of alum. Injection volume in all the case was 250 μL. Rats were injected intramuscularly with 5 Lf TT microencapsulated TT. Animals were bled at different time interval through retro-orbital plexus and serum anti-TT antibody titers were determined by ELISA.

2.2.5.3. ELISA protocol. Anti-TT IgG antibodies in rat sera were estimated as reported previously (10). Briefly 1 μg of TT in 100 μL of PB (50 mM, pH 7.4) was coated in each well of 96 well flat bottom Nunc immunoplates. The plates were then washed with PBS-T and blocked with 1% BSA solution. The plates were again washed with PBS-T and different dilutions of rat serum in PBS-T were added to the wells and incubated for 1 h at 37 °C. After washing thrice with PBS-T, 100 μL of goat-anti-rat-HRPO conjugate diluted in PBS-T was added into each well and incubated for 1 h at 37 °C. Finally, 100 μL of *O*-phenyl diamine (OPD) dissolved in citrate–phosphate buffer (pH 4.5) along with H_2O_2 was added to each well and incubated for 20 min at room temperature. Reaction was stopped by adding 5N H_2SO_4 (50 μL/well) and the absorbance was measured at 492 nm. The titers of the anti-TT antibody were defined as μg of antibody/mL of the sera.

2.2.5.4. Statistical analysis. Anti-TT antibody titers were defined as μg of antibody/mL of the sera employing affinity purified antibody as references in ELISA. Antibody titers of individual animal ($n = 6$) were estimated in duplicates and their concentrations (μg/mL) were determined as geometric mean. To analyze the statistical significance of the antibody titers students' *t*-test at 95% confidence level was carried out.

3. Results and discussion

3.1. Preparation and characterization of TT loaded PLA particles

PLA polymer was used in all the formulation as it elicits improved antibody titer in comparison to hydrophilic PLGA polymers (Raghuvanshi et al., 2001). Two sets of particles entrapping TT were prepared using w/o/w multiple emulsion solvent evaporation method. In the first set, microparticles of different sizes were prepared by varying energy input and organic phase to external aqueous phase volume ratio during secondary emulsification step. Four formulations of PLA particle entrapping TT were prepared having size ranges between 50 and 150 μm, 10 and 70 μm, 2 and 8 μm and less than 2 μm (Table 1). Encapsulation efficiency and TT load of these formulations are presented in Table 1. These size ranges were selected to delineate the role of macrophage uptake as an essential requirement for immune response from single point immunization. The large size particles (50–150 μm) will not be taken by macrophages where as the particle having size ranges 10–70 μm will have very little chance to be taken up as 5 μm has been reported to be the upper limit for phagocytosis by macrophages (Howie et al., 1993; Horisawa et al., 2002). In the

group of 2–8 μm, more than 90% of the particles have diameter less than 5 μm so they will be taken up by antigen presenting cells (Thiele et al., 2001) where as in the size group of <2 μm there will be further enhanced cellular uptake due to submicron size ranges of polymer particles (Desai et al., 1996). All the particles used for size dependent immunogenicity experiments have appreciable TT load, thus the amount of polymer injected in different groups were not very high to affect the antibody response. In fact the amount of particles used for immunization with 2–8 μm microparticles and less than 2 μm size particle were almost same.

Second sets of particles were prepared exclusively to evaluate the effect of antigen load of the PLA microparticles on antibody response. Microparticles with size ranges of 2–8 μm but TT loadings of 94.2, 28.2, 16.7, 5.8 and 1.3 μg TT/mg polymer were formulated. Except for the particle size experiments, PLA particle of size ranges between 2 and 8 μm were used in all other immunization studies. Scanning electron micrographs of the microparticles reveal spherical shape with uniformly porous morphology (Fig. 1). Presence of sucrose reduces the extent of protein aggregation during lyophilization of proteins (Lee and Timasheff, 1981) and thus was used as an excipient in the internal aqueous phase during particle formulation. Equivalent concentration of sucrose was also used in the external aqueous phase during secondary emulsification step to take care of the osmotic imbalance. Similar strategy of osmotic balance has been reported to improve the entrapment efficiency and release profile of lysozyme from polymer particles (Srinivasan et al., 2005). TT released from the particles was immunoreactive (data not shown).

