

Correlation Between In Vitro Release and In Vivo Immune Response from Biodegradable Polymer Particles Entrapping Tetanus Toxoid

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Release of immunoreactive antigen from biodegradable polymer particles in a manner mimicking the normal vaccination schedule is one of the most important requirements for the successful development of single dose vaccine formulation. Tetanus toxoid (TT) was used as a model antigen to develop single dose vaccine formulations using poly(lactide-co-glycolide) and poly(lactide) polymers. An amphiphilic stabilizer such as RSA or PVA was used at an optimal concentration in the internal aqueous phase during preparation of immunoreactive TT particles using the multiple emulsion solvent evaporation method. Particles made from different polymers released immunoreactive TT continuously for more than 4 months in vitro and release profiles were in accordance with the degradation characteristics of the polymer. Initial burst release of antigen from the particles was controlled by incorporation of different concentrations of PVA in the internal aqueous phase during particle formulation. The extent of antigen release from the particles was varied by changing the aqueous to organic phase volume during the primary emulsification stage of particles formulation. Use of above formulation variables resulted in the formulation of polymer particles having different in vitro release characteristics. Anti-TT antibody titers in vivo were also in accordance with the in vitro release characteristics of immunoreactive TT from the particles. Anti-TT antibody titers from the stabilized particles were much better than that observed from particles made without stabilizers. These results indicate the importance of stabilizers and different formulation variables for the preparation of polymer particles having desired in vitro release characteristics.

Keywords Anti-TT Antibody Titer, In Vitro Release, Microparticles, PLA, PLGA, Poly(Lactic Acid), Stability, Tetanus Toxoid

Biodegradable polymers such as poly(lactide-co-glycolide) (PLGA) and poly(lactide) (PLA) entrapping vaccine/antigens

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using the multiple (W/O/W) emulsion technique have been extensively used for the development of single dose vaccines (Cleland 1998, 1999; Hanes et al. 1997). One of the most important requirements for the successful development of single-dose vaccines is that the protein/antigen entrapped and released from particles remain competent as an immunogen and produce desirable levels of immune response. In the past few years, protein instability has become recognized as a major hurdle toward the development of single dose vaccine (Johansen et al. 1998a; Putney and Bruke 1998; Weert et al. 2000a). Even though the polymers degrade due to bulk erosion and hydrolysis (Gopferich 1996), in vitro release of the entrapped antigen from particles is frequently incomplete (Crofts and Park 1997; Gupta et al. 1997).

In many reports dealing with polymer particle based immunization, a great deal of ambiguities exist due to antigen stability related problems (Johansen et al. 2000). Very little has been reported on the correlation between in vitro release kinetics of the entrapped antigen and the matching in vivo immune response using polymer particle based immunization. Immune response from particles is often not predictable due to the erratic and incomplete in vitro release profile of the entrapped antigen (Crofts and Park 1997; Gupta et al. 1997; Johansen et al. 1998b). Many immunization protocols require pulsatile release of entrapped antigen rather than a continuous release for improved immune response (Cleland et al. 1999; Spiers et al. 2000). Thus it is essential to demonstrate that the entrapped antigen inside the polymer matrix preserves its native conformation and is released in a manner that is required to elicit the desired immune response. Designing a formulation which could address protein instability problems associated with particle formulation and predict in vivo immune response correlating with the in vitro release characteristic will be an important consideration for the successful development of single dose vaccines. This will not only help in preparing immunoreactive polymer particles having desired antigen release characteristics but also will serve as a quality control parameter for their subsequent evaluation and validation for clinical use.

Studies with the use of PLGA microspheres have been carried out to explore the possibilities of developing a single shot vaccine for tetanus toxoid (TT) with little success (Audran et al. 1998; Diwan et al. 2001; Gupta et al. 1997; Higaki et al. 2001; Men et al. 1995; Sachwendeman et al. 1998; Tobio et al. 2000). The low antibody response produced by PLA or PLGA encapsulated TT particles in most of the reports have been attributed to protein instability problems inside the polymer particles (Alonso et al. 1993; Chang and Gupta 1996; Sasiak et al. 2001). Dichloromethane (DCM) used for solubilization of polymer denatures protein during the primary emulsification step of particle formulation leading to its aggregation and nonspecific adsorption on to the polymer matrix (Johansen et al. 1998b; Raghuvanshi et al. 1998; Sanchez et al. 1999). This results in an incomplete and erratic release profile of the entrapped antigen from the polymer particles and is the main cause of ambiguities that arise during the in vivo evaluation of particle based immunization. In spite of extensive reports on the development of single dose TT vaccines, few studies have been reported on in vitro release characteristics of TT from polymer particles and its matching antibody response in experimental animals (Sasiak et al. 2001). We have recently reported the formulation of polymer particles entrapping immunoreactive tetanus toxoid (Raghuvanshi et al. 2001). The objective of the present work is to formulate particles entrapping immunoreactive TT in a biodegradable polymer matrix and evaluate their performance both in vitro and in vivo.

