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Single shot tetanus vaccine manufactured by a supercritical fluid encapsulation technology

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ABSTRACT

Single shot vaccines of tetanus toxoid (TT) were manufactured using the NanoMix™ process – a low temperature solvent free encapsulation technology using supercritical fluids. The formulations were injected into mice, and compared to multiple injections of a commercially available alum adsorbed TT vaccine. After 5 months the antibody titres were found to be similar for both the alum adsorbed and microparticle formulations, demonstrating for the first time the potential of formulating antigens in PLA microparticles using the supercritical fluid (NanoMix™) technique to produce single shot vaccines. The results are likely to be due to the maintenance of toxoid bioactivity and some degree of sustained release of the encapsulated antigens, resulting in repeated stimulation of antigen presenting cells eliminating the need for multiple immunisations. This demonstrates the potential of this supercritical fluid processing technique to reduce the need for booster doses in a vaccine regimen.

1. Introduction

The use of biodegradable polymers to encapsulate and deliver injectable controlled release products of high value pharmaceuticals such as recombinant proteins, synthetic peptides and DNA represents a major application for drug delivery systems (O'Hagan et al., 2001; Singh et al., 2007). One area of active pharmaceutical research is the development of single dose controlled release vaccines. At present, vaccines often require the administration of multiple doses over the course of several months in order to develop a robust and protective immune response (Feng et al., 2006). However, the compliance of patients in receiving the appropriate booster injections, especially in developing countries, is less than optimal. Therefore, the concept of developing a single dose controlled release vaccine is highly appealing. The benefits include improved compliance, a lower manufacturing cost from the reduced use of expensive recombinant proteins, and fewer vaccine administrations should promote wider use, particularly in the third world.

Existing marketed vaccine products generally use adjuvants based on aluminium mineral salts (commonly called alum) to generate strong immune responses (O'Hagan et al., 2001). Alum is one of the few adjuvants to receive approval for use in vaccines (Singh and O'Hagan, 2002) but has limited antigen compatibility and an inability to induce cytotoxic T-cell responses, important in parasitic and viral infections (Lima and Rodrigues, 1999).

Microparticle based vaccines have received much interest due to their potential ability to co-encapsulate multiple antigens, generate immune responses against weakly immunogenic antigens, induce cytotoxic T cell responses and be stable enough to be administered orally, nasally or parenterally (Katare and Panda, 2006a; Lima and Rodrigues, 1999; Ahire et al., 2007; Tabassi et al., 2008; Mohana et al., 2010; Bowey and Neufeld, 2010). Controlled release vaccines are most frequently formulated using the multiple emulsion solvent evaporation method based on the biodegradable and FDA approved poly-(lactic acid) (PLA) and poly-(lactic-co-glycolic acid) (PLGA) polymers (Shi et al., 2002; van de Weert et al., 2000; Jaganathan et al., 2005). These polymers have already seen extensive use in injectable sustained release drug products such as Decapeptyl™ (Ipsen), Zoladex™ (AstraZeneca), Enantone™ (Takeda) and Risperdal Consta™ (Janssen Cilag).

Altering the particle size of a microparticle vaccine system has been shown to affect antigen release rate and immunogenecity. Particles larger than 10 μm will normally act as a depot after injection, producing a sustained release of the antigen, whilst particles smaller than 10 μm may be phagocytosed by macrophages (Jaganathan et al., 2005; Lima and Rodrigues, 1999; Singh et al.,

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1998, 2007). Recent studies have shown that vaccine microparticles can act as a synthetic adjuvant to stimulate dendritic cell and T-cell immunity, and furthermore be bioconjugated with immune specific molecular 'danger' signals (Reddy et al., 2006; Raghuvanshi et al., 2002). The use of nanoparticles (~20 nm) to specifically target dendritic cells in the lymphatic vessels for improved T cell immunostimulation has also been proposed (Reddy et al., 2006).

Several studies have researched the effect of combining polymer formulations with alum. Katare and Panda (2006a) found that alum present in the formulation adsorbed the antigen released as a burst from the particle surface and was able to potentiate a superior immune response under low dose regimens (0.1 Lf) compared to those elicited by soluble antigen (10 Lf). Alum/polymer formulations have also been shown to generate more potent antibody titres than alum only (Wack et al., 2008), whilst Raghuvanshi et al. (2002) found that immunisation with polymer nanoparticles in combination with alum resulted in a very strong and early immune response (15 days).

Protein instability during the microencapsulation process is one of the major hurdles for vaccine delivery systems based on biodegradable polymer particles (Katare and Panda, 2006b). The presence of high shearing forces, lengthy exposure to organic solvents and presence of aqueous/organic interfaces can result in protein degradation and loss of immunogenecity (Baras et al., 2000; van de Weert et al., 2000; Katare and Panda, 2006b).

Tetanus toxoid vaccine is used to prevent tetanus, a serious illness that causes convulsions (seizures) and severe muscle spasms, which may in severe cases result in bone fractures of the spine. Tetanus causes death in 30–40 percent of cases. Tetanus toxoid adsorbed vaccine manufactured by Aventis Pasteur Inc., for intramuscular injection, is a sterile suspension of alum-precipitated (aluminium potassium sulphate) toxoid in an isotonic sodium chloride solution. Tetanus toxoid is normally given as a triple vaccine in a formulation together with vaccines against diptheria and acellular pertussis, the socalled DtaP vaccine. Immunisation against tetanus is recommended for all infants 6–8 weeks of age and older, as either 3 or 4 injections, depending on which type of tetanus toxoid is used.