3.2. Effect of particle size on immune response

Serum anti-TT IgG titers varied extensively while immunizing with different sized PLA particles (Fig. 2). Antibody titers from microparticles in the range of 50–150 μm were significantly lower (peak titer value 34.2 ± 4.6 μg/mL) than that obtained from smaller size particles through out the post immunization period (P value ≤ 0.05). Microparticles with size range of 2–8 μm elicited highest antibody titers (peak titer value 145.2 ± 48.6 μg/mL) and decreasing the particle size

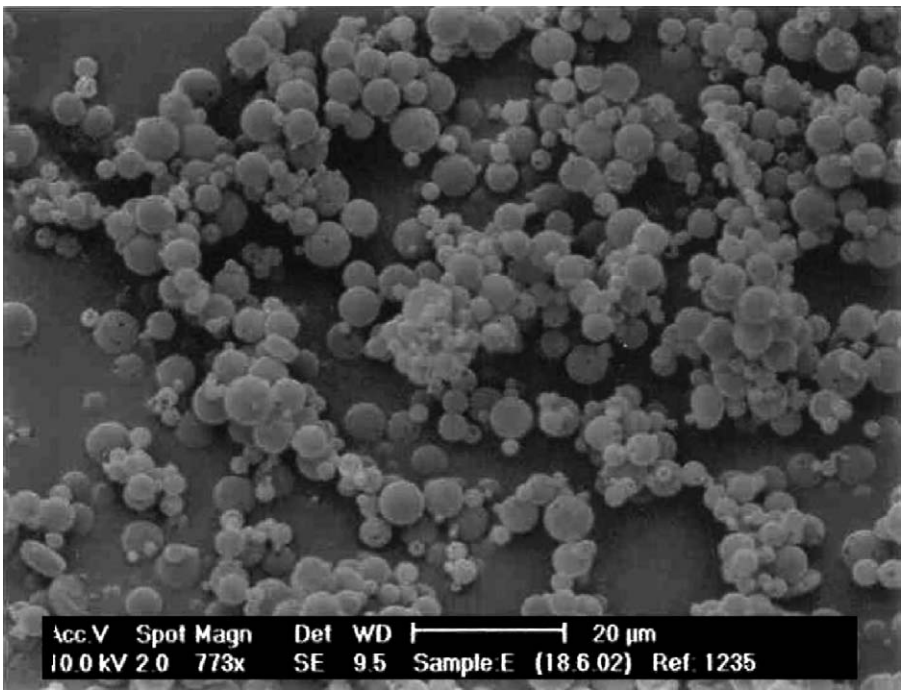

Fig. 1. Scanning electron micrographs of TT entrapped PLA particles of 2–8 μm size ranges.

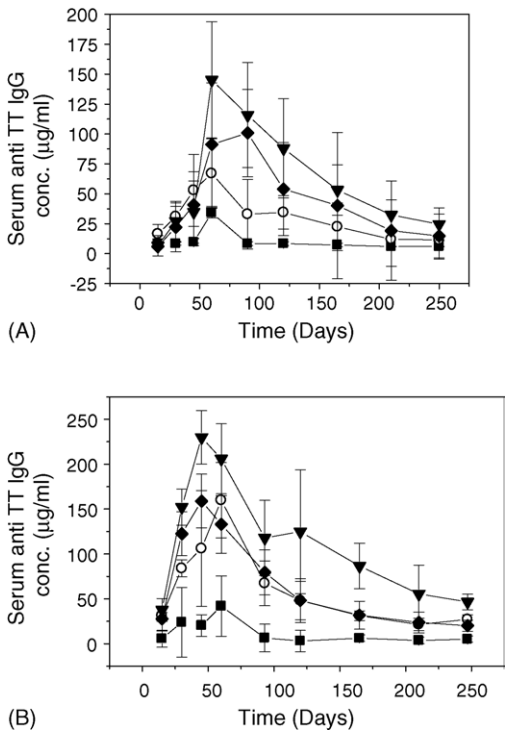

Fig. 2. Geometric means of Anti-TT IgG concentrations elicited by immunization with different sizes of PLA particles entrapping TT. PLA particles having size ranges 50–150 μm (-■-), 10–70 μm (-◯-), 2–8 μm (-▼-), <2 μm (-◆-) were immunized without alum (A) and with alum (B). Two months old female wistar rats (six in each group) were used for intramuscular immunization. Immunization dose was 5 Lf TT in all cases. One Lf of TT was equivalent to 2.6 μg of TT. TT loads in PLA microparticles were between 26.4 and 53.2 μg/mg polymer, details are given in Table 1.

further (lower than 2 μm) resulted in lower peak antibody titer (Fig. 2A). Anti-TT antibody titers from 2 to 8 μm sized particles were sustained at higher level till 250-days study period. This suggests that optimum particle size for eliciting antibody response is between 2 and 8 μm. Immunization with intermediate particle size range (10–70 μm) resulted in antibody response (67.8 ± 29.8 μg/mL) higher than the larger sized particles (50–150 μm) but lower than microparticles within size range 2–8 μm ($P > 0.95$). Similar effect of particle size on immune response has been observed previously during intraperitonial immunization of polymer-entrapped ovalbumin (Nakaoka et al., 1996).

