RESULTS AND DISCUSSION
4. RESULTS AND DISCUSSION

4.1 Microparticle formulation and preliminary results

Polymer particles entrapping TT were prepared using water in oil in water (w/o/w) multiple emulsion solvent evaporation method. Aqueous solution of TT (Internal aqueous phase: IAP) containing rat serum albumin as stabilizer was emulsified with solution of polymer in dichloromethane (Organic phase: OP). Polymer concentration in the organic phase was optimized at 50 mg/ml to yield solution with workable viscosity. External aqueous phase (EAP) consisted of 1% PVA solution. Sonication of the internal aqueous phase and organic phase resulted in stable primary emulsion, which was again emulsified with 1% PVA solution with the aid of homogenization or sonication. This resulted in the generation of multiple emulsion, with globules of organic solvent dispersed within external aqueous phase. Within these globules of organic phase, tiny droplets of internal aqueous phase are dispersed. Overnight stirring of this multiple emulsion at room temperature leads to the evaporation of dichloromethane, leaving behind micron or submicron sized polymeric spheres called microparticles or nanoparticles respectively. Polymer particles were pelleted and separated from external aqueous phase and washed with ice-cold water to remove adhering PVA molecules from the surface. Lyophilization resulted in free flowing polymeric particle powder entrapping TT. Particles in micron (microparticles, 2-10 µm) or sub micron (nanoparticles, < 1 µm) size ranges could be obtained by varying energy input during secondary emulsification. Scanning electron micrographs showed that TT entrapped PLA microparticles were spherical with smooth surface and were free from aggregates and other artifacts (Figure 4.1 A and B).

Characterization of these particles revealed around 20-30% encapsulation of TT (data not shown). PLA entrapped TT particles showed low burst release followed by slow and continuous in vitro release of TT into 50 mM PBS (pH7.4). Intramuscular immunization with microencapsulated TT generated antibody response higher than that generated by the equivalent amount of soluble TT.
Figure 4.1 (a) Scanning Electron micrograph of PLA microparticles entrapping TT.
Figure 4.1 (b) Scanning Electron micrograph of PLA microparticles entrapping TT.
Immunization studies revealed that microparticles made up of poly lactide (PLA) were capable of inducing higher antibody responses than that made up of Poly lactide-co-glycolide (PLGA) polymers (Raghuvanshi et al., 2001). It was also observed that single dose immunization of polymer particle entrapping TT elicited antibody titers lower than that observed with two divided doses of alum adsorbed TT (Raghuvanshi et al., 2002). Microparticles made from 45 KD PLA polymer was used in this study. To improve the immunogenicity of the polymer entrapped TT, detailed investigations on effect of different excipients on formulation of immunoreactive PLA particles were carried out. Optimized particle formulation was used to analyze the effect of particles size, antigen load, dose and use of additional adjuvant for improved immune response from single point immunization.

4.2. Formulation and evaluation of TT entrapped PLA particles with different excipients.

Microparticles using PLA polymers entrapping TT in the size range 2-8 μm were prepared using sonication during primary emulsification and homogenization during secondary emulsification (Raghuvanshi et al., 2001). Size of the microparticles is governed largely by ratio of organic phase to that of external aqueous phase and energy inputs during emulsification and these parameters were kept constant during particle formulation with different excipients. Formulations containing different excipients resulted in similar size ranges of PLA particles entrapping TT and were used for both in vitro and in vivo evaluation.

4.2.1 Effect of excipients on encapsulation efficiency and surface morphology of polymer particles

Incorporation of excipients in the internal aqueous phase affected the encapsulation efficiency of TT (Table 4.1) and surface morphology of particles
Table 4.1: Encapsulation efficiency of TT in different formulations with different excipient combinations in IAP and EAP.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Excipients in IAP</th>
<th>Excipients in EAP</th>
<th>Encapsulation Efficiency** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-P</td>
<td></td>
<td>PVA</td>
<td>28.6±2.4</td>
</tr>
<tr>
<td>TR-P</td>
<td>RSA</td>
<td>PVA</td>
<td>43.8±4.3</td>
</tr>
<tr>
<td>TRS-P</td>
<td>RSA+Sucrose</td>
<td>PVA</td>
<td>27.3±3.6</td>
</tr>
<tr>
<td>TRSN-P</td>
<td>RSA+Sucrose+NaHCO₃</td>
<td>PVA</td>
<td>34.3±3.2</td>
</tr>
<tr>
<td>TR-PS</td>
<td>RSA</td>
<td>PVA+Sucrose</td>
<td>44.7±3.6</td>
</tr>
<tr>
<td>TRS-PS</td>
<td>RSA+Sucrose</td>
<td>PVA+Sucrose</td>
<td>63.1±4.2</td>
</tr>
<tr>
<td>TRSN-PS</td>
<td>RSA+Sucrose+NaHCO₃</td>
<td>PVA+Sucrose</td>
<td>69.2±5.1</td>
</tr>
</tbody>
</table>

*Concentration of excipients - In IAP: RSA (2.5% w/v), Sucrose (10% w/v), NaHCO₃ (2% w/v); EAP: PVA % w/v, Sucrose (10% w/v).

** Mean of three values with standard deviations
profoundly (Figure 4.2). Particle formulations without any excipients in the internal aqueous phases (IAP) resulted in loss of immunogenicity of TT, which has been attributed to the denaturation of TT at aqueous organic interface (Raghuvanshi et al., 1998). Addition of 2.5 % w/v rat serum albumin (RSA) to IAP improved the encapsulation efficiency of TT from 28.6±2.4 % to 43.8±4.3%. Scanning Electron micrograph (SEM) of these particles revealed a compact and smooth spherical surface (Figure 4.2. a). These indicated the formation of stable emulsion while using serum albumin in primary emulsification step. Addition of sucrose (a lyoprotectant added to stabilize antigen during freeze drying) along with RSA in IAP decreased the encapsulation of TT in PLA particles from 43.8±4.3% to 27.3±3.6 %. SEM of this formulation revealed irregular surface morphology marked with numerous bumps like projections indicating a multivesicular system resulting from swelling and coalescence of inner aqueous phase droplets (Figure 4.2. b). Use of NaHCO₃ (2 % w/v) in the aqueous phase during particle formulation improved the entrapment efficiency of TT slightly (Table 4.1). However particles prepared with sucrose, RSA and sodium bicarbonate in IAP resulted in lower encapsulation of TT in PLA particles (34.3±3.2%) in comparison to that prepared with only RSA as stabilizer in the internal aqueous phase (43.8±4.3%). It was hypothesized that addition of high solute concentrations to IAP droplets in these formulations induced osmotic gradient. This caused influx of water molecules from external aqueous phase (EAP) resulting in swelling of IAP droplets. As large droplets of primary emulsion are more likely to coalesce and escape to the external aqueous phase, this resulted in loss of encapsulation of TT. Many such swollen droplets get captured in the precipitating and hardening polymer during organic solvent evaporation and give bumps like appearance, which was clearly visible in scanning electron micrographs. This was confirmed by the preparation of another formulation with equal concentration of sucrose in EAP. By maintaining osmotic balance between IAP and EAP, encapsulation efficiency was improved to 63.1±4.2% and particles having smooth surface morphology were obtained (Fig. 4.2.d). Addition of similar concentrations of sucrose in EAP to formulation containing only RSA (no sucrose

49
Figure 4.2 (a) SEM of TT containing PLA microparticles having different excipients in IAP and EAP. (IAP: TT, and RSA; EAP: PVA)

Figure 4.2 (b) SEM of TT containing PLA microparticles having different excipients in IAP and EAP. (IAP: TT, RSA and Sucrose; EAP: PVA)
Figure 4.2 (c) SEM of TT containing PLA microparticles made with different excipients in IAP and EAP (IAP: TT, RSA, Sucrose, and Sodium bicarbonate; EAP: PVA).

Figure 4.2 (d) SEM of TT containing PLA microparticles made with different excipients in IAP and EAP. (IAP: TT, RSA and Sucrose; EAP: PVA and Sucrose)
Figure 4.2(e): SEM of TT containing PLA microparticles prepared with different excipients in IAP and EAP. (IAP: TT, RSA, Sucrose and Sodium bicarbonate; EAP: PVA and Sucrose)
or sodium bicarbonate) in IAP, did not lead to such enhanced encapsulation (group TR-PS in table 4.1). This indicated the requirement for minimum solute gradient between IAP and EAP for better encapsulation, smooth and compact surface morphology of polymer particles.

Similarly, microspheres containing RSA, sucrose and sodium bicarbonate in IAP with only PVA in EAP exhibited irregular surface with large pores of heterogeneous size as well as lower encapsulations (Figure 4.2.c). Addition of sucrose to EAP in this formulation led to formation of regular spherical particles with uniform distribution of small and similar sized pores all over the surface (Figure 4.2.e). This also improved the encapsulation efficiency of TT in the PLA particles. Optimization of excipients (group TRSN-PS in table 4.1) resulted in 69.2±5.1 % encapsulation of TT during particle formulation (Table 4.1).