In this paper, a single shot vaccine of tetanus toxoid was manufactured using the NanoMix™ process. NanoMix™ is a solvent free, ambient temperature encapsulation technology that uses supercritical carbon dioxide ($scCO_2$) to encapsulate high potency, low dose drugs into microparticle matrices produced from regulatory approved polymers (Whitaker et al., 2005; Davies et al., 2008). Supercritical fluids have been used previoulsy to micronise vaccines (Sieverrs et al., 2007), but as yet they have not been used to produce controlled release vaccines.

The NanoMix process exploits that fact that when polymers, such as PLGA or PLA are exposed to supercritical carbon dioxide ($scCO_2$) the $scCO_2$ dissolves into the polymer, lowering the glass transition temperature and plasticizing (liquefying) the polymer. Under these conditions the dry powdered drug can be mixed effectively into the liquefied polymer at near ambient temperatures and in the complete absence of a conventional solvent (Howdle et al., 2001). The mixture is atomised through a nozzle into a lower pressure environment, whereby the CO_2 returns to a gaseous state, the polymer solidifies as microparticulate droplets containing the drug or antigen. This process produces injectable polymer microparticles with encapsulated drug or antigen suitable for subcutaneous or intra-muscular administration. The aim of the current study was to assess the feasibility of encapsulating tetanus toxoid within PLA microparticles, to test a range of formulations *in vivo* in mice and to compare the induced immune response to that after multiple injections of a commercially available alum adsorbed vaccine. The induced immune responses in the mice were followed for 154 days.

2. Materials and methods

2.1. Materials

Tetanus toxoid (TT) was provided by Statens Serum Institut (Copenhagen, Denmark) and the alum obtained from EDQM (France). Polylactic acid (PLA) (R202H) was purchased from Boehinger Ingelheim (UK). The micro protein BCA assay used in the experiments was from Thermo Fisher Scientific (UK). All other reagents used were at least of analytical grade and used as received.

2.2. Microparticle manufacture

Tetanus toxoid (TT) was encapsulated in PLA microparticles using the NanoMix™ process (Davies et al., 2008). Briefly, TT was concentrated from the commercially available vaccine, using Microcon YM-10 centrifugal filter devices (Millipore, US) according to manufacturer's instructions, to 75 mg/ml. To generate a 1% (w/w) mixture, 15 mg (200 μl) TT was freeze dried onto 1485 mg of PLA in the presence and absence of 1% (w/w) trehalose. The TT polymer slurry was initially frozen in dry ice for 30 min before freeze drying for 24 h. In the presence of trehalose the starting amount of PLA was adjusted accordingly. The potential aggregation of the TT was assessed by size exclusion chromatography (SEC) as described below. Non-freeze dried, commercially available TT was used as control. The polymer/toxoid freeze dried 'cake' was placed into a high pressure mixing chamber and exposed to supercritical carbon dioxide at >75 bar, >32 °C. These conditions liquefied the polymer, but left the TT in the dry state, allowing the antigen and excipients to be efficiently mixed by a stirrer at 150 rpm for 1 h. Spraying the mixture through a nozzle yielded PLA microparticles containing the antigen. All TT microparticles used for the subsequent *in vitro* and *in vivo* studies contained trehalose. Placebo microparticles were generated by processing PLA through the same conditions, substituting the TT solution for phosphate buffered saline. The size of the vaccine-microparticle formulation should ideally be below 100 μm in diameter in order to enable injectability of the finished formulation through a size 21G needle. Hence, the size fraction above 100 μm was removed by sieving.

2.3. In vitro release of TT from microparticles

The release of the TT from the microparticles was investigated by suspending 20 mg of each formulation (in triplicate) in 1 ml release buffer (0.01% Tween-20, 0.01% sodium azide in PBS) in a 2 ml microcentrifuge tube. Each tube was sealed in Nescofilm and placed on a rotor at 37 °C. Samples (0.8 ml) were taken at 30 min, 4 h, 1, 3, 6, 14, 21 and 28 days and spun at 8000 rpm for 3 min to pellet the microparticles. The removed sample volume was replaced with fresh release buffer. The release experiment was stopped at 28 days due to a minimal change in released TT being observed. Samples were quantified by micro protein BCA assay in triplicate.

2.4. Microparticle sizing

Microparticles were sized by laser diffraction (HELOS/BF, Sympatec, Germany). Microparticles were mixed in 0.1% Tween-80 to form a paste, transferred to a 50 ml cuvette and diluted with distilled water to reach an obscuration of 15–20%. Microparticles were sized in triplicate.

2.5. Extraction of tetanus toxoid from microparticles

Microparticles were dissolved in acetonitrile, centrifuged (8000 × *g* for 5 min at RT) and the supernatant discarded. This step was repeated twice and the samples dried in an oven at 40 °C. Sam-

ples were reconstituted in 750 μl of PBS, centrifuged 8000 × *g* for 5 min and the supernatant analysed by SEC as described below. Any insoluble TT pelleted material was dissolved in 400 μl of 6 M urea and was also analysed by SEC. Placebo microparticles and placebo microparticles containing spikes of known amounts of freeze dried TT were used as controls.

2.6. Micro protein BCA assay

The micro protein BCA assay (Thermo Fisher Scientific, UK) was performed according to the manufacturer's instructions, using the microplate assay protocol.