Decrease in antibody titers in the groups immunized with <2 μm particle was surprising as particles in the submicron range are taken up more efficiently by the antigen presenting cells. The possible reasons for this may be enhanced exocytosis (Panyam and Labhasetwar, 2003) or less efficient antigen processing and presentation (Brewer et al., 2004; Fifis et al., 2004) than the particles in the micron range. Although enhanced uptake by antigen-presenting cells is one of the parameters, which improve the immunogenicity of particulate antigen, processing of the antigen in cellular compartment and presentation to lymphocyte dictates the magnitude of immune response (Brewer et al., 2004; Fifis et al., 2004). Rapid escape of nano-sized particles from endo lysosomal compartment to cytosol (Panyam et al., 2002) makes them suitable for cellular immune response (Raychaudhuri and Rock, 1998), which competes with generation of antibody response through MHC class II presentation pathways. As both cellular and humoral immune response complements each other, immunization with nanoparticles in general elicits lower antibody titer from single point immunization. This is indirectly supported by the fact that most of the time nanoparticle based immunization results in cellular immune response. Similar low antibody response has been reported for very small size particles (100–500 nm) in comparison to larger size particles (1000 nm) while immunizing with BSA loaded particles (Gutierro et al., 2002b).

It was also observed that very large size microparticles (50–150 μm), which are least likely to be taken up by antigen presenting cell elicit very low antibody response. Formulation with size ranges 10–70 μm still contains large populations of microparticles, which cannot be taken up by cells however showed high antibody response from single point immunization. Earlier studies have reported considerably antibody responses from microparticles of 10–90 and 15–60 μm (Johansen et al., 2000, 2001). Singh et al. (1997a,b) used microparticles within range 26–37 μm along with those with less than 10 μm for achieving immune responses equivalent to three doses of alum adsorbed TT (Singh et al., 1997b). Improved immune response using large particles have also been reported for polymer entrapped ovalbumin (O'Hagan et al., 1993) and Influenza A vaccine (Hilbert et al., 1999). Improved immune response form large size particles (10–70 μm) in the present case and from many reported cases indicated that particle phagocytosis is not absolute requirement for achieving high serum antibody titers.

Large size particle are preferentially attached to the macrophage surface (Horisawa et al., 2002), which thus can act as a depot system for continuous release of the entrapped antigen from the cell surface for antigen processing and presentation. For very large size particles (50–100 μm) surface adsorption and presentation of antigen will be low due to small size of the macrophages i.e. 10–15 μm sizes. Immunization with particles of the size ranges of 2–8 μm probably helped both in macrophage uptake and depot formation at the cell surface resulting in high serum antibody response from single point immunization.

3.3. Effect of co-administration of alum on antibody response from different sized PLA particles

Positive role of alum in improving antibody response by immunization with admixture of alum and antigen loaded microparticle has been reported previously for TT (Singh et al., 1997b; Johansen et al., 2000; Katare and Panda, 2001; Raghuvanshi et al., 2002; Katare et al., 2003). Skewing of immune response towards Th2 type due to co-administration of alum has been suggested to be responsible for improving antibody response elicited from microencapsulated antigens (Johansen et al., 2000). Besides, it has been observed that amounts of antigens released from microparticles in vitro in presence of alum are always lower than that from particles alone (Katare and Panda, 2001). Since polymeric formulations are always associated with burst release of antigen, presence of alum helps in presenting the released antigen to antigen presenting cell, which otherwise could have been lost in circulation. This suggests that antigen released from microparticles is adsorbed on to the alum in vivo and presented in a better way for improved antibody response. Apart from this, alum also helps in tissue inflammation leading to macrophage activation thus enhanced the antibody response when used along with particle for immunization.

In the present study it was observed that the co-administration of alum along with different sized microparticles led to differential degree of enhancement in antibody titers (Fig. 2B). Microparticles in the range 2–8 μm exhibited improved antibody titers when co-administered with alum (145.8 ± 43.8 to 229.6 ± 49.8 μg/mL). The enhancement of antibody titer using admixture of alum and <2 μm size particles was lower (91.2 ± 51.6 to 133.8 ± 31.6 μg/mL) than that observed for 2–8 μm size particles ($P < 0.95$). Immunization of particles of size between 10 and 70 μm along with alum also improved the serum antibody titers considerably (67.2 ± 29.6 to 159.9 ± 41.6 μg/mL). In the case of largest sized microparticles (50–150 μm) the improvement in antibody response was the least (34.2 ± 4.4 to 41.9 ± 33.4 μg/mL). Improvement in antibody titers while immunizing admixture of particles and alum was higher when the particles were in the micron range. Differential improvement of antibody response elicited by different sized microparticle on co-administration with alum showed that particle size influences the beneficial impact of additional adjuvants. Alum forms network type structure when used along with particles and holds particles in form of small clumps. Because of this, particles of micron ranges are held together by alum while getting attached to the surface of antigen presenting cells. This results in improved immune response while immunization with admixture of alum and particles. Very big sized particle (50–150 μm size) have large surface area in comparison to macrophage thus are neither taken up by macrophage nor get effectively attached to the cell surface for antigen presentation. Detail analysis of alum and polymer particles association both in vitro and in vivo are under investigation to elucidate the mechanism of potentiating effect of alum on improved immune response from particle based immunization.