MATERIALS AND METHODS

Materials

Poly(D,L-lactide-co-glycolide), (PLGA, 50:50) 45 KD and Poly(D,L-lactide) (PLA, 45 KD) were purchased from Birmingham Polymer Inc. USA, PLGA (65:35) 22 KD and PLGA, (50:50) 14 KD were prepared as reported earlier (Mehta et al. 1995). TT (3000 Lf/ml, protein content 8 mg/ml) was purchased from Serum Institute of India, Pune. Rat serum albumin (RSA) [A-6272] and Polyvinyl alcohol, MW 30,000 (PVA) were 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.

Preparation of Polymer Particles

Poly(lactide) and poly(lactide-co-glycolide) microspheres were prepared using the solvent evaporation method (Raghuvanshi et al. 2001). The Primary emulsion between the internal aqueous phase (TT in PBS, pH 7.4) and organic phase (polymer solution in DCM) was prepared by sonication (20 watts, 80% duty cycle, 20 cycles) (Brason, Sonifier 450, USA). Secondary emulsification was carried out either by sonication to obtain nanoparticles or by homogenization (10,000 rpm for 10 min-

utes) (Virtis, Cyclone I.Q., USA) for microparticles. The resulting polymeric particles were washed twice with cold PBS and lyophilized to get free flowing powder. The size of the particles was measured using GALAI-CIS-1 particle size analyzer. All particles were prepared using RSA as a stabilizer in the internal aqueous phase. To evaluate the effect of the internal aqueous phase to organic phase ratio on the in vitro release of TT from the particles, different batches of particles entrapping TT using an aqueous to organic phase ratio of 0.2, 0.3, 0.4, and 0.5 were prepared. To see the effect of PVA concentration on in vitro release, particle batches with different concentrations (0.5, 1, and 2%) of PVA as stabilizer in the internal aqueous phase were prepared.

Estimation of Protein Content of Polymer Particles

To measure the protein content of particles, a known weight of particles were dissolved in acetonitrile to solubilize the polymer while precipitating the encapsulated protein. The precipitated protein was pelleted by spinning at 5,000 rpm for 10 minutes and was dissolved in 1% SDS solution. The protein content of the solution was determined by the micro BCA assay. The protein loading was calculated as the percent weight of protein per unit weight of polymer.

In Vitro Release Experiments

In vitro release experiments of the entrapped TT from particles were carried out in Eppendorf tubes by taking known amounts of particles in 1 ml PBS (pH, 7.4) containing sodium azide (0.05%). The tubes were kept in an orbital shaker at 37°C. At regular time intervals, the supernatant was removed from the tubes by centrifugation (5000 rpm, 10 min) and was replaced with fresh buffer. The amount of protein released into the medium at different time points was determined by analyzing its protein content using the micro BCA assay. Immunoreactivity of the released TT was determined by ELISA.

In Vivo Animal Studies

Immunogenicity of the encapsulated TT in particles was checked in an animal model, using Wistar rats. Rats were injected intramuscularly with 30 µg of either free, alum adsorbed, or particle encapsulated TT. Both PLGA and PLA polymer particles entrapping TT were used for animal experiments (six animals in each group). Animals were bled at different time intervals through retro-orbital plexus and the serum was analyzed for anti-TT antibody by ELISA. Antibody titers were estimated in duplicate with reference to a standard antibody and their concentration (µg/ml) determined as the geometric mean.