2.7. Size exclusion chromatography (SEC)

The SEC analysis technique was adapted from a method by Steere and Eisenberg (2000). An Agilent 1100 series (Agilent Technologies Ltd., UK) was equipped with a TSK-Gel 3000PW$_{XL}$ column and TSK-Gel PW$_{XL}$ guard column (Tosoh Bioscience GmbH, Germany). The mobile phase was 25 mM sodium phosphate monobasic monohydrate and 100 mM sodium sulphate, pH 6.9, run at a flow rate of 0.5 ml/min. Sample volume per injection was 20 μl and TT was detected at 214 nm.

2.8. In vivo study

The ability of single shot injections of the NanoMix™ sustained release tetanus vaccine formulations to induce an immune response was assessed in mice over a 5 month period. Thirty six female Balb/c mice aged six weeks were split into six groups of six mice for injection. The type of formulations administered, the injection schedule and the number of immunisations given are summarized in Table 1. The mice were injected subcutaneously in the scruff of the neck with 0.1 ml of the formulation. The effectiveness of a single injection of the microparticle formulations was compared to immunisation of the mice with a commercially available alum adsorbed vaccine, injected at three occasions. Group 1, was administered with a placebo formulation, comprising PLA mixed with phosphate buffered saline, processed in an identical manner to that containing TT. Group 2, was administered with a formulation containing a dose of 3 Lf TT/mouse, which comprised 1% TT: 98% PLA and 1% trehalose [w/w] (TT-MP). Group 3, consisted of mice administered with a formulation made up of 2 Lf TT-MP, dispersed in 1 Lf TT adsorbed to alum to give a total dose of 3 Lf per mouse (TT-MP-alum). As control groups, Group 4, was administered with TT adsorbed to alum (TT-alum) (EDQM, France) (3 doses of 1 Lf/mouse) and Group 5, with soluble TT (EDQM, France) (3 Lf/mouse). Group 6 was injected with a similar formulation to TT-MP but with increased dose of TT at 7.5 Lf/mouse (TT-MP-INC). The microparticles were suspended in an injection vehicle (27 mg in

Table 1
Treatment groups for the testing of TT formulations *in vivo*. Placebo: PLA microparticles in PBS; TT-MP: PLA microparticles with encapsulated TT. TT-MP-alum: PLA microparticles with encapsulated TT and mixed with alum adsorbed TT; TT-MP-INC: PLA microparticles with an increased dose of TT.

Group	Treatment	Dose (limits of flocculation [Lf])	Number of immunisations
Group 1	Placebo	0.0	1 (day 0)
Group 2	TT-MP	3.0	1 (day 0)
Group 3	TT-MP-alum	3.0	1 (day 0)
Group 4	TT-alum	3 × 1.0	3 (days 0, 28, 56)
Group 5	Soluble TT	3.0	1 (day 0)
Group 6	TT-MP-INC	7.5	1 (day 0)

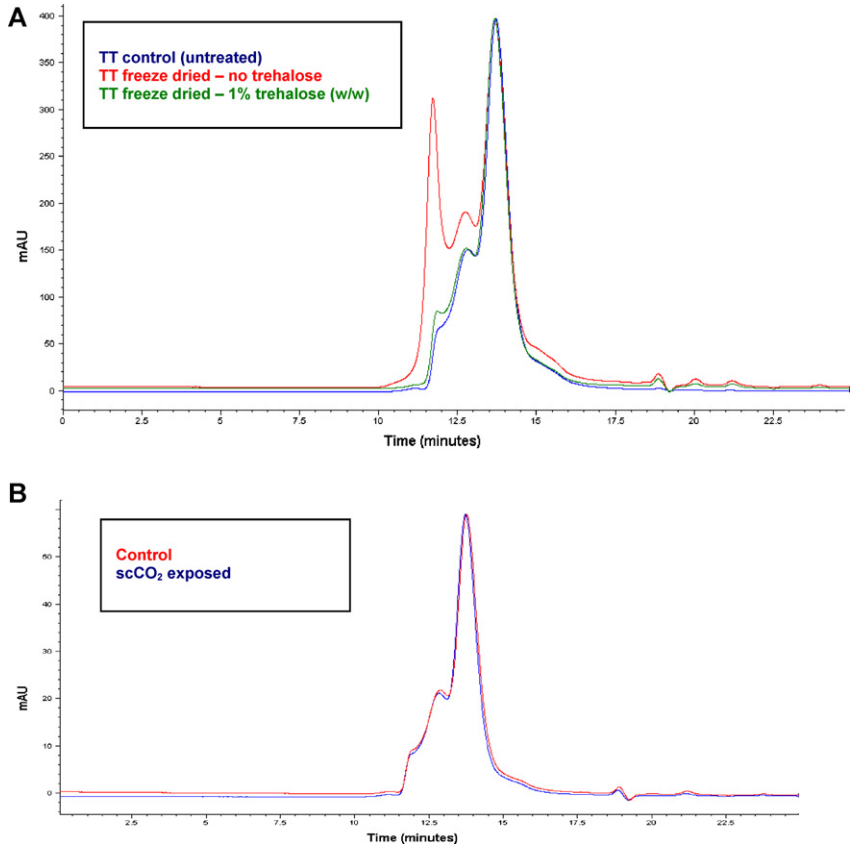

Fig. 1. Tetanus toxoid analysis by size exclusion chromatography. A comparison of untreated TT control and treated with- and without the presence of 1% (w/w) trehalose. (A) Freeze drying of TT causes aggregation of antigen, however this aggregation can be reduced by the addition of excipients such as trehalose. (B) Exposure of TT to scCO$_2$ causes little or no change to the antigen.