These results indicated that excipients used for improving stability of antigen affect surface properties of polymer particles and encapsulation efficiency of the protein antigen. Co-encapsulation of excipients like albumin, trehalose, γ-hydroxypropyl cyclodextrin has been reported to enhance the encapsulation of tetanus toxoid, where as other excipients like calcium salts reduce the encapsulation of TT (Johansen et al., 1998). As emulsion stability is a major parameter for the preparation of polymer particles (Schugens et al., 1994, Pistel and Kissel, 2000, Kiyoyama et al., 2003), it is necessary to analyze the role of excipients both on emulsion stability and antigen stability during particle formulation. Effect of primary emulsion stability on surface characteristics of micro particles leading to formation of deformed structure has been reported for PLA particles (Schugens et al., 1994). Maintenance of similar sucrose concentrations in both internal and external aqueous phases helped in better emulsion and protein stability, which resulted in high lysozyme entrapment in polymer particles (Srinivasan et al., 2005). It is thus desirable to use additives, which can improve polymer particle formulation in multiple ways such as
improved protein and emulsion stability, regular surface morphology and high entrapment efficiency.

4.2.2 In vitro release of TT from polymer particles prepared with different excipients

Polymer particles prepared with incorporation of different excipients both in IAP or EAP during formulation showed varying in vitro release of TT during burst phase as well as over the entire study period (Figure 4.3). Poly-lactide particle formulation containing only TT in IAP exhibited low antigen release during burst phase (12.6±2.4%) with cumulative percent release of 24.1±3.4% at the end of the 120-day study period (Figure 4.3 A). The release of immunoreactive TT from particles prepared without any excipients can be attributed to the high concentrations of TT used in IAP (60 mg/ml) during particle formulation. It has been reposted that at high concentration of protein solution, a fraction of total protein gets preferentially adsorbed at the aqueous organic interface and helps in reducing protein denaturation (Arshady, 1994, Raghuvanshi et al., 1998). A very large fraction of antigen being encapsulated thus remains in the interior of globules, shielded from denaturing effects of the interface. Use of high concentration of TT (60 mg/ml) during particle formulation helped in entrapment of immunoreactive TT which was released during in vitro experiments.

Incorporation of RSA improved the amount of TT released in vitro significantly (28.4±2.6 % during burst phase and 40.9±4.1% at the end of the study period). When the same formulation was prepared with inclusion of sucrose in EAP along with PVA, no significant change in release profile of polymer entrapped TT was observed. Addition of sucrose in formulation containing TT and RSA in IAP decreased the burst release of TT from particles but the over all release remained unaffected. Sucrose is a lyoprotectant, which inhibits aggregation of proteins during lyophilization and helped in release of more amount of TT during the post burst phase of in vitro release study. Sodium bicarbonate present in the
Figure 4.3: Comparison of *in vitro* release profiles of immunoreactive TT from TT loaded microspheres containing various excipients in IAP. (A): Formulations prepared with only PVA in the EAP. IAP constituents are: TT (-□-); TT and RSA (-O-); TT, RSA and sucrose (-▲-); TT, RSA, sucrose and sodium bicarbonate (-▽-). (B): Formulations prepared with EAP containing sucrose and PVA. IAP constituents are: TT and RSA (-■-); TT, RSA and sucrose (-●-); TT, RSA, sucrose and sodium bicarbonate (-▲-).
IAP neutralizes the acidic microenvironment inside the degrading microspheres and keeps the pH favorable for maintaining antigen integrity. Polymer particles prepared using TT, RSA, sucrose and sodium bicarbonate in IAP and PVA in EAP exhibited extremely low release during burst phase (2.2±1.2 %) although this formulation exhibited uniform release of immunoreactive antigen throughout the rest of the study period (Figure 4.3 A). The cumulative release of TT at the end of study period was 28.9±3.2 %. The lower encapsulation and presence of large heterogeneous pores on the microsphere surface of this formulation indicated complete loss of surface associated TT.

Polymer particles containing TT, RSA and sucrose in IAP and PVA and sucrose in EAP exhibited very high burst release (39.6±3.6 %) although release after one week was very low (Figure 4.3 B). Similar effect was observed with formulation containing TT, RSA, sucrose and sodium bicarbonate in IAP and PVA and sucrose in EAP. In this formulation, burst release was 44.3±3.4 % and cumulative percent release at the end of the 120-day study period was 50.2±3.7 %. Large burst releases of TT from the formulations maintaining osmotic balance of sucrose between IAP and EAP was observed (Figure 4.3 B) and was always associated with improved encapsulation of TT during particle formulation. This showed that presence of sucrose in the external aqueous phase controlled the osmosis induced emulsion instability. These results indicated that excipients not only affect the entrapment efficiency of the protein antigen but also modulate the in vitro release of entrapped protein from the particles. By optimal use of various excipients, a tailored particle formulation having desired in vitro release profile can be prepared which will suit specific controlled release applications.

4.2.3 Immune response from polymer particles having different excipients

TT microparticles prepared using different excipients (described in Table 4.1) were used for immunization studies in the wistar rats. PLA entrapped TT particles having different excipients generated wide varieties of antibody response from
single point immunization. A 150-fold variation was found in the peak anti-TT antibody titers elicited by different formulations.

4.2.3.1 Effect of protein concentration on Immune response

It has been previously reported that absence of RSA results in loss of immunogenicity of TT during microparticle preparation (Raghuvanshi et al., 1998). It was observed that microencapsulation using dilute TT solutions (8 mg/ml) in absence of any stabilizer led to complete loss of the immunogenicity of TT (Figure 4.4). Co-encapsulation of RSA (2.5% w/v) improved the antibody response from PLA particles from non-detectable to 65 μg/ml in serum from single point immunization. However, formulation in which concentrated TT (60 mg/ml) was used without any excipient in IAP also showed improved antibody titer from single point immunization (peak titer value 55.4±21.3 μg/ml). Incorporation of RSA was useful in further improving the peak antibody titres to 81.3±29.1 μg/ml from single point immunization (Figure 4.4). This suggested that investigation on the effects of antigen concentration can provide valuable insights on in vivo performance of polymeric formulations. This can help in clearing the ambiguities related to protein stability associated with microparticle based vaccine delivery system. Earlier, Audran et al., 1998 have reported that when microencapsulated antigens are administered at higher doses, the residual amount of antigens escaping denaturation is sufficient in small animals like rodents to induce optimal immune response (Audran et al., 1998). However, when polymer particle based immunization is used for higher animals or humans, it is essential to completely prevent the denaturation of entrapped antigen. Amphiphilic stabilizers not only reduce the denaturation of vaccine at the aqueous organic interface but also help in stabilizing the primary emulsion droplets. Combination of high concentration of protein antigen and emulsion stabilizer was useful in protecting protein denaturation at aqueous organic interface and resulted in improved antibody response from single point immunization. Similar improved performances using very high concentration of
Figure 4.4: Effects of RSA on the immune response elicited by TT loaded biodegradable PLA particles prepared with dilute or concentrated TT in the IAP. Animals were immunized with PLA particles prepared using dilute TT (8 mg/ml) e (-•-); Concentrated TT (60 mg/ml) (-▲-); Dilute TT (8mg/ml) and RSA (-●-); Concentrated TT (60 mg/ml) and RSA (-▼-).
protein solution during primary emulsion step has been reported for human growth hormone encapsulation using PLGA polymer (Kim and Park, 2004).

4.2.3.2 Effect of sucrose and NaHCO₃ on immune response

Immunization with polymer particles containing RSA and sucrose along with TT in IAP generated antibody titres (peak value 20.8±16.4 µg/ml) lower than those generated from particles having only TT and RSA in IAP (peak value 81.3±29.1 µg/ml) through out the study period (Figure 4.5). This was surprising as addition of sucrose was expected to prevent antigen aggregation during lyophilization and therefore should have resulted in improved immune response from single point immunization. This observation was also consistent with the hypothesis of swelling of IAP droplets while using sucrose during emulsion. RSA has been shown to protect antigen integrity by preferentially adsorbing on aqueous organic interface (Raghuvanshi et al., 1998). The swelling of internal aqueous phase droplets increased the aqueous organic interface area more than that which RSA could saturate and would have exposed a large fraction of antigen to organic solvent at aqueous organic interface. This resulted in denturatrion of TT at the interface and subsequently the particle formulation elicited lower antibody response. On incorporation of sucrose both in IAP and EAP, the peak anti-TT antibody titers improved five fold (108.7±22.1µg/ml). Use of sucrose only in EAP had no effect on antibody response from polymer entrapped TT. Highest antibody titers (mean peak tires 164.2±59.6 µg/ml) were obtained with formulation containing TT, RSA, sucrose and sodium bicarbonate (all the stabilizing excipients) in IAP and sucrose and PVA in EAP. This formulation also exhibited the highest titers at the end of 247-day study period. However, deletion of sucrose from EAP of this formulation, while keeping all other excipients in external and internal aqueous phases same (which also resulted in complete loss of burst release of TT from the particles) led to the decrease in the peak antibody titers by 150 fold (Figure 4.5). During the entire post-immunization period, the antibody titres were practically non detectable, although this formulation was
Figure 4.5: Serum anti-TT IgG concentrations from immunization with TT loaded PLA particles containing different excipients in IAP and EAP. Excipients used in different formulations were: TT and RSA in IAP and PVA in EAP (■); TT and RSA in IAP and PVA and sucrose in EAP (○); TT, RSA and sucrose in IAP and PVA in EAP (▲); TT, RSA and sucrose in IAP and sucrose and PVA in EAP (▼); TT, RSA, sucrose and sodium bicarbonate in IAP and PVA in EAP (▽); TT, RSA, sucrose and sodium bicarbonate in IAP and PVA and sucrose in EAP (□).
having all the desirable excipients and exhibited a continuous release of immunoreactive antigen in \textit{in vitro} release study. This demonstrated that the presence of excipients in EAP can exert powerful effect on the \textit{in vivo} performance of an antigen loaded polymeric formulation. High burst release of the antigen probably helped in generating strong immune response from polymeric antigen delivery system by better priming of the immune system. Large amounts of surface associated antigen result in the availability and presentation of high concentrations of antigen to antigen presenting cells, which helped in eliciting high antibody titers from single point immunization.