3.4. Effect of antigen load on immune response

Our current understanding of the mechanism of antibody response suggests that the antigen content of microparticles has the potential to affect immune response. Still there are no reports on effect of antigen load on elicitation of antibody titers from particle-based immunization. It has been suggested that when microparticles with lower antigen loads are used for immunization, the same antigen dose is distributed over a larger number of microparticles. In such conditions, transport of phagocytosed microparticles containing antigen to secondary lymphoid tissues become saturated due to limited homing and transport capacity of the antigen presenting cells. This would create a situation of persisting rather than pulsatile antigen release and will help in improve immune response (Johansen

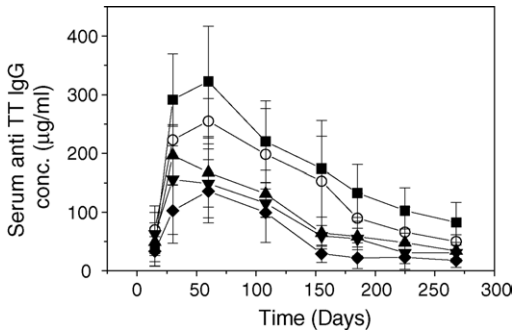

Fig. 3. Anti-TT IgG concentrations elicited by immunization of TT loaded PLA microparticles having TT loads of (μg/mg) 94.2 (-■-), 28.2 (-○-), 16.7(-▲-), 5.8 (-▼-), 1.3 (-♦-). Groups of six female wistar rats 2 months old were immunized with 5 Lf microencapsulated TT along with alum intramuscularly. One Lf of TT was equivalent to 2.6 μg of TT. Geometric means of serum antibody titers are presented with standard deviations.

et al., 2000). If this hold true, low antigen load particles should give better immune response than particle having high antigen load.

In the present investigation, it was observed that as the antigen load was enhanced from 1.3 to 28.2 μg/mg of PLA particles, peak antibody titers enhanced from 135.8 ± 53.4 to 254.6 ± 38.4 μg/mL. Further increase in antigen load to 94.2 μg/mg enhanced peak antibody titers to 320.0 ± 94.1 μg/mL (Fig. 3). The titers achieved by microparticles with higher antigen loads (thus lower number of microparticles) resulted in higher and more sustained antibody titers throughout the study period. This indicates that number of microparticles within a fairly large range is not the limiting factor in generation of immune responses particularly from intramuscular immunization. In the case of microparticles with high antigen loads, their interaction with antigen presenting cells result in delivering higher amounts of antigen inside antigen presenting cells. This subsequently leads to generation of high antibody titers.

It has been reported that immune response from exogeneous antigen is dependent on concentrations of antigen inside the antigen presenting cells (Vidard et al., 1996; Raychaudhuri and Rock, 1998). Priming of immune response with high initial load of antigen has been advocated for getting long lasting immune response through activation of T cell help (Slifka and Ahmed, 1998). It was observed that for high antibody titers from particles based immunization, the initial load of antigen delivered should be sufficiently high. This also helps in reducing the amount of polymer for single dose vaccine formulation. It has been previously reported that polymer degradation products affect antigen adversely (Weert van de et al., 2000), so lower concentration of antigen in polymer matrix will be more destabilizing to the antigen and will subsequently results in lower antibody titers. Thus polymeric particle with high antigen load is more beneficial for long lasting antibody response from single point immunization.

3.5. Dose response studies using TT entrapped PLA particles

Dose response studies were carried out by immunizing female wistar rats with 15, 10, 5 and 1 Lf of microencapsulated TT along with alum. Dose dependent increase in antibody titers was observed at all the time points through out the 9 months study period (Fig. 4). Maximum antibody titers of 400 μg/mL was achieved from single point intramuscular immunization with 15 Lf of PLA entrapped TT. Perceptible early titers were observed on 15th day with dose as low as 1 Lf of microencapsulated TT when co-administered with alum, which were significantly higher than 10 Lf of soluble TT ($P \leq 0.05$); immunization with admixture of PLA entrapped TT and alum thus improved the immune response almost 100 times. Peak antibody