ELISA Protocol

To determine the levels of anti-TT antibodies in rat sera, 1 µg of TT in 100 µl of PBS (50 mM, pH 7.4) was coated in each well of a 96 well flat-bottomed Nunc immunoplates (Raghuvanshi

et al. 1998). The plates were then 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 three times with PBS-T, 100 μ l 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 μ l of O-phenyl diamine (OPD) dissolved in citrate-phosphate buffer (pH 4.5) with H₂O₂ was added to each well and incubated for 20 minutes at room temperature. The reaction was stopped by adding 5N H₂SO₄ (50 μ l/well) and the absorbance was measured at 492 nm. The titers of the anti-TT antibody were defined as μ g of antibody/ml of the sera. To measure the immunoreactivity of TT released from particles in in vitro experiments, a different ELISA protocol was used (Raghuvanshi et al. 1998).

RESULTS AND DISCUSSION

Continuous Release of Immunoreactive TT from Stabilized Particles

Immunoreactive TT particles were made by incorporating polyvinyl alcohol (PVA, 0.5% w/v) or rat serum albumin (RSA, 2.5% w/v) as stabilizer in the internal aqueous phase during the primary emulsification step of particle formulation (Raghuvanshi et al. 2001). The average TT loadings in polymer particles were around 4% of the polymer weight. Continuous release of immunoreactive TT from particles made from PLGA (50:50, 14 KD) was observed after an initial burst phase (Figure 1). Particles made without the incorporation of stabilizers did not release TT continuously; only 20% of the encapsulated TT was released during the burst phase. This is in accordance with the recently published observation that very little immunoreactive TT was released from particles prepared without the use of stabilizers (Johansen et al. 1998a). Use of PVA resulted in more uniform release of the antigen in comparison to RSA. Incorporation of

serum albumin resulted in more than 90% release of entrapped antigen from polymer particles over a period of 140 days in comparison to 50% release while using PVA. Burst release of TT from particles made using PVA was low in comparison to that of particles made using RSA. Use of bovine serum albumin has been reported to reduce the denaturation of TT during preparation of PLGA particles (Johansen et al. 1998a). However using bovine serum albumin, only 40% of the total entrapped TT was released in vitro, whereas, in the present study most of the entrapped antigen was released continuously and uniformly over a long period of time. The more uniform release of TT was probably due to incorporation of an optimal amount of serum albumin in the internal aqueous phase, which prevented interaction of DCM with antigen at the interface during the primary emulsification step (Raghuvanshi et al. 1998, 2001). Such beneficial effects of PVA or albumin in promoting protein stability during their entrapment in polymer particles have also been reported for lysozyme (Weert et al. 2000b).

In Vitro Release of TT from PLA and PLGA Particles

Polymer particles made from different polymers were tested for the in vitro release experiments. Continuous release of immunoreactive TT was observed from particles made from different polymers (PLGA, 50:50, 45 KD, and PLA 45 KD) using RSA as a stabilizer (Figures 2 and 3). A burst release effect of antigen was observed from all three polymer particle preparations where around 15–20% of the entrapped antigen was released. It also was observed that the initial burst effect was mostly in early hours of in vitro experiments where nearly 15% of the encapsulated antigen is released in two hours and the remaining 5% is released over a period of one day. This has the implication that the time lag between the rehydration of the lyophilized TT particle and in vivo administration should be as short as possible to minimize their release from the particles before injection.

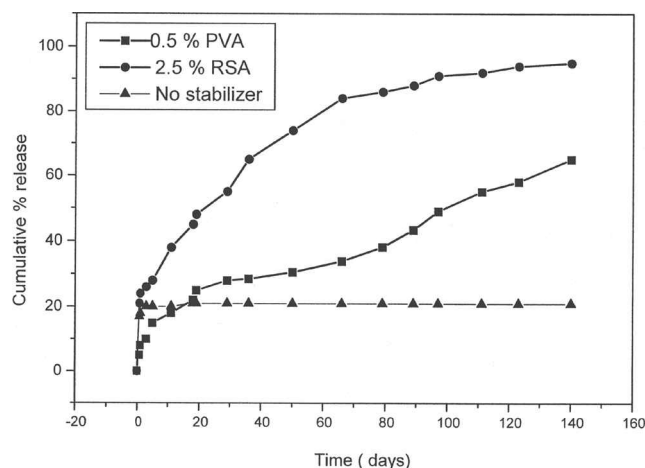


FIG. 1. In vitro release of TT from PLGA (50:50, 14 KD) polymer particles made with RSA (—●—), PVA (—■—) or without stabilizers (—▲—) in the internal aqueous phase during particle formulation.