Comparison of kinetics of antibody responses in different formulations revealed that serum antibody titres induced on day 15 were low in all the cases. The anti-TT antibody titres peaked during 7th week in all the formulations. Sustainability of antibody concentrations in serum was also differential. To compare the sustainability independent of peak antibody titres, ratio of antibody concentration at the end of study period to peak antibody concentration was calculated as sustainability index of that group. It was observed that formulation with all optimal excipients also induced highest peak and sustained titres with sustainability index of 0.36. Formulations lacking sucrose in EAP, exhibited lower sustainability index of 0.16. Higher sustainability was observed in formulations showing higher burst release and higher peak antibody titres. PLA particles with lower burst release of TT and higher cumulative release of TT during post burst phase period showed low sustainability index. So it was concluded that in case of microparticle based vaccine delivery system, sustainability of antibody titres is governed by the initial priming. Peak titres were higher in the case of formulations with higher burst release. The formulations with lower burst release resulted in low priming of the immune system and subsequently lower peak titres could not sustain the antibody titres as efficiently in spite of releasing higher fraction of dose during post burst period. Use of optimal quantities of sucrose in internal and external aqueous phases of emulsion during particle formulation thus improved immune response from single point immunization.
4.2.4. Correlation between *in vitro* release of antigen and *in vivo* immune response from different formulations

It was observed that alterations in the physical characteristics caused by the addition of stabilizers also affected the performance of antigen loaded biodegradable TT microparticles tremendously depending upon the mechanism by which they act. Comparison of the results of *in vitro* release study and *in vivo* immunization experiments revealed that the formulations prepared with sucrose and PVA in EAP and only sucrose or sucrose and sodium bicarbonate in IAP exhibited highest burst release of TT. This formulation also elicited highest antibody titers, which were maintained at a higher level throughout the study period. The formulation, displaying continuous release of antigen throughout the study period without any burst release did not elicit high antibody titer. It was observed that in all the formulations, antibody titers correlated with the fraction of encapsulated antigen released during burst phase and not with antigen released slowly in later phase. This suggested that large amount of surface associated antigens, which became readily available to the antigen presenting cells as a bolus dose dictated the antibody response.

Use of sucrose in both internal and external aqueous phases helped in generating highest antibody response from single point immunization. Use of sucrose leads to higher amounts of surface associated antigen, which probably helped in better priming of the immune system for antibody response. High and sustained antibody response from single point immunization depends on the initial priming of the immune system. Particularly for T cell dependent antigen, it has been widely accepted that the initial bolus of antigen has profound effect on generation of immune response (Kaech and Ahmed, 2001). Thus, an immunization modality resulting in high initial bolus of antigen following continuous release will be more useful for development of single dose vaccine for TT as it is a T cell dependent antigen.
4.2.5 Immune responses from admixture of alum and antigen loaded polymer particles

Positive role of alum in improving antibody response, when co-administered with antigen loaded microparticle has been reported previously for TT (Raghuvanshi et al., 2002). In order to assess the extent to which antibody response induced by microparticles is affected by alum, immunizations with microparticles having variable excipients in IAP and EAP were carried out. It was observed that co-administration of alum enhanced the peak antibody titers from microparticles by 1.5 to 5 folds in different formulations (Figure 4.6). Enhancement was more in those microparticles which induced lower titres when administered without alum. In the case of microparticles exhibiting large burst release and generating high antibody titres in absence of alum, the extent of enhancement of antibody titers on co-administration with alum was less. Upon co-administration of alum the greatest difference was seen in peak antibody titres, which subsequently diminished. Co-administration of alum did not improve sustainability of antibody response and despite eliciting much higher early and peak antibody titres; titres at the end of 8-month study period were almost similar to those induced in absence of alum. It suggested that although initial titres were influenced by presence of alum, long term responses and sustainability are controlled to a greater extent by the characteristics of the microparticles. The improvement of initial and peak antibody titers on co-administration of alum indicated that alum plays a crucial role in priming of the immune system. Formulations with higher burst release and capable of generating higher initial and peak titres, exhibited lesser degree of improvement in antibody response on immunization with admixture of particles and alum.

Amounts of antigen released from microparticles in vitro in presence of alum were always lower than those from the particles alone (Katari and Panda, 2001). It is possible that the initially released antigen gets adsorbed on to the alum and improves the presentation of antigen to antigen presenting cells. However, more
Figure 4.6: Effect of alum on antibody response induced by different microparticle formulations. All the formulations were having TT in IAP and 1% PVA in EAP. Other components in different formulations are A: RSA in IAP co-administered with alum (-▲-) and without alum (-■-), RSA and sucrose in IAP with alum (-▲-) and without alum (-■-).
B: RSA, sucrose and sodium bicarbonate in IAP with alum (-▲-) and without alum (-■-).
C: RSA and sucrose in IAP and sucrose in EAP co-administered with alum (-▲-) and without alum (-■-); RSA, sucrose and sodium bicarbonate in IAP and sucrose in EAP with alum (-▲-) and without alum (-■-).
enhancements in immune response from microparticles with lower burst release and low antibody titers suggest some other or additional mechanism responsible for improving the immune response on immunization with admixture of particle and alum. Better dispersibility of particles leading to improved phagocytosis and thus improved immune response has also been suggested as one of the reasons for improving the immune response on co-administration with alum (Johansen et al., 2001). Polymer particles release soluble antigen and it is well known that soluble antigen are not immunogenic. Presence of alum skews immune response towards Th2 type of response and augments antibody titer. Continuous release of immunoreactive antigen and presence of additional adjuvants act cooperatively to improve the immune response from single dose vaccine preparation from biodegradable polymer particles.

4.2.6. Conclusions from formulation experiments

In the present investigation it was observed that the addition of excipients in IAP influenced the encapsulation and in vitro release profiles of TT from poly lactide microspheres. Presence of serum albumin and use of high concentration of TT during primary emulsification stage helped in reducing antigen denaturation at the interface and improved the immunogenicity of the polymer entrapped TT particles. Poor encapsulation, lower surface associated antigen (as reflected in lower burst release) and irregular surface characteristics associated with large pores suggest the instability of multiple emulsions. These resulted in reduction and even complete loss of immunogenicity of TT loaded microspheres.

Improvement in encapsulation was observed on addition of sucrose in external aqueous phase only in the presence of other excipients in internal aqueous phase. This observation showed that the presence of osmogen in the internal aqueous phase affected the encapsulation of protein antigen adversely, which could be improved by balancing the concentration of osmogen between the two phases. Use of sucrose in both the phases resulted in higher amount of surface
associated antigen (as reflected in higher burst effect) and improved immune response from single point immunization. Sucrose molecules dissolved quickly in \textit{in-vitro} release media creating large pores, similar to pore formation in polymer scaffold using salt (Murphy \textit{et al.}, 2002). Use of sucrose thus helped in better release of the entrapped protein apart from reducing the extent of protein aggregation during lyophilization. High initial burst release associated with sucrose formulation resulted in long lasting sustained antibody response from single point immunization. Thus it was concluded that excipients, which are necessary to protect the integrity of proteins or antigen during formulation and during \textit{in vitro} or \textit{in vivo} release affected the physical characteristics of the microspheres profoundly. This necessitates corrective measures to be taken in order to get optimal performance from microparticles. Antibody titers from PLA entrapped TT particles were further improved by using small amount of alum during immunization. Immunization with admixture of TT particles and alum improved the peak antibody titer considerably from single point immunization.

\textbf{4.3 Effect of formulation variables on the immune response from TT entrapped PLA particles}

Research on controlled release vaccine delivery system has mainly focused on stability and immunogenic potential of the released antigen (Raghuvanshi \textit{et al.}, 1998, van de Weert \textit{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 such as particle size, load of antigen, presence of additional adjuvants and route of immunization have major influence on the quality and magnitude of immune response from single point immunization. Current understanding of immune system and research on antigen entrapped polymer particles suggests that these parameters may be very crucial for the presentation of antigen to immune cells and for controlling the overall
immune response (Esparaza and Kissel, 1992, Nakaoka et al., 1996, Igartua et al., 1998; Cleland, 1999, Johansen et al., 2000, Johansen et al., 2001, Gutierro et al., 2002a, Gutierro et al., 2002b). In this part of study, TT entrapped in PLA particles were used to evaluate the influence of size, use of additional adjuvant, antigen load, and doses on antibody titer 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. Except for the experiments on effect of particle size on immunogenicity, PLA particle of size ranges between 2-8 μm were used in all immunization experiments.