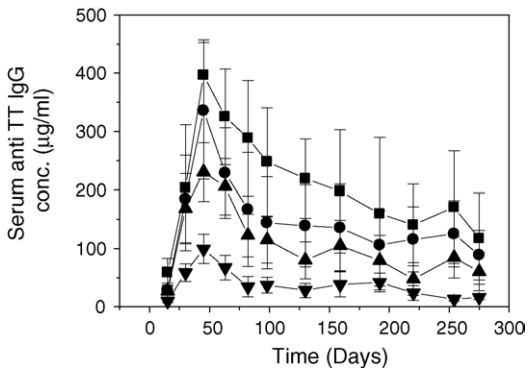

Fig. 4. Serum anti-TT IgG concentrations elicited by immunization with different doses of microencapsulated TT co-administered with alum in wistar rats. TT loaded microparticles equivalent to different dose of TT were co-administered along with alum intramuscularly to wistar rats. Doses of microencapsulated TT immunized for different groups of animals were 15 Lf (-■-), 10 Lf (-●-), 5 Lf (-▲-) and 1 Lf (-▼-) TT. One Lf of TT was equivalent to 2.6 μg of TT.

titers from particle-based immunization were observed between 7 and 9 weeks of post immunization.

Among the groups immunized with varying doses of PLA entrapped TT, peak antibody titers showed larger magnitude of dose dependent enhancement at lower doses. As the antigen doses were increased, corresponding rate of enhancement of peak antibody titers decreased. On the contrary, rate of change in early titers, kept on increasing with increasing doses of antigen. This implies that enhancing antigen doses increases the magnitude of early titers but does not leads to proportionate enhancement in peak titers. A dose dependent enhancement in the antibody titers was observed till the end of 250 days of study period.

Antibody sustainability index, which is defined as the ratio of serum antibody concentration on the last day of study period to the peak titers observed in a particular group was used to evaluate the influence of doses on immune response. Comparison of sustainability indices in groups immunized with different doses reveals that in the group immunized with 1 Lf antigen, sustainability index was 0.15 while the higher doses exhibited the sustainability indices between 0.26 and 0.29. Higher doses of microencapsulated antigen leads to better priming and makes enhanced amount of antigen available during post burst period thus helps in sustaining antibody concentrations at higher values. These results demonstrate that with proper formulation, antibody response can be achieved in dose dependent manner within a fairly large range of dose. This apart from demonstrating improved immunogenicity of the entrapped antigen at lower doses also serves as quality control parameter for the clinical evaluation of the particle-based formulation.

3.6. Comparison of immune response from single dose polymer entrapped TT with two doses of alum adsorbed TT

For evaluating the performance of the polymeric formulation against conventional two dose regimen of alum adsorbed TT; two groups of rats were immunized with single dose of 10 and 5 Lf microencapsulated TT along with alum where as two other groups were administered with two doses each of 5 and 2.5 Lf alum adsorbed TT at day 0 and 30 (Fig. 5A and B). Immunization with 10 Lf of PLA entrapped particles along with alum resulted

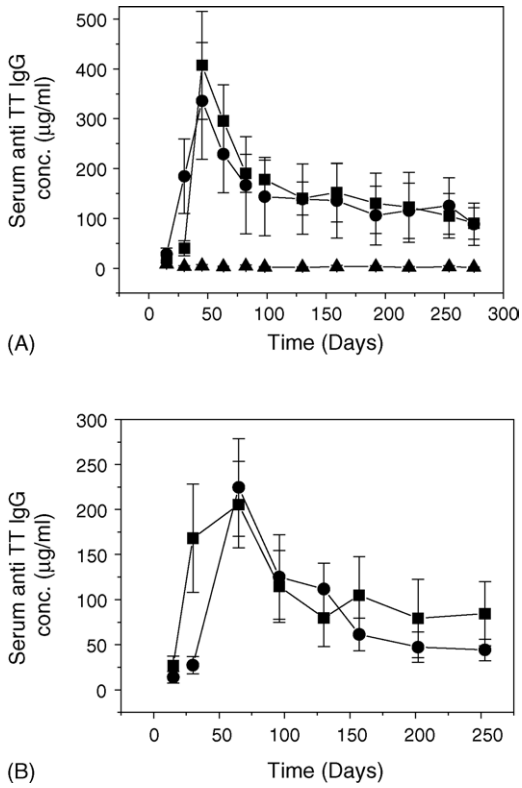

Fig. 5. Comparison of anti-TT IgG concentrations from immunization of single doses of TT microparticles co-administered with alum and two doses of alum adsorbed TT. (A) Two doses of 5 Lf alum adsorbed TT administered at day 0 and 30 (-■-), single dose of 10 Lf microencapsulated TT co-administered with alum (-●-), 10 Lf plain TT in saline (-▲-). (B) Single dose of 5 Lf (-■-) microencapsulated TT co-administered with alum and two divided doses each of 2.5 Lf (-●-) alum adsorbed TT given on day 0 and 30.

in comparable serum antibody response ($P > 0.95$) as observed from two doses of alum adsorbed TT (Fig. 5A). Antibody titers achieved in the group immunized with single dose microparticles based vaccine (184.64 ± 74.88 μg/mL) was higher than than the group immunized with the first dose of alum-adsorbed vaccine (40.3 ± 14.7 μg/mL). However, after the second dose of alum-adsorbed vaccine on day 30, antibody titers were higher (407.4 ± 108.2 μg/mL) than that observed with microparticles administered along with alum (335.8 ± 117.1 μg/mL). Immunization with 10 Lf of soluble TT did not resulted in appreciable antibody titers.