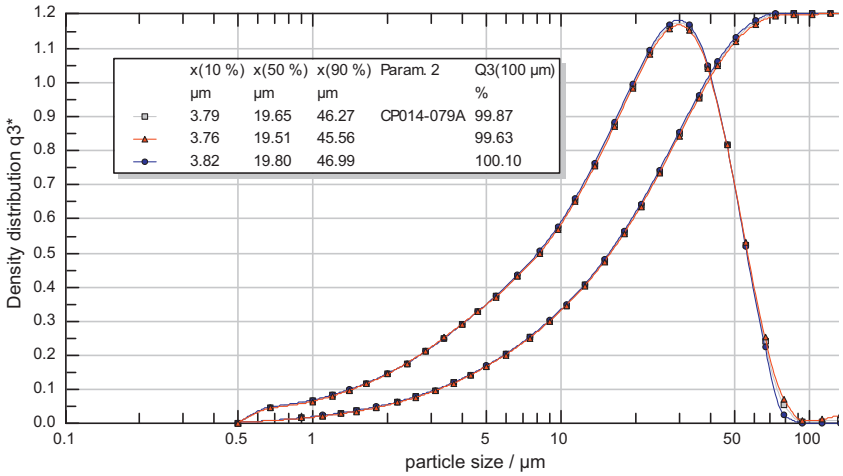

Fig. 2. Particle size distribution of the TT formulations after sieving, showing the density distribution (A) and the cumulative distribution (B). More than 90% of the particles are seen to have a particle size <100 µm.

500 µl) consisting of 3% CMC, 0.9% NaCl, 0.1% Tween 20 before injection through a 21G needle. Blood samples were taken pre-dose and after 14, 42, 70, 112 and 154 days. Anti-tetanus toxoid IgG response to each of the treatments was determined by ELISA.

2.9. Enzyme linked immunosorbent assay (ELISA)

Samples containing TT were pipetted, in triplicate (100 µl per well), onto MAXISORP ELISA plates (Nunc A/S, Denmark) and incubated at 4 °C overnight. The plate was washed with PBS, pH 7.2 containing 0.05% Tween-20 four times, followed by a wash in PBS, pH 7.2. The plate was blocked by adding 200 µl of 5% non-fat milk in PBS, pH 7.2, to each well and incubating for 2 h at RT. The blocking solution was removed and 100 µl of primary antibody (anti-tetanus toxoid goat polyclonal antibody, 1:20,000 [v/v] in blocking solution) was added and incubated for 2 h at RT. Following washing, 100 µl of the secondary antibody (rabbit anti-goat HRP, 10 µg/ml) was added and further incubated for 2 h at RT. After washing, 100 µl of substrate, two tetramethylbenzidine tablets (2 mg) dissolved in 20 ml of 0.05 M phosphate citrate buffer, was added to each well. After incubating for 15–20 min at RT, the reaction was stopped by the addition of 25 µl 2 M sulphuric acid. Results were analysed by spectrophotometer (BIO TEK Synergy HT, USA) at 450 nm wavelength, corrected at 540 nm. A positive control was prepared from commercially available mice serum from clotted whole blood (Sigma) which was spiked with a known quantity of TT. The negative control consisted of mice serum only and PBS. The role of the controls was to assess whether the ELISA was optimised and working correctly, and to determine that any negative results were valid.

2.10. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2003 edition. Results from the 1:10k dilutions were analysed by one-way ANOVA and p-values ≤ 0.05 was considered statistically significant.

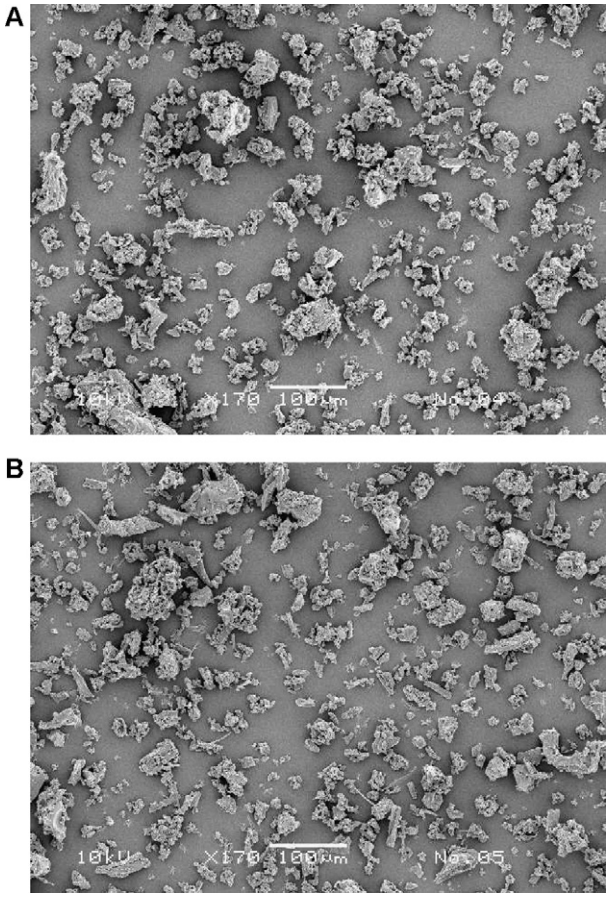

Fig. 3. SEM images of the TT microparticles at 170× magnification. (A) TT microparticles no trehalose and (B) TT microparticles with 1% trehalose (w/w).