### 4.3.1 Effect of particle size on immune response

#### 4.3.1.1 Preparation and characterization of TT loaded PLA particles

For formulation of single dose TT vaccine, PLA polymer was used for particle preparation as they elicit improved antibody titer in comparison to hydrophilic PLGA polymers (Raghuvanshi et al., 2002). Four formulations of PLA particles containing TT were prepared having size ranges between 50-150 μm, 10-70 μm, 2-8 μm and 0.5-1.98 μm (Table 4.2). These size ranges were selected to delineate the role of macrophage uptake in generation of immune response from single point immunization. The largest size particles (50-150 μm) are not expected to be taken up by macrophages. Particle having size ranges 10-70 μm will have very low probability of being taken up by macrophages 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 diameters less than 5 μm so they are expected to be taken up by antigen presenting cells readily (Thiele et al., 2001). Particles with size less than 2 μm (0.5 – 1.98 μm) were expected to be taken up more efficiently by antigen presenting cells due to submicron size ranges of polymer particles (Desai et al., 1996). Scanning electron micrographs of the microparticles reveal spherical shape with uniform morphology similar to as described in earlier figures (Figure...
Table 4.2: Preparation and Characterization of different sized TT entrapped PLA particles

<table>
<thead>
<tr>
<th>Formulation</th>
<th>P.E.</th>
<th>S.E.</th>
<th>OP: EAP</th>
<th>Particle size (μm)</th>
<th>Encapsulation Efficiency (%)</th>
<th>Antigen load (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Son.</td>
<td>Stirring</td>
<td>1:100</td>
<td>110.4 (50-150)</td>
<td>30.5</td>
<td>26.4</td>
</tr>
<tr>
<td>B</td>
<td>Hom.</td>
<td>Hom.</td>
<td>1:50</td>
<td>48.7 (10-70)</td>
<td>32.5</td>
<td>31.2</td>
</tr>
<tr>
<td>D</td>
<td>Son.</td>
<td>Hom. *</td>
<td>1:4</td>
<td>4.1 (2-8)</td>
<td>60.4</td>
<td>53.2</td>
</tr>
<tr>
<td>E</td>
<td>Son.</td>
<td>Son.</td>
<td>1:4</td>
<td>0.75 (0.50-1.98)</td>
<td>56.8</td>
<td>49.3</td>
</tr>
</tbody>
</table>

P.E.: Primary Emulsification, S.E.: Secondary Emulsification, O.P.: Organic Phase (: 50 mg/ml Poly lactide in Dichloromethane), EAP: External Aqueous Phase (1 % (w/v) Poly vinyl alcohol in Water) Son.: Sonication (30W, 0.2 output control, 40%duty cycle), 1min.; Hom.: Homogenization 5000 rpm, 5 min. Hom *: 10,000 rpm, 10 min. Stirring: Thermolyne Cimarac-2 magnetic stirrer at 200 rpm, 10 minutes.

Table 4.3: Characteristics of microparticles with different TT loading.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle size (μm)</th>
<th>Encapsulation efficiency (%)</th>
<th>TT load (μg/mg) on PLA particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>4.2±2.1</td>
<td>69.9±6.1</td>
<td>94.2</td>
</tr>
<tr>
<td>C2</td>
<td>4.2±1.7</td>
<td>79.8±8.2</td>
<td>28.2</td>
</tr>
<tr>
<td>C3</td>
<td>4.3±1.8</td>
<td>49.2±5.1</td>
<td>16.7</td>
</tr>
<tr>
<td>C4</td>
<td>4.3±1.8</td>
<td>68.2±4.2</td>
<td>5.8</td>
</tr>
<tr>
<td>C5</td>
<td>4.7±1.6</td>
<td>60.7±3.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>
4.1 and 4.2). All these particle formulations exhibited an initial burst release followed by little release of TT over the period of time during *in vitro* release experiments (Figure 4.7). TT released from the particles was immunoreactive. Large sized PLA particles released lower amount of TT during burst phase in comparison to that of smaller sized particles. During formulation, protein antigens migrate towards the surface and thus considerable amount of TT accumulate at the particle surface. As large size particles have less surface area per unit weight of polymer particles, TT released from those were low in comparison to smaller particles.

### 4.3.1.2. Antibody response from different sized PLA particles

Serum anti-TT IgG titres varied extensively on immunizing wistar rats with different sized PLA particles (Figure 4.8). Antibody titers generated by microparticles in the range of 50-150 μm were significantly lower (peak titre value 34.2±4.6 μg/mL) than those generated by smaller size particles. Microparticles with size range of 2-8 μm elicited highest antibody titres (peak titre value 145.2 ±48.6 μg/mL) and decreasing the particle size further (lower than 2 μm) led to reduction in peak antibody titre (Figure 4.8 A). This indicated that optimum particle size for eliciting antibody response is between 2-8 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. Anti-TT antibody titers from immunization with particle size of 2-8 μm were more sustained than other formulations till the end of the 250-days study period. Similar effect of particle size on antibody response has been observed previously following intraperitonial immunization of polymer-entrapped ovalbumin (Nakaoka *et al.*, 1996).

Decrease in antibody titres in the groups immunized with smallest sized particles (0.5 – 1.98 μm) was surprising as particles in the submicron range are taken up more efficiently by the antigen presenting cells. The possible reasons for this
Figure 4.7: *In vitro* release profiles of TT from different size ranges of PLA particles. Different size ranges are 50-150 µm (■), 10-70 µm (○), 2-8 µm (▼), 0.50-1.98 µm (○).
Figure 4.8: Anti-TT antibody response elicited by different sizes of TT entrapped PLA particles. Particles having size ranges 50-150 μm (■), 10-70 μm (○), 2-8 μm (▲), 0.5-1.98 μm (◆) were immunized without alum (A) and with 25 μl of alum (B). Groups of 6 female wistar rats 2 months old were immunized with single dose of 5 Lf PLA entrapped TT along with alum intramuscularly. Geometric means are presented with standard deviations.
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. Enhanced uptake of antigen by antigen presenting cells is one of the parameters, which improves the immunogenicity of the particulate antigen. However processing of the antigen in cellular compartment and presentation to lymphocyte dictates the magnitude of antibody response (Brewer et al., 2004, Fifis et al., 2004). Rapid escape of PLA nanoparticles from endolyosomal compartment to cytosol (Panyam et al., 2002) triggers 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, immunizations with nanoparticles in general elicit 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 also been reported for very small size particles (100 to 500 nm) in comparison to larger size particles (1000 nm) using BSA loaded particles (Gutierro et al., 2002b).

It was observed that very large size microparticles (50-150 μm), which are least likely to be taken up by antigen presenting cell elicited very low antibody response. Formulation with size ranges 10-70 μm containing large populations of microparticles, which cannot be taken up by antigen presenting cells showed high antibody response from single point immunization. Earlier studies have reported appreciable antibody responses from microparticles of 10-90 μm and 15-60 μm (Johansen et al., 2000, Johansen et al., 2001). Singh et al., 1997b have 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). Appreciable 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 the above
mentioned reports indicated that particle phagocytosis and transport to lymph nodes were not the absolute requirement for achieving high serum antibody titers. Large size particles remain preferentially adhered to the macrophage surface (Horisawa et al., 2002) and probably act as a depot system for continuous release of the entrapped antigen for processing and presentation. For very large size particles (50-150 \( \mu \)m) surface adsorption and presentation of antigen will be low due to small size (10-15 \( \mu \)m) of the macrophages. Immunization with particles within the size ranges of 2-8 microns helps in both macrophage uptake and depot formation at the cell surface resulting in high serum antibody response from single point immunization. Preliminary investigation on particle uptake by macrophages in the laboratory has indicated that particles within the size range 2-8 \( \mu \)m preferably remain adherent to the cell surface (unpublished data). Thus apart from depot effect as well as uptake by macrophages, the polymeric particles may be acting in alternative way of delivering soluble antigen while remaining adhered to the surface of the antigen presenting cells. Further studies are underway to explore the molecular mechanism of antigen presentation from polymer-entrapped antigens.

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

Positive role of alum in improving antibody response on immunization with admixture of alum and antigen loaded microparticle has been reported previously for TT (Singh et al., 1997, Johansen et al., 2001, Raghuvanshi et al., 2002, Katare et al., 2003). In the present study, the extent to which alum augments the antibody response from microparticles of different sizes was explored.

Immunization experiments revealed that the co-administration of alum along with different sized microparticles led to differential degree of enhancement in antibody titres (Figure 4.8 B). Microparticles in the range 2-8 \( \mu \)m exhibited improved antibody titres when co-administered with alum (145.8±43.8 to
229.6±49.8 μg/mL). The enhancement of antibody titer with nanoparticles was lower (91.2±51.6 μg/mL to 133.8±31.6 μg/mL) than that observed for 2-8 μm size particles. Immunization with particles having size range 10-70 μm along with alum also improved the serum antibody titers considerably (67.2±29.6 μg/mL 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 μg/mL to 41.9±33.4 μg/mL). Improvement in antibody titres while immunizing admixture of particles and alum was highest when the particles are in the micron range. Differential improvement of antibody response elicited by different sized microparticle on co-administration of alum proved that particle size also contributed to the beneficial impact of adjuvant while immunized along with particle.

High antibody response from the mixture of smaller size particles (0.5-1.98 μm) and alum may be due to enhanced phagocytosis and inflammatory response to co-administered alum (HogenEsch 2002). Alum forms network type structures when used along with particles, which hold particles in form of small clumps. Because of this, particles in submicron and micro ranges are held together by alum while remaining adhered to the surface of antigen presenting cells. This results in improved immune response on immunization with admixture of alum and particles. Very large sized particles (50-100 μm size) have huge surface area in comparison to macrophage and therefore are neither taken up by macrophage nor get enough chance to adhere to the cell surface for antigen presentation. Presence of alum probably helps in better presentation of the soluble antigen released from the particles (HogenEsch 2002, Rimaniol et al., 2004) and both polymer particles and alum acted cooperatively to improve the antibody response from single dose of polymer entrapped TT vaccine preparation. Similar potentiation of immune response while using multiple adjuvants has been reported for both TT and hepatitis B vaccine (Diwan et al., 2002, Wang et al., 2003). Alum does not potentiate the immune response of many antigens and in such cases combined adjuvant effect of both the particles and alum can be exploited for generation of improved immune response.
4.3.2 Effect of antigen load on immune response

Current understanding of the mechanism of antibody response suggests that the antigen content of microparticles affect the magnitude of 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 (Johansen et al., 2001). If this holds true, low antigen load particles should give better immune response than particles having high antigen load.