In the groups immunized with two divided doses (day 0 and 30) of 2.5 Lf of alum adsorbed TT vaccine

(Fig. 5B); antibody titers before second dose were considerably lower (27.3 ± 9.6 μg/mL) than those observed with microencapsulated antigen (168.1 ± 60.1 μg/mL). After the second doses of alum adsorbed TT at day 30, antibody titers enhanced rapidly. Difference in peak antibody titers between the two groups was not significant on day 60 (205.5 ± 48.1 μg/mL for the microparticles along with alum and 224.6 ± 54.2 μg/mL for two divided doses of alum adsorbed TT) as well as through out the study period ($P > 0.95$). Serum antibody concentrations were slightly higher in the case of two divided doses of alum-adsorbed vaccines.

Immunization with microparticles along with alum resulted in very high early titers as compared to the conventional vaccination. It was observed from the comparison of the four groups that microparticles performed better at lower dose than alum based conventional vaccines. The better performance of microparticles at lower doses shows their potential as single dose vaccine. Such differences were not observed at higher antigen doses probably because of saturation of the immune system. It was also observed that microparticles with higher peak titers elicited sustained high antibody response. Previous efforts to develop single dose vaccine have utilized mixing microparticles of varying sizes or degradation profiles. This study demonstrated that single population of microparticles having optimal size; antigen load and immunization along with alum can result in high and sustained antibody responses from single point immunization. Out of many studies on single dose TT vaccine using biodegradable polymer particles, very few have compared the antibody response from single dose polymer entrapped antigen with that of two divided doses of alum adsorbed TT (Kersten et al., 1996; Singh et al., 1997a,b; Johansen et al., 2001; Diwan et al., 2001). Most of them have shown improved antibody response from single dose polymer entrapped TT but very rarely the titers have been shown to be better or equal to that achieved from two divided doses of alum adsorbed TT. In the present study we have demonstrated that long lasting antibody response comparable to that observed from two divided dose of alum adsorbed TT can be achieved from single dose of admixture of polymer particles and alum in experimental animals. Improved antibody response particularly at lower doses of TT immunization from admixture of particle and alum was probably because of the use of hydrophobic polymer, excipients during particle formulation to reduce protein denaturation, optimal size and load of antigen used in this study. All of these parameters acted co-operatively towards the improved immune response from single point immunization.

4. Conclusion

In the present investigation it was observed that immune response from microencapsulated antigen was dependent on particle size, antigen load, dose and presence of additional adjuvant. Thus apart from taking care of protein stability problems associated with polymeric vaccine formulation, it is essential to optimize these above parameters for improved and sustained immune response. Differential influence of alum on the performance of different size microparticles, along with above observations suggests that both uptake and surface attachment of these particles by antigen presenting cells does have a major influence on generation of immune response. Using these inputs one could generate an optimal formulation, which when immunized in presence of alum elicit antibody titers comparable to two divided doses of alum-adsorbed vaccine. Immunogenicity of the entrapped antigen was found to be more at lower dose regime indicating the suitability of polymer entrapped antigen for development of single dose vaccine. The results are of indication that, optimal particle size in micron ranges, high load of antigen per unit amount of polymer particles and use of additional adjuvant are very important for generation of long lasting immune response from single point immunization.

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References

Boehm, G., Peyre, M., Sesardic, D., Huskisson, R.J., Mawas, F., Douglas, A., Xing, D., Merkle, H.P., Gander, B., Johansen, P., 2002. On technological and immunological benefits of multivalent single-injection microsphere vaccines. Pharm. Res. 19, 1330–1336.

Brewer, J.M., Pollock, K.G., Tetley, L., Russell, D.G., 2004. Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles. J. Immunol. 173, 6143–6150.

Carcaboso, A.M., Hernandez, R.M., Igartua, M., Rosas, J.E., Patarroyo, M.E., Pedraz, J.L., 2004a. Potent, long lasting systemic antibody levels and mixed Th1/Th2 immune response after nasal immunization with malaria antigen loaded PLGA microparticles. Vaccine 22, 1423–1432.

Carcaboso, A.M., Hernandez, R.M., Igartua, M., Rosas, J.E., Patarroyo, M.E., Pedraz, J.L., 2004b. Enhancing immunogenicity and reducing dose of microparticulated synthetic vaccines: single intradermal administration. Pharm. Res. 21, 121–126.

Cleland, J.L., 1999. Single-administration vaccines: controlled-release technology to mimic repeated immunizations. Trends Biotechnol. 17, 25–29.