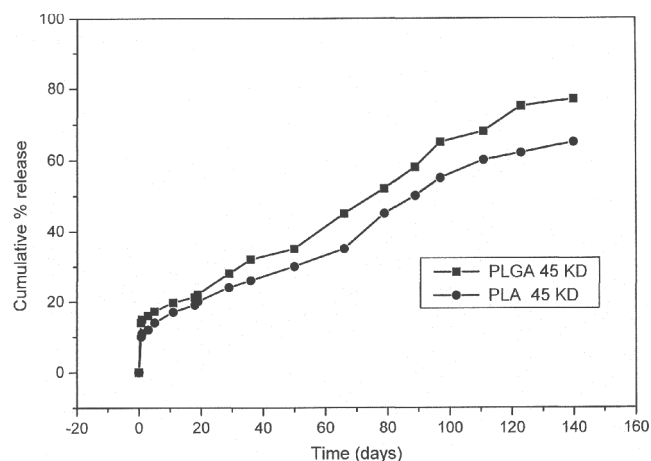


FIG. 2. In vitro release of TT from PLGA 50:50, 45 KD (—■—) and PLA, 45 KD (—●—) polymer particles incorporating RSA as stabilizer during particle formulation.

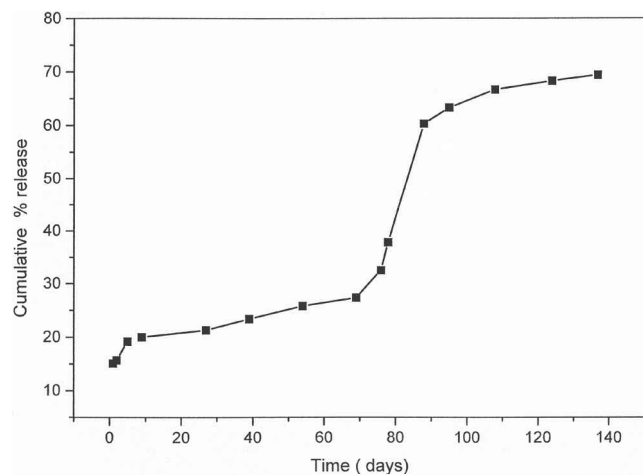


FIG. 3. In vitro release of encapsulated TT from RSA stabilized microparticles made from PLGA (65:35), 22 KD polymer.

The burst effect from the polymer matrix is because of two main reasons: one, the surface of the particles hydrate on contact with the aqueous medium and, second, when the polymer particles are at 37°C, the hydrated surface of the particles loosen because the temperature is close to glass transition temperature (T_g). The combined effect of both phenomena lead to the release of encapsulated TT in the burst phase. It has been proposed that protein release from PLGA/PLA polymer particles occurs via two different mechanisms (Wu 1995). The first mechanism involves the leaching of protein molecules from the water soluble protein domains that exist on and near the surface of the microparticles. After the protein molecules are released from these domains, aqueous channels or pores are generated. For water soluble protein drugs, aqueous channels generated by this protein leaching mechanism facilitate the release of protein molecules on and near the surface of a microparticle. The second mechanism of release involves degradation of the polymer, which is associated with the generation of microporosity, enhanced water uptake and ultimately disintegration of the polymer matrix. This phase is related to polymer composition, molecular weight, and molecular weight distribution and, consequently, the water uptake and the swelling of the polymer matrix. When these two phases of release do not overlap, a discontinuous release is observed, whereas when these two phases of release overlap, a continuous release profile is obtained.

Release of antigen from PLA particles was slow in comparison to the release from PLGA particles (Figure 2). Release of TT from PLGA 45 KD was less in comparison to that from PLGA 14 KD particles incorporating RSA as stabilizer (Figure 1). This was in accordance with the chemical characteristics of the polymers. The more hydrophobic the polymer, the slower water uptake and degradation will be, thus protein release from such polymer particles will be low. PLA being more hydrophobic than PLGA released the antigen at a slower rate than PLGA particles. Such slower degradation rate of PLA polymers