To exclusively evaluate the effect of PLA microparticles with different TT load on antibody response, microparticles with size ranges of 2-8 μm but different TT loadings (94.2, 28.2, 16.7, 5.8 and 1.3 μg TT/mg polymer) were used. It was observed that as the antigen load was enhanced from 1.3 μg/mg to 28.2 μg/mg of PLA particles, peak antibody titres enhanced from 135.8±53.4 μg/mL to 254.6±38.4 μg/mL (Figure 4.9). Further increase in antigen load to 94.2 μg/mg enhanced peak titres to 320.0±94.1 μg/mL. The titres 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, uptake of few microparticles results in higher amount of antigen inside antigen presenting cells. This leads to better antigen presentation and subsequently improved the antibody responses. It has been previously reported that immune response from exogenous antigen is dependent on antigen concentration in the antigen presenting cells (Vidard et al., 1996, Raychaudhari and Rock, 1998). Priming of immune response with high
Figure 4.9: Anti-TT antibody response induced by PLA microparticles with different TT loading. TT loads (µg/mg) in different particles were 94.2 (-■-), 28.2 (-○-), 16.7 (-▲-), 5.8 (-▼-), 1.3 (-◆-). Groups of 6 wistar rats were immunized with 5 Lf microencapsulated TT along with alum intramuscularly. Geometric means are presented with standard deviations.
response through activation of T cell help (Slifka and Ahmed, 1998). Our results also indicated that for generation of high antibody titers from particle based immunization, the initial load of antigen delivered should be sufficiently high. This strategy 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 (van de Weert et al., 2000). This suggests that lower concentration of antigen in polymer matrix will be denatured more and will subsequently elicit lower antibody titers. Thus polymeric particle of optimal size range with high antigen load was more beneficial for elicitation of long lasting antibody titers from single point immunization.

4.3.3 Dose response studies using TT entrapped PLA particles

Dose response studies were carried out by immunizing female wistar rats with 15, 10, 5, 1, 0.5 and 0.1 Lf of microencapsulated TT along with alum. As the immunization dose was increased from 0.1 Lf to 15 Lf, dose dependent increase in antibody titres was observed through out the 9 months study period (Figure 4.10 A & B). Maximum antibody titers of 400 μg/mL was achieved from single point intramuscular immunization with 15 Lf of PLA entrapped TT. Perceptible early titres 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>0.95); immunization with admixture of PLA entrapped TT and alum thus improved the immune response almost 100 times. Peak antibody titres were observed between 7-9 weeks post immunization. Although there was appreciable difference between the successive doses on day 15, larger differences were observed in the peak antibody titre values. Among the groups immunized with varying doses of microencapsulated TT, peak antibody titres showed larger magnitude of dose dependent enhancement at lower doses. Immunization with microparticles containing 1 Lf TT along with alum showed considerable anti-TT antibody titer from single point immunization.
Figure 4.10: Serum Anti-TT IgG induced by different doses of microencapsulated TT co-administered with alum in wistar rats. TT loaded microparticles equivalent to different dose of TT were co-administered with alum intramuscularly to wistar male rats. Details of doses are A: Microencapsulated TT equivalent to 15 Lf (■), 10 Lf ( ○), 5 Lf (▲), 1 Lf (▼) TT; B: Microencapsulated TT equivalent to 1.0 Lf ( ■), 0.5 Lf (○), 0.1 Lf (▲) TT and 10 Lf (▼) soluble TT in saline.
Dose response curves in terms of antibody titres elicited by different doses of microencapsulated antigen in the range 0.1 to 15 Lf were prepared for early titres (15\textsuperscript{th} day), peak titres (45 day) and late titres (275 day) (Figure 4.11 A). It was observed that peak antibody titres increased profoundly with increasing the doses, while early and late titres exhibit smaller degree of improvement within this range. The dose dependent kinetic of antibody response can be better comprehended by finding the rate of enhancement of serum antibody concentration with respect to dose. The first order derivative of dose response curve revealed that the rate of change of peak antibody titers per Lf of TT administered was higher at lower doses (Figure 4.11 B). As the immunization dose increased, the rate of enhancement in peak antibody titers per Lf of TT kept on decreasing. This was also evident from the tapering effect towards the higher end of the dose range in figure 4.11A. This reflected the suitability of lower dose regime on enhancement of peak antibody titers while using polymer entrapped immunization. For early and late antibody titres, rate of antibody enhancement remains either constant or increased slightly on increasing the dose. Rate of change of peak antibody titers were more dependent on antigen doses than the rate of change of early or late antibody titers.

Dose response study within the range 0.1 Lf – 15 Lf with microencapsulated antigen co-administered with alum showed dose dependent increase in antibody titres through out 9 months study period. Microparticles, when co-administered with alum were capable of eliciting high early antibody titres. Since the most desirable objective of vaccination is to achieve neutralizing antibody titres above the prescribed limit for a very long period, it is important to take this into account while defining the ideal dose. Antibody sustainability index, defined as the ratio of serum antibody concentration on the last day of study period to the peak titres observed in a particular group, was used as a tool for determine the efficacy of dose response studies during immunization. Comparison of sustainability indices in groups immunized with different doses revealed that for doses lower that 1 Lf, sustainability indices were between 0.12-0.16 while for doses 5, 10 and 15 Lf,
Figure 4.11: (A) Dose response curves represented in terms of antibody titres induced by different doses of TT loaded microparticles co-administered with alum; early titres on day 15 (-■-), peak titres on day 45 (-○-) and last day titres on day 275 (-▲-). (B): Rate of antibody titres enhancement with respect to dose represented as first order derivative of dose response curves during early titres on day 15 (-■-), peak titres on day 45 (-○-) and the last day titres on day 275 (-▲-).
indices were between 0.26-0.29. Higher doses of microencapsulated antigen leads to better priming and makes enhanced amount of antigen available during post burst period (as larger number of microparticles are administered per rat). This helped in sustaining antibody concentrations at higher values. Thus the results demonstrated 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 serve as a quality control parameter for the clinical evaluation of the particle based vaccine formulation.

4.3.4 Comparative antibody response from single doses of alum adsorbed antigen and admixture of microencapsulated TT and alum

For comparison with conventional vaccine, different groups of animals were immunized with single dose of 10 Lf, 5 Lf, and 1Lf alum adsorbed TT and with equivalent doses of microencapsulated TT co-administered with alum. It was observed that immunization with admixture of microencapsulated TT and alum resulted in higher and more sustained antibody titers than alum adsorbed TT of similar dose throughout the 9 months of study period (Figure 4.12 A, B, C). Antibody responses peaked earlier in case of single dose alum adsorbed vaccine (between 4-6 weeks) than in the case of microencapsulated antigen (7-9 weeks). Presence of alum along with microencapsulated TT improved the antibody titers 2-3 times higher than those observed with similar doses of alum adsorbed TT. At the end of 250 days of post immunization, antibody titers from particle based immunization were higher than that observed with alum adsorbed TT immunization.

4.3.5 Conclusions from formulation parameters experiments

From these formulation parameter experiments, it was concluded that particles size, use of additional adjuvant, antigen load on polymer particles, and doses influenced antibody response from single point immunization. For PLA entrapped
Figure 4.12: Primary antibody response induced by single dose of equal amount of TT loaded microparticles co-administered with alum and alum adsorbed TT. A: 10 Lf TT microparticles co-administered with alum (●), 10 Lf alum adsorbed TT (○); B: 5 Lf TT microparticles co-administered with alum (●), 5 Lf alum adsorbed TT (○); C: A: 1 Lf TT microparticles co-administered with alum (●), 1 Lf alum adsorbed TT (○).
TT, size ranges between 2-8 µm elicited maximum antibody titers. Use of proper excipients to take care of protein instability at different stages of particles formulation helped in generation of high and sustained antibody titers from single point immunization. Antibody titers were also dependant on load of antigen per unit weight of polymer and doses of immunization. It was observed that high load of antigen in polymer matrix resulted in improved antibody response at equivalent doses of antigen. This also helps in reducing the amount of polymer per unit immunization dose. Even though polymeric particles entrapping TT gave rise to substantial antibody responses upon immunization, the levels of antibody titers increase significantly when small amount of alum was co-administered along with particles. Immunization of admixture of PLA particle and alum improved the peak antibody response and maintained the antibody response at a higher level throughout 250 days of post immunization period. Use of optimal parameters such as particle size (2-8 µm), high antigen load (> 28 µg TT/mg of polymer) and immunization of admixture of particles and alum improved the antibody titers significantly from single point immunization. Particularly at lower doses, immunogenicity of polymer entrapped TT was much higher than that observed with similar doses of alum adsorbed TT.