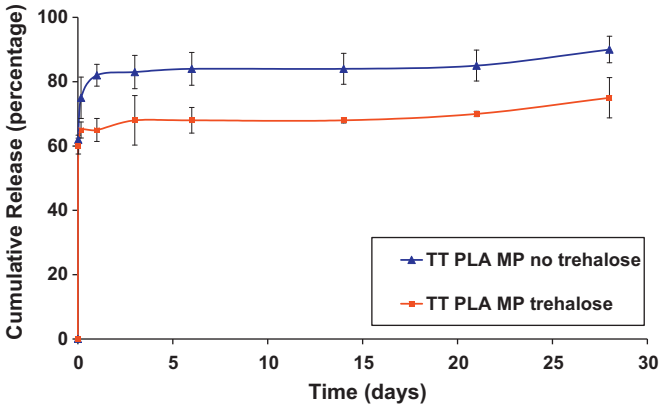

Fig. 4. *In vitro* release of Tetanus toxoid from PLA microspheres.

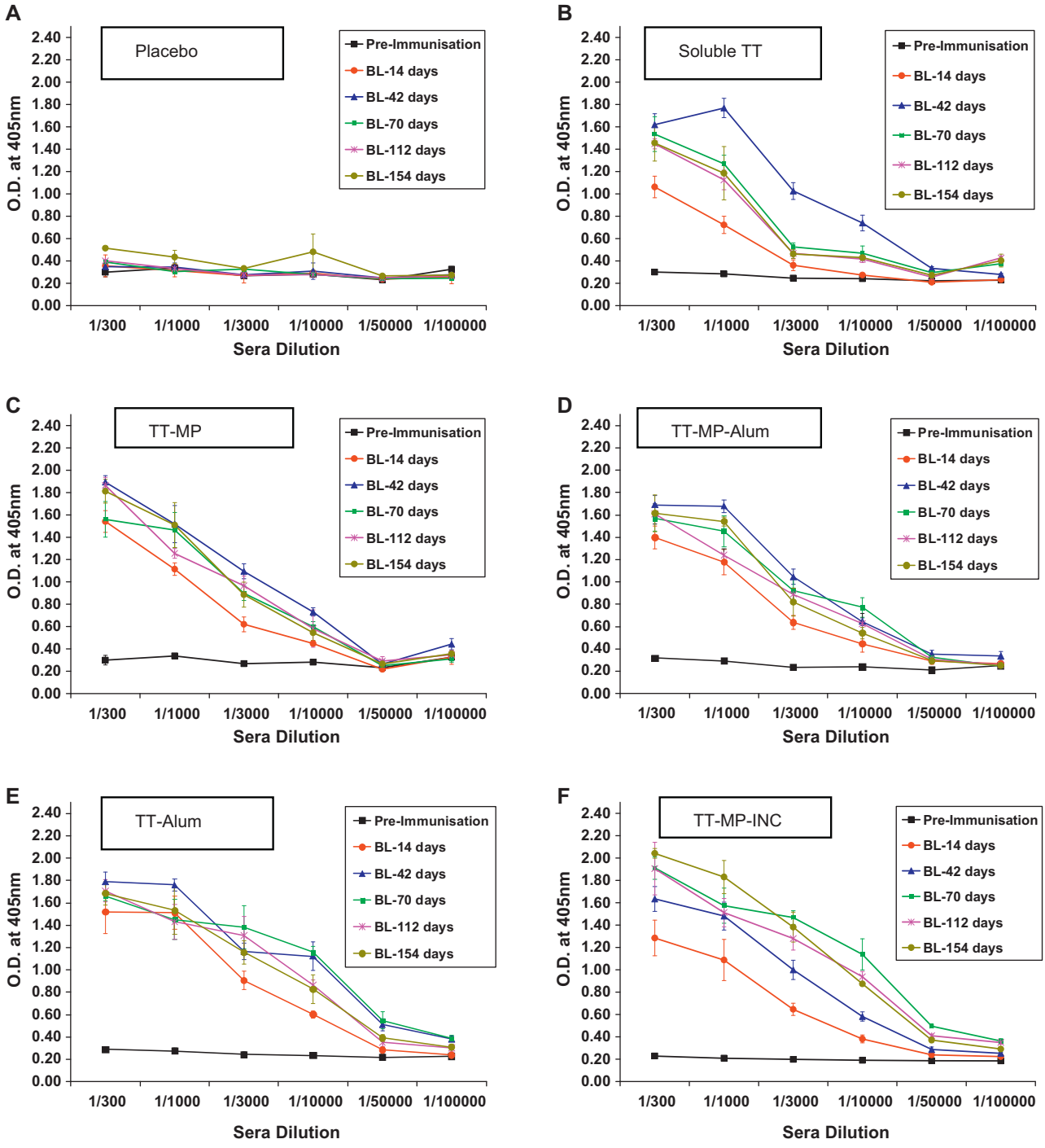

Fig. 5. Anti-tetanus toxoid IgG titres of immunised mice with time bleeds (BL) up to 154 days. (A) Placebo formulation, TT control (B) soluble TT (C) TT-MP (D) TT-MP alum (E) TT-alum (3 doses of 1 Lf/mouse) (F) TT-MP-INC.