particles have been reported recently (Lin et al. 2000). Release of TT from PLGA (50:50) particles was continuous, whereas it was biphasic from PLGA (65:35, 22 KD) particles (Figure 3). The biphasic release observed with 65:35 PLGA could be due to the non-overlapping release of protein from the two possible mechanisms discussed above. Incorporation of an optimal amount of RSA helped in improving the stability of antigen and thus the in vitro release profile was in accordance with the degradation characteristics of the polymer. Though the present formulation with PLGA (50:50) showed a continuous release of encapsulated protein, a hydrophilic polymer like PLGA (50:50) also can be formulated in such a way so as to generate two different groups of aqueous channels which can lead to two different non-overlapping release profiles resulting in a biphasic release pattern. Such bi-phasic in vitro release profiles of TT has also been reported from PLGA particles (Alonso et al. 1993; Cleland et al. 1999).

Effect of PVA Concentration in the Internal Aqueous Phase on In Vitro Release of TT from the Particles

The formulation with PVA showed a lower burst and cumulative release of antigen over a period of 140 days in comparison to that of RSA (Figure 1). This could be because of the gelling properties of PVA which act as an additional barrier for antigen release. Incorporation of different concentrations of PVA in the internal aqueous phase leads to different types of release profiles of TT from PLGA, 50:50, 14 KD polymer particles (Figure 4). It was observed that by increasing the PVA concentration in the internal aqueous phase (0.5, 1, and 2%), the burst release of TT from particles decreased from 3.7 to 1.1%. With the use of 0.5%, 1%, and 2% PVA in the internal aqueous phase, the cumulative release after 22 days was 22%, 12%, and 9%, respectively. This indicated that, with the incorporation of

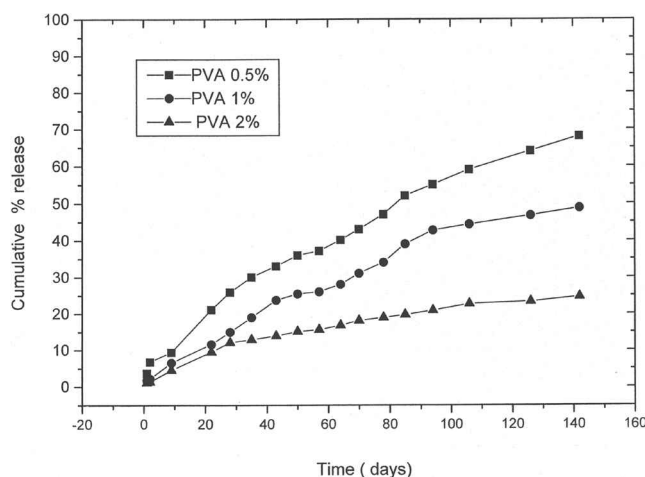


FIG. 4. Effect of varying PVA concentrations on in vitro release of TT from PLGA, 50:50, 14 KD nanoparticles (0.62 nm). PVA concentration (w/v) of 0.5% (—■—), 1% (—●—), and 2% (—▲—) was used in the internal aqueous phase for particle formulation.

different concentrations of PVA in the internal aqueous phase, particles exhibiting different in vitro release kinetics could be prepared. Higher concentrations of PVA form a comparatively stronger gel, which leads to slower release of encapsulated material. Such changes in formulation variables can be used for the application of controlled release of non-vaccine therapeutic proteins, where minimum release of the encapsulated protein is desired in the burst phase.

Effect of Internal Aqueous Phase to Organic Phase Ratio on the In Vitro Release of TT from the Particles

One of the significant formulation parameters which affects the release of entrapped protein was the aqueous to organic phase ratio during the primary emulsification step of particle formulation, as it controls the porosity of the particles. Four different batches of particles were prepared with internal aqueous to organic phase ratios of 0.2, 0.3, 0.4, and 0.5, and were used for in vitro release studies (Figure 5). It was observed that particles made from high aqueous to organic phase ratios resulted in a

high burst release of antigen. Particles made from an aqueous to organic phase ratio of 0.5, released almost 35% of the entrapped antigen on day 1 in comparison to 7% burst release using 0.05 aqueous to organic phase. With the increase in internal aqueous to organic phase volume ratio, the amount of internal aqueous phase per unit weight of polymer increased. The tiny droplets of internal aqueous phase evaporated during lyophilization leaving behind tiny pores containing the dry protein in the polymer matrix. Increased amount of this aqueous phase resulted in increased number of pores in the polymer matrix leading to more porous particles. Different types of in vitro release profiles obtained by changing the ratio of internal aqueous to organic phase indicated the importance of the above factor for the formulation of different types of particles. Particularly, by the varying internal aqueous phase, the burst effect can be increased or decreased as per the requirement of the protein being encapsulated. Similar effects of varying the internal aqueous to organic phase ratio on porosity of the particle during encapsulation of BSA in PLGA polymer matrix has been reported (Crofts and Park 1995).