4.4 Efficacy of single dose polymeric vaccine formulation for TT

4.4.1 Comparison of immune response from single dose polymer entrapped TT with two divided doses of alum adsorbed TT

For evaluating the performance of the polymeric formulation against conventional two dose regimen of alum adsorbed TT; three groups of rats were immunized with single dose of 10 Lf, 5 Lf and 2 Lf of microencapsulated TT along with alum where as three other groups were administered with two divided doses each of 5 Lf, 2.5 Lf and 1 Lf of alum adsorbed TT at day 0 and 30. Antibody response from single dose polymer entrapped TT and two divided doses of alum adsorbed TT at different doses are presented in Table 4.4.
Table 4.4: Serum anti TT IgG concentrations elicited following immunization with single dose of microparticle entrapped TT (MPTT) co administered with alum and two divided doses of alum adsorbed TT at different doses regimens.

<table>
<thead>
<tr>
<th>Immunization modality and dose</th>
<th>Serum anti TT IgG concentrations (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 Day</td>
</tr>
<tr>
<td>10 Lf MPTT +Alum</td>
<td>184.6±74.8</td>
</tr>
<tr>
<td>5Lf+5Lf alum TT</td>
<td>40.3±14.7</td>
</tr>
<tr>
<td>5 Lf MPTT +alum</td>
<td>168.1±60.1</td>
</tr>
<tr>
<td>2.5 Lf+2.5 Lf alum TT</td>
<td>27.3±9.6</td>
</tr>
<tr>
<td>2 Lf MPTT +Alum</td>
<td>85.2±26.4</td>
</tr>
<tr>
<td>1Lf+1Lf alum TT</td>
<td>13.2±5.4</td>
</tr>
</tbody>
</table>
Considerably higher antibody titres (P<0.05) were achieved in the group immunized with single dose microparticles based vaccine (184.64±74.88 μg/mL) than the group immunized with the first dose of alum-adsorbed vaccine (40.3±14.7 μg/mL). After administration of the second dose of alum adsorbed vaccine in the groups immunized with conventional two dose vaccine, the titres were higher (407.4±108.2 μg/mL) than that observed with microparticles administered along with alum (335.8±117.1 μg/mL). However, the difference in antibody titres between the two groups was comparable after the second month till to the end of the study period. In the groups immunized with two divided doses (day 0 and 30) of 2.5 Lf TT as alum adsorbed vaccine; antibody titres before second dose were considerably lower (27.3±9.6 μg/mL) than those observed with microencapsulated antigen (168.1±60.1 μg/mL). But after the second doses at day 30, antibody titres enhanced rapidly and on day 60 it was 224.6±54.2 μg/mL in comparison to that of 205.5±48.2 μg/mL from admixture of single dose of microparticles and alum. Serum antibody concentrations were slightly higher in the case of two divided doses of alum-adsorbed vaccines. However at lower dose regimes: immunization with 2 Lf of admixture of PLA entrapped TT and alum elicited similar antibody titer than that observed with two doses of 1Lf of alum adsorbed TT immunization (Figure 4.13 A, B). Even after the second dose of alum adsorbed TT, the peak antibody titer was around 72.4±13.3 μg/ml in comparison to that of 111.2±34.2 μg/ml observed from single doses of polymer entrapped TT. At lower dose regimes, antibody titers were higher for polymer entrapped TT immunization throughout the post immunization days.

Immunization with microparticles along with alum resulted in very high early titres as compared to the conventional two dose vaccination. However it should be noted that the dose administered on day 0 was half of the total dose in case of alum adsorbed vaccine. Since overall dose were similar in the two groups, microparticles based vaccine can be used as an excellent modality to induce high early antibody titers. Comparison of antibody titers at different doses revealed that microparticles performed better at lower dose than alum based
Figure 4.13: Comparison of anti-TT IgG response induced by 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 (-●-), 10 Lf plain TT given in saline (-▲-). B: Single doses of 5 Lf (-■-) and two doses each of 2.5 Lf Alum adsorbed TT (-●-), 2 Lf microencapsulated TT co-administered with alum (-▲-), and two doses each of 1 Lf alum adsorbed TT (-▼-).
conventional vaccines. The better performance of microparticles at lower doses proved 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 titres elicited high antibody response, which was sustained throughout the study period.

Microparticles based vaccine performed increasingly better at lower doses. Lower values and sustainability of antibody titres induced by alum adsorbed vaccine suggest the possibility of relatively inferior priming of conventional vaccine as well as poorer depot effect compared to microencapsulated antigens administered along with alum. On comparing the antibody titers in group immunized with conventional two dose vaccine with that immunized with the single dose of polymer particle based vaccine along with additional adjuvant alum, higher early titres were observed in the first month itself (before administration of booster) along with better antibody titres during the late phase of study. Higher sustainability of microparticle based vaccine in comparison to two divided doses of alum adsorbed vaccine was very promising. Previous efforts to develop single dose vaccine have utilized mixing microparticles of varying sizes or degradation profiles. It was demonstrated in this study that single population of microparticles having optimum parameters can induce high and sustained antibody responses.

4.4.2 Determination of antibody affinity and antibody isotyping in different groups

Antibody affinity of anti-TT IgG present in sera was determined by analyzing the binding of sera to varying concentrations of antigen. Amount of unbound antibody was assayed by ELISA. Higher was the ELISA OD, lower was the affinity of antibodies. It was observed that microencapsulated antigen when co-administered with alum generated antibodies having comparable affinity to that observed from two divided doses of alum adsorbed antigen (Figure 4.14). Antibodies affinity matured around 90 days of primary immunization in both the
Figure 4.14: Comparison of affinitiy of anti-TT antibody in rats immunized with single dose of 10 Lf Microencapsulated antigen alone (Δ-) or coadministered with alum (-○-) and two divided doses each of 5Lf alum adsorbed TT (□-) administered at day 0 and 30. Pooled rat serum were normalized for anti TT IgG and incubated with equal volumes of antigen solution containing TT equivalent to 100μ g /ml and fraction of unbound antibody were analyzed by ELISA.
case. High affinity antibodies were observed throughout the study period for both two divided doses of alum adsorbed TT and single dose of microencapsulated TT. Affinity of antibody to the native antigen is a very important parameter of immune response and lower affinity of antibody are ineffective in rendering protection instead of high titers. Immunization with polymeric particles resulted in generation of higher affinity antibody indicating the importance of antigen stabilization during particle formulation (Raghuvanshi et al., 1998, Schwendeman et al., 2002).

Antibody isotyping revealed the similar concentrations of IgM in animals immunized with single dose of microparticles along with alum as well as conventional immunization with two doses of alum adsorbed antigen (Table 4.5). Immunization with microparticle alone generated lower serum IgG1 concentrations than those observed with alum based immunization, presence of alum along with particles improved the IgG1 levels. Levels of IgG2a were more or less similar for alum or particle based immunization. The IgG2a / IgG1 ratio was high for immunization with only particles, and was lower in alum adsorbed TT and admixture of particle and alum groups. Immunization with particles alone resulted in higher concentrations of IgG2a types antibody while alum based immunization resulted in more of IgG1 isotypes. Immunization with admixture of particles and alum not only improved the antibody response but also shifted the immune response towards Th2 types as indicated by lower IgG2a / IgG1 ratio as compared to only particle based immunization. As for tetanus the serum antibody titers are the main requirements, immunization with admixture of alum is the ideal combination for generation of long lasting high affinity antibody response.

4.4.3 Conclusions

Single point immunizations with microparticles having optimal characteristics in terms of particle size and antigen loads along with alum resulted in improved and sustained antibody responses. The enhancement of antibody response was
Table 4.5: Different antibody isotypes elicited in rats on different days following immunization with admixture of particle and alum and two divided doses of alum adsorbed antigen.

<table>
<thead>
<tr>
<th>Antibody isotypes</th>
<th>Dilution of serum at which ELISA O.D.s are above cut off values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5Lf+5Lf alumTT</td>
</tr>
<tr>
<td>IgM</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>10^7</td>
</tr>
<tr>
<td>IgG1</td>
<td>3X10^7</td>
</tr>
<tr>
<td>IgG2a</td>
<td>10^7</td>
</tr>
<tr>
<td>IgG2b</td>
<td>200</td>
</tr>
<tr>
<td>IgG2a/IgG1</td>
<td>3</td>
</tr>
</tbody>
</table>
observed in lower doses indicating that such formulation can be used at lower
doses for achieving protective immunity for many multidose vaccines. Single
dose of tetanus toxoid entrapped in PLA polymeric formulation when immunized
in presence of alum elicited antibody titres comparable to two divided doses of
alum adsorbed vaccine. Both the peak anti-TT antibody titers and its
sustainability were comparable with that observed from immunization with two
divided doses of alum adsorbed TT. In fact, immunogenicity of the entrapped
antigen was found to be better at lower dose regime indicating the suitability of
polymer entrapped antigen for development of single dose vaccine. These
results indicated that particle size favoring cellular uptake/attachment and
presentation, high load of antigen and use of additional adjuvant are very
important for generation of long lasting immune response from single point
immunization. Such immunization parameters need careful investigation for
maximizing immune response from single point immunization using polymer
entrapped antigen.