3. Results

3.1. Effect of freeze drying and exposure of tetanus toxoid to $scCO_2$

The addition of trehalose in a 1:1:98 ratio (trehalose: tetanus toxoid: polymer [w/w/w]) was shown to prevent or at least reduce aggregation of TT during freeze drying, whilst not causing excessive damage to the antigen as measured by SEC, whilst no change in SEC profile was observed following exposure to $scCO_2$ (Fig. 1). Hence, trehalose was used in all further preparations of the TT microparticles. Furthermore, TT encapsulated into PLA microparticles by means of the NanoMix® method using $scCO_2$ maintained its antigenicity *in vivo* as shown below.

3.2. Determination of microparticle size and loading efficiency

The average mean diameter of microparticles (Vmd) was measured to be 22.8 μm with >90% of microparticles being below the desired size 100 μm in diameter (Fig. 2). The morphology of the TT microparticles shows that under the stated production conditions, the particles were irregularly shaped with some pores, caused by the rapid loss of CO_2 gas bubbles (Fig. 3). Furthermore, the addition of 1% trehalose (w/w) resulted in no detectable change in particle

morphology and size. The actual load of TT within the microparticles was determined by acetonitrile extraction and the amount of isolated TT then quantified by micro BCA. The theoretical load of TT was calculated to be 8.0 μg/mg microparticles. Using the BCA assay, the particle load was found to be 6.3 μg/mg microparticles. Loading efficiency was calculated to be 78.1%.

3.3. In vitro release of TT from microparticles

The *in vitro* release of TT from the microparticles produced with and without trehalose was investigated. Initially a significant burst effect (80% of the loading) and a high release was observed for the TT loaded PLA microparticles without the trehalose, which was significantly higher than for the TT microparticles containing trehalose, TT and PLA 1:1:98 [w/w/w], (65%). Both formulations followed a similar release profile for the remaining part of the study (Fig. 4).

3.4. In vivo studies

The anti-tetanus toxoid IgG titres induced from the immunisation of the mice with the different formulations are shown in Fig. 5. No immune response was detected in the placebo group and only a minimal response was detected in mice vaccinated with soluble TT (Fig. 5(A and B)). Mice injected with soluble TT showed a maximum antibody titre optical density (O.D.) at 42 days (Fig. 5(B)). The antibody titre O.D. from 70 days to 154 days decreased rapidly with sera dilution. Both microparticle formulations TT-MP and TT-MP-alum showed a peak in antibody titres at 42 days, followed by a slight decrease at 70 days and 112 days, and then improved titres at 154 days (Fig. 5(C and D)). Statistical analysis showed that the immune responses for the two microparticle formulations were not significantly different ($p > 0.05$). Similar antibody titres were generated at each bleed. This was also confirmed by the 1:10k dilution data shown in Figs. 6 and 7.

Statistical analysis confirmed that TT-MP-alum gave a significantly greater immune response throughout the study than soluble TT alone. Similar results were obtained for the TT-MP formulation, although the result was not significant at 42 days and 112 days. The antibody titres in the TT-alum samples showed a similar O.D. trend to the TT-MP and the TT-MP-alum formulations (Fig. 5(E)). However antibody titres in the mice vaccinated with TT-alum were significantly greater across the sera dilution range. For example, the samples collected from mice injected with TT-alum showed a steady decline in antibody O.D. across the sera dilution range, whilst samples collected from mice injected with TT-MP and TT-MP-alum showed a sharp decrease in concentration to the 1:50,000 dilution. Antibody titres for the mice injected with TT-alum increased rapidly at 42 days and peaked at 70 days for the 1:10k data (1.12 and 1.16. O.D. at 405 nm, respectively) (Figs. 5(E) and 6). Concentrations of TT antibodies in blood sera dilutions from mice injected with TT-MP-INC were shown to induce a significantly greater immune reaction than the placebo and soluble TT formulations across the study. Furthermore, the anti-tetanus toxoid IgG titres induced after a single injection of TT-MP-INC, increased successively and significantly with each bleed (Figs. 5(F) and 6). Antibody titres decreased in O.D. steadily across the entire dilution range. Analysis of the 1:10k data clearly showed that formulation TT-MP-INC almost produced a doubling in antibody titres from day 42 to 70 (0.58–1.14 O.D. at 405 nm, respectively) (Fig. 6). Although this formulation generated a slower immune reaction than the TT-alum, the decline in antibody titres was much slower throughout the remainder of the study (Figs. 6 and 7). Hence, it was shown that the highest dose TT microparticle formulation (TT-MP-INC) injected at day 0 was able to induce similar or higher anti-tetanus toxoid IgG titres to

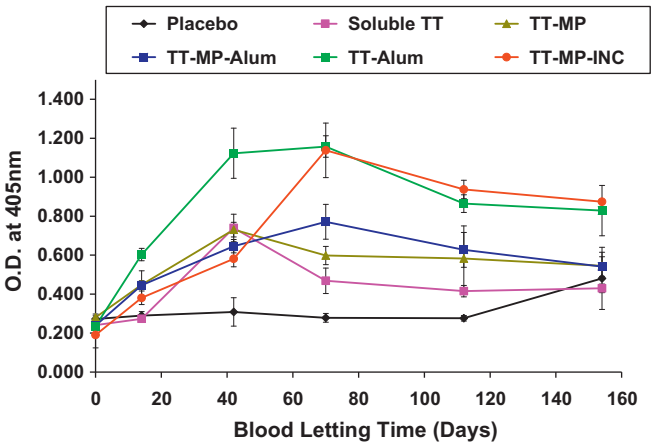

Fig. 6. Anti-tetanus toxoid IgG titres of immunised mice with time bleeds up to 154 days. sera diluted 1/10k.