Coombes, A.G., Lavelle, E.C., Jenkins, P.G., Davis, S.S., 1996. Single dose, polymeric, microparticle-based vaccines: the influence of formulation conditions on the magnitude and duration of the immune response to a protein antigen. Vaccine 14, 1429–1438.

Desai, M.P., Labhasetwar, V., Amidon, G.L., Levy, R.J., 1996. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. Pharm. Res. 13, 1838–1845.

Diwan, M., Khar, R.K., Talwar, G.P., 2001. Tetanus toxoid loaded preformed microsphere of cross-linked dextran. Vaccine 19, 3853–3859.

Esparza, I., Kissel, T., 1992. Parameters affecting the immunogenicity of microencapsulated tetanus toxoid. Vaccine 10, 714–720.

Fifis, T., Gamvrellis, A., Crimeen-Irwin, B., Pietersz, G.A., Li, J., Mottram, P.L., McKenzie, I.F., Plebanski, M., 2004. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J. Immunol. 173, 3148–3154.

Gupta, R.K., Singh, M., O'Hagan, D.T., 1998. Poly(lactide-co-glycolide) microparticles for the development of single dose controlled-release vaccines. Adv. Drug Del. Rev. 32, 225–246.

Gutierro, I., Hernandez, R.M., Igartua, M., Gascon, A.R., Pedraz, J.L., 2002a. Influence of dose and immunization route on the serum IgG antibody response to BSA loaded PLGA microspheres. Vaccine 20, 2181–2190.

Gutierro, I., Hernandez, R.M., Igartua, M., Gascon, A.R., Pedraz, J.L., 2002b. Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine 21, 67–77.

Hanes, J., Cleland, J.L., Langer, R., 1997. New advances in microsphere-based single dose vaccines. Adv. Drug Del. Rev. 28, 97–119.

Hilbert, A.K., Fritzsche, U., Kissel, T., 1999. Biodegradable microspheres containing influenza A vaccine: immune response in mice. Vaccine 17, 1065–1073.

Horisawa, E., Kubota, K., Tuboi, I., Sato, K., Yamamoto, H., Takeuchi, H., Kawashima, Y., 2002. Size-dependency of D,L-lactide/glycolide copolymer particulates for intraarticular delivery system on phagocytosis in rat synovium. Pharm. Res. 19, 132–139.

Howie, D.W., Menthey, B., Hay, S., Roberts, B.V., 1993. The synovial response to intraarticular injection in rats of polyethylene wear particles. Clin. Orthop. 292, 352–357.

Igartua, M., Hernandez, R.M., Esquisabel, A., Gascon, A.R., Calvo, M.B., Pedraz, J.L., 1998. Enhanced immune response after subcutaneous and oral immunization with biodegradable PLGA microspheres. J. Control. Rel. 56, 63–73.

Johansen, P., Estevez, F., Zurbriggen, R., Merkle, H.P., Gluck, R., Corradin, G., Gander, B., 2001. Towards clinical testing of a single-administration tetanus vaccine based on PLA/PLGA microspheres. Vaccine 19, 1047–1054.

Johansen, P., Gander, B., Merkle, H.P., Sesardic, D., 2000. Ambiguities in the preclinical quality assessment of microparticulate vaccines. Trends Biotechnol. 18, 203–211.

Jones, D.H., 2003. Microencapsulation of vaccine antigens. Meth. Mol. Med. 87, 211–222.

Katare, Y.K., Panda, A.K., 2001. Effect of alum on in vitro release profile and immunogenicity of microencapsulated tetanus toxoid. Proc. Int. Symp. Control. Rel. Bioact. Mater. 28, 1059–1060.

Katare, Y.K., Panda, A.K., Lalwani, K., Haque, I.U., Ali, M.M., 2003. Potentiation of immune response from polymer entrapped antigen: towards the development of single dose tetanus toxoid vaccine. Drug Del. 10, 231–238.

Kersten, G.F.A., Donders, D., Akkermans, A., Beuveery, E.C., 1996. Single shot with tetanus toxoid in biodegradable polymer microsphere protects mice despite acid induced denaturation of the antigen. Vaccine 14, 1632–1672.

Lee, J.C., Timasheff, S.N., 1981. The stabilization of proteins by sucrose. J. Biol. Chem. 256, 7193–7201.

Nakaoka, R., Inoue, Y., Tabata, Y., Ikada, Y., 1996. Size effect on the antibody production induced by biodegradable microspheres containing antigen. Vaccine 14, 1251–1256.

Newman, K.D., Elamanchili, P., Kwon, G.S., Samuel, J., 2002. Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells in vivo. J. Biomed. Mater. Res. 60, 480–486.

O'Hagan, D.T., Jeffery, H., Davis, S.S., 1993. Long-term antibody responses in mice following subcutaneous immunization with ovalbumin entrapped in biodegradable microparticles. Vaccine 11, 965–969.