4.5 Memory antibody response responses from immunization
with single doses of polymer entrapped TT

High dropout rates for the second boosters in case of tetanus, lack of health care
facilities and infrastructure in developing and underdeveloped countries
necessitate that single point immunization should result in sustained primary
response as well as strong memory response. Since, microparticle based
vaccines deliver antigens to antigen presenting cells in a way which has been
observed to be different from alum based vaccines; it was of interest to
investigate the memory responses following single point immunization with
microparticles. Surprisingly in spite extensive research on polymer entrapped
vaccine formulation very little has been reported on the secondary immune
response from polymer entrapped antigen (Esparaza and Kissel, 1992, Gupta et
al., 1997). In both the cases memory response was studied for short period of
time while boosting with antigen along with adjuvant. As the real test for
development of immunological memory is the enhanced antibody response upon
challenge with very little soluble antigen, it was decided to evaluate memory response following single dose immunization of polymeric entrapped TT. This was also necessary as there were speculations that controlled release formulation using polymeric particles for vaccination may induce immunological tolerance. Preliminary investigation with immunization experiments using different combination of polymer entrapped TT when boosted with soluble antigen after six months of primary immunization resulted in very high secondary antibody response (Table 4.6). It was observed that single dose immunization with PLA entrapped TT either in nanoparticles or microparticles when boosted with 0.5 Lf of soluble TT after six month of primary immunization resulted in high antibody titers. Immunization with admixture of particles and alum resulted in very high secondary antibody responses upon boosting with soluble TT (Table 4.6) which were significantly higher than that observed from two doses of alum adsorbed TT immunization (P<0.05). Immunization with physical mixture of nanoparticles and microparticles alone or along with alum also gave rise to improved secondary antibody response upon boosting with soluble TT. To further explore this phenomenon, secondary antibody responses were evaluated extensively. Animals used for the evaluation of primary antibody responses in different experiments were boosted with very low amounts of soluble TT after 8-10 months and secondary antibody responses were analyzed.

4.5.1 Memory response from PLA entrapped TT particles having different excipients both in internal and external aqueous phase

Groups of wistar rats immunized with TT loaded microparticles (2-8 \( \mu \)m size) prepared with different excipients in IAP and EAP were boosted with 0.5 Lf of soluble TT after eight months of primary immunization. It was observed that most of groups which elicited considerable amount of primary antibody response also exhibited high secondary antibody responses (Figure 4.15). Secondary antibody responses varied widely in groups immunized with different formulations. Highest secondary responses were observed in formulations prepared with sucrose both in IAP and EAP. Very high burst release of these formulations, along with higher
Table 4.6: Primary and secondary anti-TT IgG response in rats following immunization with different immunization protocols.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum anti TT IgG concentrations (µg/ml) during primary response</th>
<th>Peak</th>
<th>Last day</th>
<th>Peak serum anti TT IgG concentrations (µg/ml) during secondary response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two doses of Alum adsorbed TT (5Lf+5Lf)</td>
<td></td>
<td>354.6</td>
<td>68.9</td>
<td>327.2</td>
</tr>
<tr>
<td>Microparticles entrapping TT (10 Lf)</td>
<td></td>
<td>23.3</td>
<td>3.0</td>
<td>347.7</td>
</tr>
<tr>
<td>Microparticles entrapping TT (10 Lf) with alum</td>
<td></td>
<td>169.0</td>
<td>18.2</td>
<td>869.9</td>
</tr>
<tr>
<td>Nanoparticles entrapping TT (10 Lf)</td>
<td></td>
<td>26.1</td>
<td>3.86</td>
<td>348.1</td>
</tr>
<tr>
<td>Admixture of nanoparticles and microparticles entrapping TT (5 Lf each, total 10 Lf)</td>
<td></td>
<td>58.8</td>
<td>19.8</td>
<td>366.7</td>
</tr>
<tr>
<td>Admixture of nanoparticles and microparticles entrapping TT (5 Lf each, total 10 Lf) with alum</td>
<td></td>
<td>189.9</td>
<td>14.7</td>
<td>800.1</td>
</tr>
</tbody>
</table>
Figure 4.15: Primary and secondary antibody responses elicited by TT loaded microparticles containing different excipients in IAP and EAP. Dose of TT equivalent to 5Lf was administered. All formulations were prepared using $1\%$ PVA as EAP. Other excipients in different formulations were TT, RSA, sucrose and sodium bicarbonate in IAP and sucrose in EAP ($\nabla$); TT, RSA and sucrose in IAP and sucrose in EAP ($\circ$); TT, RSA, sucrose and sodium bicarbonate only in IAP ($\triangle$); TT, RSA and sucrose only in IAP ($\square$); Alum adsorbed TT ($\cdot$).
early and peak primary response suggests that magnitudes of secondary responses are closely related to the degree of priming during primary immunization. Secondary responses induced by these formulations were higher than those induced by equivalent dose of alum adsorbed TT. Group of animals immunized with formulation containing TT, RSA, sucrose and sodium bicarbonate in IAP without sucrose in EAP, which induced very low antibody titres during primary responses, exhibited lower secondary responses upon boosting with soluble TT. This observation showed that absence of burst release resulted in very low secondary antibody responses. The secondary antibody titers improved 6-8 times upon boosting with soluble TT on 240 days after the primary immunization. Extents of enhancement in antibody titers upon boosting were very high from groups of animals whose antibody titers were low on the day of boosting. The extent of increase in secondary antibody titers from groups having high early titers was low although the absolute value of secondary antibody titers was high. High levels of circulating antibodies lead to neutralization and clearance of antigens and probably the reason for lower increase of antibody titers upon boosting.

Co-administration of alum, which was observed to have positive impact on the primary antibody responses, also improved secondary antibody responses significantly in all the formulations (Figure 4.16). The degree of enhancement of antibody titres during secondary responses was not as diverse as it was in primary responses. Peak antibody titers during secondary responses improved almost ten times in formulations with sucrose both in IAP and EAP upon boosting with 0.5 Lf soluble TT. Co-administration of alum helped to improve priming and subsequently early and peak titres with great degree in the formulations with lower burst release, thus leveling the extent of improvement in secondary antibody responses. The extent of increase in secondary antibody titer in the groups of animals was high where the residual antibody levels were low at the time of boosting. However animal immunized with formulation which gave highest primary antibody response also elicited very high secondary antibody titers.
Figure 4.16: Primary and secondary antibody responses induced by TT loaded microparticles containing different excipients in IAP and EAP co-administered with alum. Dose of TT equivalent to 5 Lf TT were used for immunization. All formulation were prepared having 1 % PVA in EAP. Other excipients in different formulations were TT, RSA, sucrose and sodium bicarbonate in IAP and sucrose in EAP (△); TT, RSA and sucrose in IAP and sucrose in EAP (○); TT, RSA, sucrose and sodium bicarbonate in IAP (▲); TT, RSA and sucrose in IAP (□); Alum adsorbed TT (◊).
the group immunized with single dose of alum adsorbed TT, the ratio of secondary to primary peak titres were lower than those observed in the case of microparticles groups irrespective of co-administration of alum. Formulation containing RSA, Sucrose and NaHCO₃ in the internal aqueous phase and sucrose in the external aqueous phase elicited around 800 µg/ml of peak secondary antibody titer upon boosting with soluble TT.

4.5.2 Effect of microparticle size on memory responses from PLA entrapped TT particles

Secondary antibody response from immunization with different size ranges of TT loaded PLA particles varied extensively (Figure 4.17). Microparticles with size between 2-8 µm induced very high secondary antibody responses, which persisted at a very high level for longer period of time. Groups of rats immunized with microparticles having sizes of 0.5 – 1.98 µm also induced high secondary responses but they were lower than those observed with microparticles with size ranges of 2-8 µm. It was observed that groups immunized with formulations having particle size range 10–70 µm and 50-150 µm exhibited very poor secondary responses. PLA particles with size ranges 10-70 µm did not result in improved secondary response in spite of reasonable primary response. Higher sized particle (50-150 µm) exhibited lower primary as well as secondary antibody response. This indicated that memory response is not only related to early and peak titers but also to the size of polymeric particles. Only those microparticles whose size ranges were best suited either for uptake or adherence to antigen presenting cells exhibited improved secondary responses upon boosting with soluble TT. These results confirmed that PLA particles of size ranges between 2-8 µm gave rise to highest secondary antibody response. Thus for both primary as well as secondary antibody response particles size ranges between 2-8 µm were found to be optimal for single dose TT immunization.
Figure 4.17: Primary and secondary antibody response induced by TT loaded PLA particles of different sizes ranges, co-administered with alum. Animals were immunized with particles sizes of 50-150 μm (■), 10-70 μm (○), 2-8 μm (△) and 0.50-1.98 μm (□).
4.5.3 Memory response from immunization with PLA particles having different antigen load

Secondary antibody titers increased with increase in antigen loads of TT loaded PLA particles (Figure 4.18). As the TT load per unit weight of PLA particles were increased from 1.3 to 94.2 µg TT/mg polymer, secondary antibody titers upon soluble boosting increased from 200 µg/ml to 780 µg/ml. All the TT microparticle formulations with different antigen loads were having size range between 2-8 µm to eliminate the effect of size on memory responses. There was a correlation between the peak primary antibody titers with that of peak secondary antibody titers upon boosting with soluble antigen. Animals with higher peak titers during the primary antibody response exhibited higher peaks during the secondary antibody response upon boosting with soluble TT. The extent of improvement of antibody titers was high in the groups of animals where the residual antibody titers at the point of boosting were low. The secondary antibody responses induced by microparticles with high antigen loads persisted at higher levels till the end of experiment (for three months after boosting with 0.5 Lf soluble TT). It seems that distribution of antigen over a large number of microparticles lower over all dose of antigen reaching antigen presenting cells thus affected both the primary and secondary antibody response. High load of antigen in polymer particles was thus found to be favorable for both primary and high secondary antibody response.