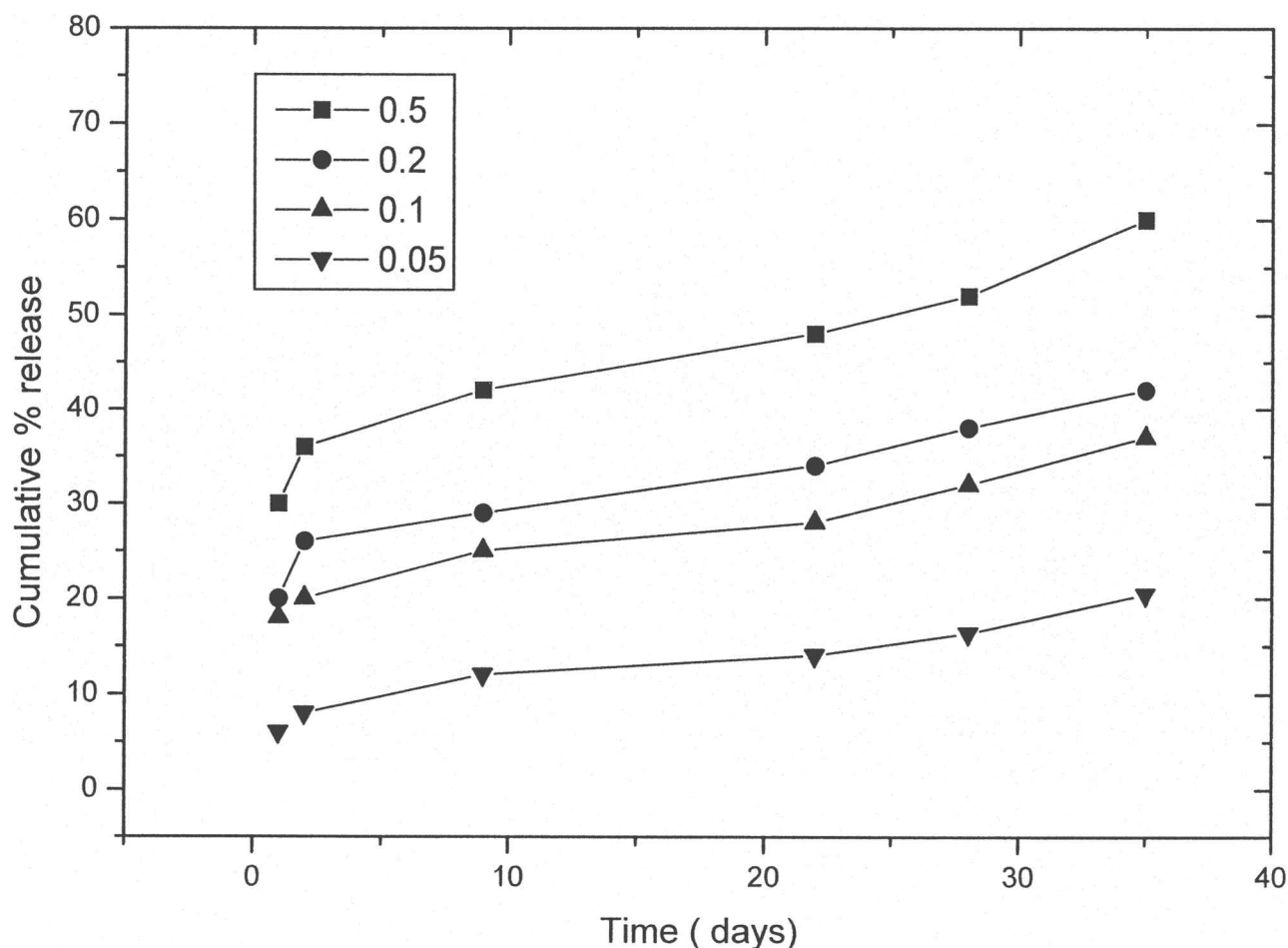


FIG. 5. In vitro release profiles of TT from PLGA 50:50 14 KD polymer particles made by varying internal aqueous phase to organic phase ratio during primary emulsion step of particle formulation. RSA was used as stabilizer and particles made from different aqueous to organic phase ratio (■- 0.5, ●- 0.2, ▲- 0.1, and ▼- 0.05) were used for in vitro studies.