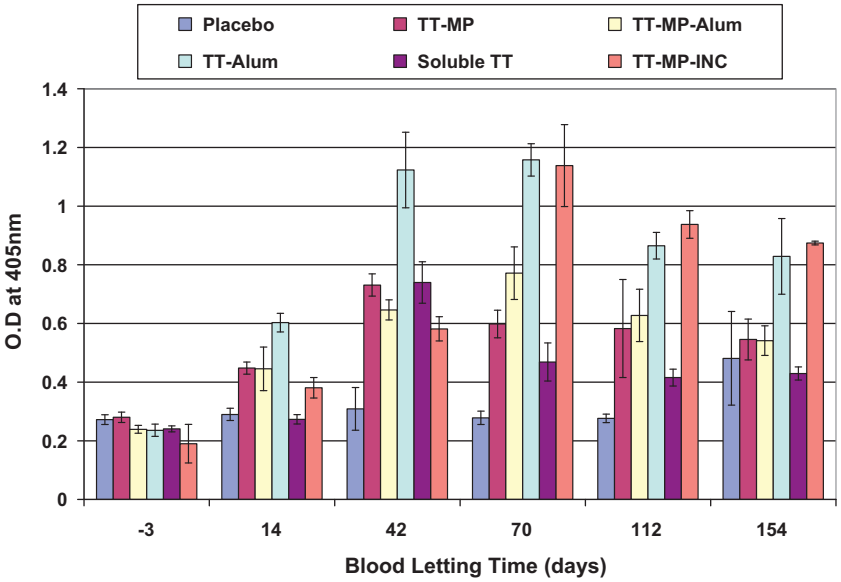

Fig. 7. Anti-tetanus toxoid IgG titres of pooled sera from six immunisation groups (sera diluted 1/10k). Blood time up to 154 days.

those induced by the TT-alum formulation that was injected at day 0 followed by two booster injections at day 28 and 56.

4. Discussion

In recent years, several new vaccine adjuvants and vaccine delivery systems have emerged, which are expected to enable the achievement of improved immune responses from different antigens without the use of alum. For example, the adjuvant, MF59, an oil in water emulsion containing squalene, was recently approved for use in the EU with the influenza vaccine Fluad (Novartis) (Sesardic and Dobbelaer, 2004; Wack et al., 2008).

Delivery of vaccines using micro and nanoparticle systems via parenteral, oral and nasal delivery routes has also been studied extensively in the last decade because of their ability to promote strong immune responses against weakly immunogenic antigens, induce cytotoxic T cell responses as well as antibody titres and the potential to co-encapsulate multiple antigens (Katare and Panda, 2006a; Lima and Rodrigues, 1999; Davis, 2001; Jabbal-Gill et al., 2001; Jaganathan et al., 2005). The most commonly used polymers for vaccine microparticle production are the FDA approved poly-(lactic acid) (PLA) and poly-(lactic-co-glycolic acid) (PLGA) polymers. The microparticles are normally produced using emulsion solvent evaporation methods. However, problems in terms of instability of the antigen during production often occur (Schwendeman et al., 1996; Shi et al., 2002; van de Weert et al., 2000; Raghuvanshi et al., 2001; Jaganathan et al., 2005; Jianga et al., 2005). Hence, TT encapsulated in PLGA microspheres were found to be partly inactivated during microsphere production and release, because of the processing of aqueous TT solutions in the presence of organic solvents, exposing lyophilised TT to moisture and incubating the vaccine in the degrading PLGA polymer at 37 °C (Schwendeman et al., 1996). The same group later reported that they had identified excipients that prevented the aggregation of the TT and retained the TT antigenicity under simulated deleterious conditions. They obtained a continuous *in vitro* release of TT for one month with retained antigen stability (Jiang and Schwendeman, 2008). Katare and Panda (2006a), apart from TT stabilisation problems, also identified a maximum entrapment efficiency of 69% dependent on the various excipients added to the formulation to improve stability of the TT. Raghuvanshi et al., 2001 encapsulated TT in PLGA particles using an emulsification process and found that denaturation of TT by the solvent dichloromethane could be prevented by the addition of rat serum albumin which also enhanced the encapsulation efficiency. As in the present studies, single injections of microparticles encapsulating stabilised TT elicited anti-TT antibodies titres for more than 5 months.

The PLA microparticle production process (NanoMix^{TM}), used, in the present study, for the production of the TT microparticles is based on $scCO_2$ processing. The freeze drying step in which the antigen in solution is spread over the polymer and freeze dried before being exposed to $scCO_2$, allowed for removal of the solvent in which the TT was suspended and a thorough mixing of the small amount of antigen with the PLA polymer.