4.5.4 Effect of antigen doses on memory response from immunization with TT entrapped polymer particles

Memory antibody responses were tested in rats immunized with different dose of microencapsulated TT with size range between 2-8 µm. Dose dependent increase in the peak secondary antibody titres were observed upon boosting with soluble TT after 275 days of primary immunization (Figure 4.19 and 4.20). Absolute secondary antibody titers were high for higher doses of PLA entrapped TT immunization. However the extent of increase in antibody titers upon boosting
Figure 4.18: Primary and secondary antibody response induced by 5 Lf of TT loaded PLA particles with different loads of antigen co-administered with alum. Different loads of TT (µg/mg) in particles were 94.2 (-■-), 28.2 (-○-), 16.7 (-△-), 5.8 (-▽-) and 1.3 (-□-).
Figure 4.19: Primary and secondary antibody responses induced following administration of different doses of TT loaded microparticles co-administered with alum. Different doses were 15 Lf (-■-), 10 Lf (-○-), and 5 Lf (-▲-).
Figure 4.20: Primary and secondary antibody responses induced by different doses of microencapsulated TT and soluble TT. Different doses of microencapsulated TT co-administered with alum were 1 Lf (-■-), 0.5 Lf (-○-) and 0.1 Lf (-▲-). For comparison 10 Lf saline TT (-▼-) was also administered to a different group of animals.
with soluble TT varied considerably. In groups of animal immunized with lower doses of PLA entrapped TT; particularly with 0.5 Lf and 0.1 Lf of PLA entrapped TT immunization, the extent of increases in antibody titers upon boosting was 40 to 50 fold as compared to that of 6 fold observed at higher dose regimes. In the group immunized with 0.1 Lf of PLA entrapped TT, secondary antibody responses were significantly higher than those observed in groups immunized with 10 Lf soluble TT in spite of having similar primary responses (Figure 4.20). It indicated that at similar dose regimes, entrapment in polymer matrix leads to almost 100 fold increase in secondary antibody response as compared to that observed with soluble TT. Another important feature observed was that in the case of groups immunized with lower doses of antigen, peak secondary antibody responses were observed after seven days of boosting. This showed that entrapment of antigen in polymer particles not only improved the immunogenicity but also helped in developing better immunological memory.

4.5.5 Comparative memory response from single dose of microencapsulated TT with that of two divided doses of alum adsorbed TT

Memory response from single point immunization of PLA particle with that of two divided dose of alum adsorbed TT was compared at different dose regimes. It was observed that at all doses, the secondary antibody response from polymer entrapped TT was higher than that observed from two doses of alum adsorbed TT immunization (Figure 4.21-23). At dose regime of 10 Lf PLA entrapped TT; the extent of increase in antibody titer upon boosting was six fold in comparison to two fold increase in antibody titer from two divided doses of alum adsorbed TT immunization (Figure 4.21). The residual antibody titers in these two groups were almost similar at the point of soluble boosting with soluble TT. This suggested that PLA entrapped TT elicited higher secondary antibody response than that observed from two doses of alum adsorbed TT vaccination. Similar improved secondary antibody titers were also observed at lower doses of TT immunization (Figure 4.22, 4.23). Surprisingly at 5 Lf dose regime, single dose of alum adsorbed TT gave better secondary response than two divided doses of alum
Figure 4.21: Primary and secondary antibody responses induced after immunization with 10 Lf microencapsulated TT co-administered with alum (-○-)
and two doses of 5 Lf alum adsorbed TT administered at day 0 and day 30 (-■-).
Figure 4.22: Primary and secondary antibody responses induced by single dose of TT loaded microparticle co-administered with alum equivalent to 5 Lf TT (-O-), two doses each of 2.5 Lf alum adsorbed TT administered at day 0 and 30 (-△-) and single dose of 5 Lf alum adsorbed TT (-▽-).
Figure 4.23: Primary and secondary antibody response induced by 2 Lf microencapsulated TT co-administered with alum (-■-) and two doses each of 1 Lf alum adsorbed TT administered on day 0 and 30 (-○-).
adsorbed TT. This observation suggests the detrimental effect of booster doses on long-term memory and needs further investigation. The groups immunized with two divided doses of alum adsorbed TT elicited higher antibody titres at the end of primary response and it might have subdued secondary response to some extent by neutralizing and clearing soluble antigen administered to check memory response. However, it should be noted that single dose of equivalent amount of antigen loaded in microparticles, when co-administered with alum induced considerably higher secondary titer than those obtained from groups immunized with single or divided doses of alum adsorbed antigen. At a dose regime of 2 Lf of PLA entrapped TT, the extent of antibody response increase upon boosting was almost 14 fold in comparison to 5 fold observed for two divided doses of alum adsorbed TT immunization. In the groups immunized with single or double doses of alum adsorbed vaccine, secondary response was marked by an early peak (around 7 days), which dwindled very rapidly. Secondary antibody response from polymer entrapped TT immunization peaked 15 days after the boosting and sustained at a higher level. This indicated that the memory response from polymer entrapped TT immunization was much better than that observed for alum adsorbed TT immunization particularly at lower dose regimes.

4.5.6 Antibody affinity and antibody isotyping studies

The affinities of antibody during memory response in case of microencapsulated TT were similar to that generated by two doses of alum adsorbed vaccine. It was observed that when different concentrations of antigen were incubated with constant amounts of antibody, the fractions of antibody unbound to antigens (higher the fraction of antibody unbound to antigen, lower the affinity of antibody and vice versa) were higher for alum adsorbed TT immunization (Figure 4. 24). This indicated the lower affinity of antibodies elicited by alum adsorbed TT immunization in comparison to that elicited with polymer particles based immunization. However at very high antigen concentration the affinities of antibody generated by both groups were same. Higher affinity antibodies of TT
Figure 4.24: Anti-TT antibody affinity to soluble TT in the serum of groups immunized with 10 Lf microencapsulated TT co-administered with alum (-O-) and two doses each of 5 Lf alum adsorbed TT (-■-) on 15 days after boosting with 1.0 Lf soluble TT.
were observed around 75 days of post immunization from both the groups (Figure 4.25). Higher affinities antibodies were maintained till 270 days of post immunization in animals boosted with soluble TT after 170 days of primary immunization. Antibody isotypes from immunization of polymer entrapped TT and alum adsorbed TT were analyzed and presented in table 4.7. Serum IgM concentrations elicited by conventional two dose alum adsorbed vaccine during primary and secondary antibody responses were similar to those elicited following single dose immunization with the admixture of polymer entrapped TT and alum. Polymer entrapped TT immunization resulted in more IgG2a isotypes after boosting with soluble TT. Concentrations of other classes of IgG in the serum were similar.

4.5.7 Conclusions on memory response from polymer particle entrapped TT immunization

Animals immunized with different microparticle based TT formulations elicited very high secondary (memory) response upon boosting with non-immunogenic amount of TT after 6-8 months of primary immunization. Memory response was found to be dependent on the factors affecting primary antibody response from single point immunization. Maximum secondary antibody response was elicited by particles with size range 2-8 µm, high antigen load and with high burst release. Memory antibody titer was also dependent on the dose of antigen administered in particulate form. Memory response generated by particle-based immunization was quicker and significantly higher than that induced by equivalent dose of alum adsorbed TT. Low doses of microencapsulated TT administered with alum also exhibited very high memory antibody response. In spite of eliciting high antibody titres after the second dose, the group of animals receiving two doses of alum-adsorbed TT elicited lower secondary antibody titers than the group receiving equivalent amount of TT as single dose in polymer particles. Memory antibody response generated after particle based immunization sustained at a higher level over a longer period of time as compared to that generated by alum adsorbed vaccine. The affinities of antibody
Figure 4.25: Unbound antibody after incubation of pooled serum from rats immunized with two doses of 5 Lf alum adsorbed TT each (-□-) and single dose of 10 Lf microencapsulated TT coadministered with alum (-○-) during different days after immunization.
Table 4.7 Different antibody isotypes in rat serum immunized with two doses (5 Lf each) of alum adsorbed TT and single dose (10 Lf) microencapsulated TT co administered with alum

<table>
<thead>
<tr>
<th>Days</th>
<th>Dilution of serum* at which ELISA O.D.s are above cut off values**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5Lf+5Lf alum adsorbed TT</td>
</tr>
<tr>
<td>Primary</td>
<td>IgM</td>
</tr>
<tr>
<td>15</td>
<td>$10^2$</td>
</tr>
<tr>
<td>69</td>
<td>$10^3$</td>
</tr>
<tr>
<td>247</td>
<td>$10^3$</td>
</tr>
<tr>
<td>Memory</td>
<td>IgM</td>
</tr>
<tr>
<td>7</td>
<td>$10^3$</td>
</tr>
<tr>
<td>21</td>
<td>$10^3$</td>
</tr>
</tbody>
</table>

*: Pooled serum from 6 rats
**: O.D.s more than three times normal rat serum
generated by two doses of alum adsorbed vaccine. Antibody generated during memory response from particle based immunization had similar IgM titres but higher IgG titres than conventional two doses of alum adsorbed TT. Single dose immunization of polymer entrapped TT apart from eliciting long lasting primary immune response demonstrated high secondary antibody response. The fact that controlled release formulation in spite of slow release of antigen results in immunological memory adds another advantage to the utility of polymeric vaccine delivery system. Uptake of particles, continuous release of antigen and high burst release of TT from polymer particles may be acting in cooperative ways to improve immunological memory from single point immunization. The mechanism underlying this phenomenon is not clear yet but it is worth investigating.