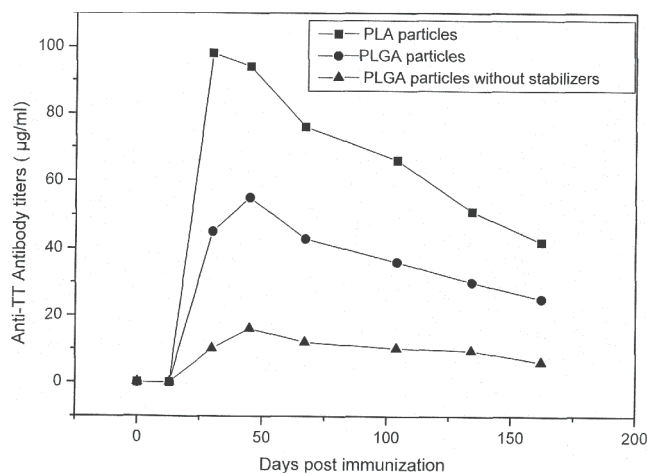


FIG. 6. Anti-TT antibody titer from group of rats immunized with (a) PLGA nanoparticles without stabilizers (Δ), (b) ($\blacksquare$) PLA particles with RSA as stabilizer and (c) PLGA particles with RSA as stabilizer ($\bullet$). In each case 15 Lf of TT entrapped in particles were immunized intramuscularly in single dose.

In Vivo Response from Particles Encapsulating TT

Since particles encapsulating TT without any stabilizer were not showing release of TT after the burst phase (Figure 1) they were tested for in vivo performance. Rats were immunized with 15 Lf of unstabilized TT encapsulated in PLGA (50:50/14 KD) microparticles. It was observed that unstabilized TT in microparticles was a weak immunogen and showed very low antibody response (Figure 6). On the other hand, immunization with 15 Lf TT stabilized by addition of RSA during the primary emulsification encapsulated in particles made from PLGA (50:50/45 KD) and PLA showed better antibody responses (Figure 6). Continuous anti-TT-antibody titers were observed for a period of 150 days. Antibody response from PLGA (65:35, 22 KD) was biphasic and was in accordance with the biphasic in vitro release profile of TT from the particles (Figure 7). The anti-TT-antibody

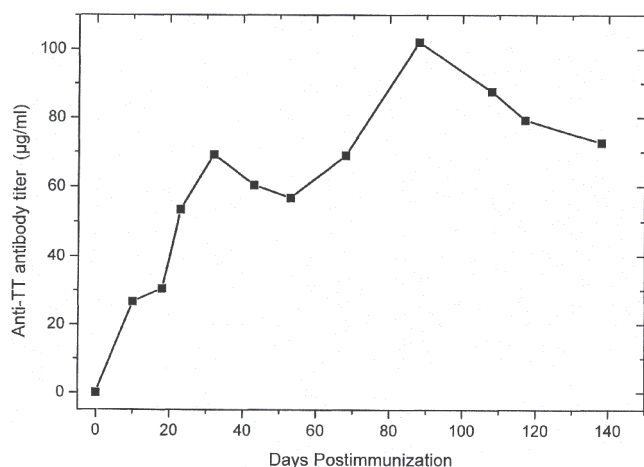


FIG. 7. Anti-TT antibody titer from group of rats immunized with a single dose of 15 Lf TT in PLGA (65:35) 22 KD particles using RSA as stabilizer during primary emulsification step of particle formulation.

titers from PLA, PLGA, 50:50, and PLGA 65:35 were not only higher than the unstabilized particles but also were in accordance with in vitro release pattern of TT from these particles (Figures 6 and 7). An early peak of anti-TT antibody titers was observed (Figure 6) which presumably could be due to the burst release of TT from particles as observed in in vitro release experiments (Figure 2). Enhanced immunoreactivity of these polymer particles encapsulating TT confirms the stabilizing role of serum albumin during the primary emulsion step of particle formulation. Enhanced immunoreactivity of TT in the presence of stabilizer also has been reported during microencapsulation in polymeric particles (Gupta et al. 1997), which supports our observation. Anti-TT antibody titers obtained from this present particle formulation were not better than the two injections of 5 Lf TT each on alum (Raghuvanshi et al. 2001). It is expected that use of a different immunization protocol along with use of an additional adjuvant such as alum will improve the immune response from these stabilized particles as reported earlier for TT (Singh et al. 1997; Tobio et al. 2000).