This technique was demonstrated to encapsulate about 80% of the TT in the polymer microparticles whilst maintaining antigenicity and bioactivity to a high degree. The interaction of the TT with $scCO_2$ did not result in any further degradation or aggregation of the TT

The immunisation of mice with the 3.0 Lf TT-MP formulation as a single shot injection induced an immune response that was similar (but for the 1:10k dilution data) to that of a conventional alum adsorbed vaccine (TT-alum) administered as three separate injections (3×1.0 Lf). The immune response with time was highest at 42 days and then remained elevated for up to 154 days. The fact that the immune response from the TT-MP was not superior to that obtained from the TT-alum is surprising in the light of results by Jaganathan et al. (2005) who found that an injection in guinea pigs with TT-PLGA stabilised with trehalose (0.5 Lf TT) resulted in a better immune response than that obtained for multiple injections of alum adsorbed TT (0.5 Lf TT + 0.5 Lf TT booster after 4 weeks). The reason for the difference results in to the present study is likely due to the different animal model, the different doses of TT administered (Kipper et al., 2006) and the different particles size distribution of the TT-PLGA (1–10 μm) compared to the TT-MP (0.5–100 μm, 50% < 20 μm, 25% < 10 μm). Particles below 10 μm would be expected to be engulfed by macrophages and dendritic cells and thus contribute more strongly to the immune response than larger particles (Eldridge et al., 1991). However, several studies have also shown that vaccinating with a mixture of particle sizes can provide the most effective immune response due to their ability to interact with antigen presenting cells whilst providing a depot effect (Katare and Panda, 2006a; Jianga et al., 2005; Jaganathan et al., 2005; Raghuvanshi et al., 2002). The fact that in the present study, the immune response stayed elevated for a long duration in the present studies is suggested to be due to the high number of larger particles (Vmd = 22.8 μm) of this formulation, that would primarily have produced a depot effect. Katare and Panda (2006a) found that very large particles (50–100 μm) generated a poor immune response due to their limited interaction with phagocytes and their small available surface area. Conversely, they identified that microparticles ranging from 10 to 70 μm generated an improved immune response, suggesting that these particles interacted more readily with the surface of antigen presenting cells. Furthermore, it should also be noted that the Jaganathan study (Jaganathan et al., 2005) showed the TT to be released from the TT-PLGA microspheres over 20 days with a low burst effect whereas the TT-MP particles had a high initial burst of TT.

The *in vitro* release studies showed that the TT was released with an initial burst in the first day of about 60–75% of the total TT, followed by a slower release phase. It would be expected that for the slowly degrading PLA polymer a second burst of release would have been seen at a later timepoint. However, due to the slow increase in release of TT, the experiment was terminated at 28 days. The high burst release observed for the TT-MP indicates that a large part of the TT was situated close to or on the surface of the particles.

The TT-MP-alum, was tested because it had previously been found that the addition of alum in diphtheria toxoid microparticle formulations primed the immune system more effectively and also generated more potent antibody responses in a shorter period of time (Singh et al., 1998). The addition of magnesium hydroxide and alum into PLA/PLGA formulations had also been found to stabilise the pH and reduce aggregation (Jaganathan et al., 2005). However, it was shown in the present studies that the addition of alum to the microparticle formulation did not significantly improve the immune response of TT-MP.

Soluble TT only gave a weak, short lived immune response, which was significantly lower than the response obtained for TT-MP-INC and TT-alum. TT-MP-alum also produced a significantly greater immune response than the soluble TT, but for TT-MP this was only the case at 14, 70 and 154 days.

TT-MP-INC showed a significant improvement in antibody titres compared to TT-MP throughout the five month study. This microparticle formulation contained 7.5 Lf TT given as a single injection compared to the 3×1 Lf injections of TT in the alum adsorbed vaccine. There was no statistical difference between the microparticle TT-MP-INC and TT-alum, indicating that a sustained release microparticle formulation was as effective in creating an immune response as a currently marketed TT formulation, even though the mice vaccinated with the latter formulation were given two additional boosters. The titre for TT-MP-INC increased slower

than the titre for the TT-alum, but reached the same value at 70 days. After this the titre slowly declined but was as high as the titre for TT-alum at 154 days. The reason for the better effect of the higher dosed formulation is likely due to some degradation of the TT in the formulations after injection and hence a relatively larger loss of bioactivity in TT-MP and TT-MP-alum than in TT-MP-INC. Similar good results have been shown by Feng et al. (2006) for a single dose hepatitis B vaccine using PLGA microspheres, with the titres in mice comparable for the microsphere formulation and the alum formulated HbsAg vaccine requiring booster injections The greater cost of producing the increased dose formulation would be easily compensated by the reduction in vaccine administrations, and the improvements in public health resulting from increased compliance and immunity of the population.

5. Conclusion

The NanoMix™ process used in the present studies for production of the TT microparticles is a simple, solvent free process able to encapsulate vaccine antigens in polymer microparticles whilst maintaining their antigenicity and bioactivity. Elevated antibody levels can be achieved for a period of over 5 months following the administration of a single dose of the NanoMix™ microparticle vaccine delivery system, and are comparable to those following three doses of the normal alum adsorbed tetanus vaccine. Although, the longer term sustained release of the TT from the TT-MP formulations is not directly demonstrated, this indicates that the bioactivity of the antigens is reasonably maintained and that a prolonged release of the antigen from the microparticles is maintained over this period. The vaccine product would be presented as a freeze dried material ready for suspension just before injection and would be expected to show a reasonable long shelf life. However, although the TT-MP-INC formulation has shown similar or superior immune responses as compared to the alum vaccine it still remains to be determined if the TT-MP-INC formulation confers the same degree of protection as the marketed alum vaccine. If this is the case, it is likely that the formulation produced here could be exploited as a single shot TT vaccine. Such a sustained release vaccine system should also be applicable to other vaccines.

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