Panyam, J., Zhou, W.Z., Prabha, S., Sahoo, S.K., Labhasetwar, V., 2002. Rapid endo-lysosomal escape of poly(D,L-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. FASEB J. 16, 1217–1226.

Panyam, J., Labhasetwar, V., 2003. Dynamics of endocytosis and exocytosis of poly(D,L-lactide-co-glycolide) nanoparticles in vascular smooth muscle cells. Pharm. Res. 20, 212–220.

Peyre, M., Sesardic, D., Merkle, H.P., Gander, B., Johansen, P., 2003. An experimental divalent vaccine based on biodegradable microspheres induces protective immunity against tetanus and diphtheria. J. Pharm. Sci. 92, 957–966.

Peyre, M., Audran, R., Estevez, F., Corradin, G., Gander, B., Sesardic, D., Johansen, P., 2004. Childhood and malaria vaccines combined in biodegradable microspheres produce immunity with synergistic interactions. J. Control. Rel. 99, 345–355.

Raghuvanshi, R.S., Goyal, S., Singh, Om, Panda, A.K., 1998. Stabilization of dichloromethane-induced protein denaturation during microencapsulation. Pharm. Dev. Technol. 3, 269–276.

Raghuvanshi, R.S., Singh, Om, Panda, A.K., 2001. Formulation and characterization of immunoreactive tetanus toxoid biodegradable polymer particles. Drug Del. 8, 99–106.

Raghuvanshi, R.S., Katare, Y.K., Lalwani, K., Ali, M.M., Singh, O., Panda, A.K., 2002. Improved immune response from biodegradable polymer particles entrapping tetanus toxoid by use of different immunization protocol and adjuvants. Int. J. Pharm. 245, 109–121.

Raychaudhuri, S., Rock, K.L., 1998. Fully mobilizing host defense: building better vaccines. Nat. Biotechnol. 16, 1025–1031.

Schwendeman, S.P., 2002. Recent advances in the stabilization of proteins encapsulated in injectable PLGA delivery systems. Crit. Rev. Ther. Drug Carrier Syst. 19, 73–98.

Shi, L., Caulfield, M.J., Chern, R.T., Wilson, R.A., Sanyal, G., Volkin, D.B., 2002. Pharmaceutical and immunological evaluation of a single-shot hepatitis B vaccine formulated with PLGA microspheres. J. Pharm. Sci. 91, 1019–1035.

Singh, M., Li, X.M., McGee, J.P., Zamb, T., Koff, W., Wang, C.Y., O'Hagan, D.T., 1997a. Controlled release microparticles as a single dose hepatitis B vaccine: evaluation of immunogenicity in mice. Vaccine 15, 475–481.

Singh, M., Li, X.M., Wang, H., McGee, J.P., Zamb, T., Koff, W., Wang, C.Y., O'Hagan, D.T., 1997b. Immunogenicity and protection in small-animal models with controlled-release tetanus toxoid microparticles as a single dose vaccine. Infect. Immun. 65, 1716–1721.

Slifka, M.K., Ahmed, R., 1998. Long-lived plasma cells: a mechanism for maintaining persistent antibody production. Curr. Opin. Immunol. 10, 252–258.

Srinivasan, C., Katare, Y.K., Muthukumaran, T., Panda, A.K., 2005. Effect of additives on encapsulation efficiency, stability, and bioactivity of entrapped lysozyme from biodegradable polymer particles. J. Microencapsul. 22, 127–138.

Tabata, Y., Ikada, Y., 1988. Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophage. Biomaterials 9, 356–362.

Tabata, Y., Inoue, Y., Ikada, Y., 1996. Size effect on systemic and mucosal immune responses induced by oral administration of biodegradable microspheres. Vaccine 14, 1677–1685.

Thiele, L., Rothen-Rutishauser, B., Jilek, S., Wunderli-Allenspach, H., Merkle, H.P., Walter, E., 2001. Evaluation of particle uptake in human blood monocyte-derived cells in vitro. Does phagocytosis activity of dendritic cells measure up with macrophages? J. Control. Rel. 76, 59–71.

Uchida, T., Martin, S., Foster, T.P., Wardley, R.C., Grimm, S., 1994. Dose and load studies for subcutaneous and oral delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin. Pharm. Res. 11, 1009–1015.

Uchida, T., Goto, S., 1994. Oral delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin as vaccine formulation: particle size study. Biol. Pharm. Bull. 17, 1272–1276.

Vidard, L., Kovacsovics-Bankowski, M., Kraeft, S.K., Chen, L.B., Benacerraf, B., Rock, K.L., 1996. Analysis of MHC class II presentation of particulate antigens of B-lymphocytes. J. Immunol. 156, 2809–2818.

Weert van de, M., Hennink, W.E., Jiskoot, W., 2000. Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm. Res. 17, 1159–1167.