In Vivo Response from Particles Made with Varying Ratios of Internal Aqueous Phase to Organic Phase in the Primary Emulsion

The ratio of internal aqueous to organic phase during primary emulsion has a marked effect on in vitro release of protein from microparticles (Figure 5). To find out its effect on the immune response, rats were immunized with particles prepared from PLGA (50:50, 14 KD) with the internal aqueous phase:organic phase ratios of 0.2 and 0.5. Antibody titers obtained from these two groups are shown in Figure 8. It was observed that particles with a higher internal aqueous phase:organic phase ratio showed significantly higher anti-TT-antibody-titers. The anti-TT-antibody titers were in accordance with the high in vitro release of TT for the particles described in in vitro section (Figure 8). A high

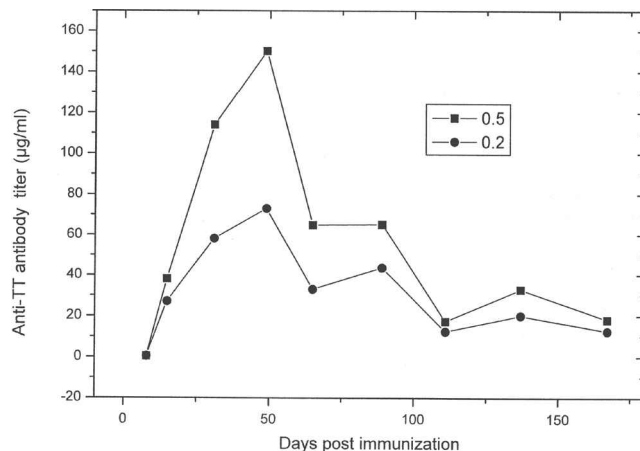


FIG. 8. Anti-TT antibody titer from group of rats immunized with a single dose of 15 Lf TT in PLGA 14 KD particles made from different organic to aqueous phase ratio [($\blacksquare$) 0.5 and ($\bullet$) 0.2] during primary emulsification step of particle formulation.

internal aqueous phase:organic phase ratio leads to more porous particles which lead to faster release of encapsulated material (TT) from the polymer matrix. This provides more antigen to the immune system resulting in high antibody titers.

Correlation between in vitro release and in vivo antibody titers have been reported for the sub-unit vaccine for HIV-1, in which polymeric formulation has provided an auto boosting effect (Cleland et al. 1999). This was achieved by using different PLGA combinations which degrade after particular times thus providing auto boosting of antigens in vivo. It is expected that by physical combination of particles made from such polymers and by making particles which have the desired burst effect, multiple vaccine schedules can be reduced to a single dose preparation. The correlation of in vivo titers with that of in vitro release also signifies that the antigen entrapped has native conformation and release governed by polymer degradation.

CONCLUSION

The stability of the antigen during its entrapment in polymer particles and its continuous release as required for immunization is of paramount importance for the successful development of single dose vaccines using biodegradable polymer particles. In spite of extensive reports on the immunogenicity of the polymer entrapped antigens, very little has been reported on the correlation between release characteristics of the entrapped antigen and its in vivo performance.

In this report we have demonstrated that incorporation of optimal amounts of RSA or PVA helped in the release of immunoreactive TT in a continuous manner from particles made from different polymers. Most importantly, in vivo TT antibody titers in a rat model from these particles were concomitant with the in vitro TT release profile in all formulations using different polymers. This demonstrated that that by using stabilizers at an optimal concentration during the primary emulsification stage of the particle formation, stability problems associated with biodegradable polymer matrix systems can be reduced substantially. This will be helpful in formulating immunoreactive polymer particles having desired release characteristics which can mimic the conventional vaccine immunization protocol.

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