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Note

PLGA-dendron nanoparticles enhance immunogenicity but not lethal antibody production of a DNA vaccine against anthrax in mice

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Abstract

Dendriplexes, complexes of dendrons and condensed plasmids containing the gene for protective antigen (PA) of *Bacillus anthracis*, were encapsulated in poly-lactide-*co*-glycolide (PLGA) particles using the double emulsion method. The two dendrons employed are a dendron with three C_{18} chains (C_{18} dendron) and one with no attached hydrocarbon chains (the C_0 dendron). Three types of particles were examined, namely PLGA-C_{18} dendriplexes, PLGA-C_0 dendriplexes and the control PLGA-naked DNA system. These were characterised by standard biophysical methods such as photon correlation spectroscopy (PCS) and scanning electron microscopy to select the complexes for *in vivo* testing. Three intramuscular immunizations were carried out using 14 μg of DNA per dose at weekly intervals in BALB/c mice. Antibodies against rPA were measured using ELISA. Results indicate that the PLGA-C_{18} dendriplex particles produced superior levels of anti-PA IgG antibodies in comparison to animals immunized with the PLGA-C_0 dendriplex particles. The level of antibody production was dependent on the number of immunizations, higher antibody levels being measured after two booster vaccinations. However toxin neutralizing antibodies were absent in all treatment groups, and it is likely that the mice lack protection against lethal toxin and anthrax infection. Further studies are needed to optimize the formulation of DNA vaccines and increase the level of anti-lethal toxin antibodies and enhance their functionality.

Keywords: Nanoparticles; PLGA; Dendriplexes; DNA vaccines; Protective antigen

1. Introduction

Particulate systems composed of biodegradable polymers are part of new vaccine technology. Particulates belong to diverse delivery systems that encapsulate proteins or DNA to increase uptake by target tissue. PLGA is a well known polymer with low toxicity and an excellent safety record. It has been used as a carrier system for DNA for the purposes of immunisation via the parenteral and mucosal routes (Jones et al., 1997). The encapsulation of DNA in poly(lactide-*co*-glycolide-acid) (PLGA) nanoparticles is a process which may be advantageous in controlling DNA release and providing protection against degradation when administered as a non-viral gene delivery carrier. We hypothesise that the dendriplexes offer a distinct advantage over naked DNA, avoiding the degradation of DNA during the formulation of the PLGA particles, facilitating rapid delivery to target antigen presenting cells and providing an additional adjuvant effect often observed by particulate systems (Jiang et al., 2005).

Bacillus anthracis, the causative agent of anthrax, produces two binary toxins: lethal toxin (LT) and oedema toxin. These toxins are produced immediately after germination of the *Bacillus* spore. They attack various cell types and organ systems, hence they are major contributors to the lethal outcome of infection in man and mammals (for review see: Turnbull, 2000). Anthrax vaccination is protective through the induction of anti-toxin antibodies and antibodies against the binding subunit, the protective antigen (PA), regarded as the most important for protection (Turnbull, 2000). However the vaccine is crude, causes side effects and the immunization schedule is lengthy, requiring annual booster doses (Turnbull, 2000; Pittman et al., 2002). Several alternatives for the current crude anthrax vaccines are therefore under investigation. Here we report on the immunogenicity of a novel formulation for DNA vaccines, namely dendriplexes encapsulated in PLGA to hopefully

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improve the protection of the DNA from degradation and ensure controlled release of DNA. Apart from these advantageous aspects, PLGA particles represent a different form of immune adjuvant (Xie et al., 2005). PLGA particles have been shown to improve uptake and processing by antigen presenting cells (Mize et al., 2000, 2003; Singh et al., 2004). We examine whether immunization by PA DNA dendriplex PLGA particles induce a superior anti-PA antibody response in comparison with PLGA-PA DNA particles without dendron complexation and whether the antibodies possess toxin neutralizing abilities.

2. Materials and methods

2.1. Materials for PLGA particle formulation

Poly(lactide-*co*-glycolide) (PLGA) (50:50), Medisorb DL 2A, (M_w 15,200, with carboxylic end groups) purchased from Alkermes. Poly(vinyl alcohol) (PVA) of M_w 13,000–23,000 (87–89% hydrolysed) was supplied by Sigma–Aldrich.

2.2. Formulation of PLGA-dendriplex particles

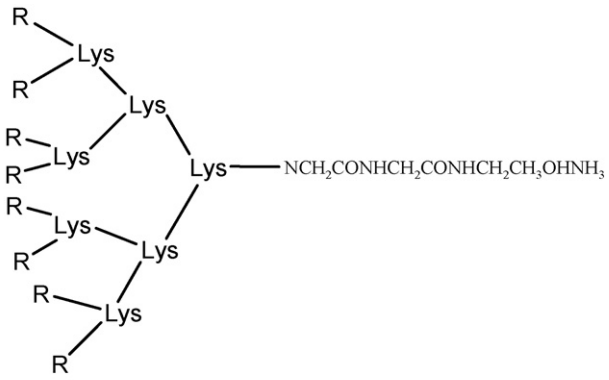
Full length PA (PA83) was cloned into the eukaryotic plasmid, pCMV/myc/ER containing a signal sequence for fusion protein secretion (Hahn et al., 2004). The plasmid DNA was purified as described by Hahn et al. (2004) and was complexed with poly-lysine dendrons to form compact nanoparticles. Two dendrons were used in the *in vivo* study, the first dendron (C_0), containing seven lysine groups and eight amino groups and no hydrocarbon chains attached to the core and the second dendron, which contained three C_{18} chains (Fig. 1).

The PLGA particles were formulated by a double emulsion method (Ribeiro et al., 2005). The dendriplexes were formed by mixing DNA and dendron at a 10:1 molar charge ratio. These particles were added to 3% (w/v) PVA. The surfactant solution containing the dendriplexes was homogenised with PLGA (50:50) in DCM to form a primary o/w emulsion. A second concentration of 1.25% (w/v) PVA was homogenised with the primary o/w emulsion giving a w/o/w emulsion. The PLGA particles were harvested by centrifuging at 20,000 × *g* at 15 °C and subsequently washed, then lyophilised to permit long term stability.

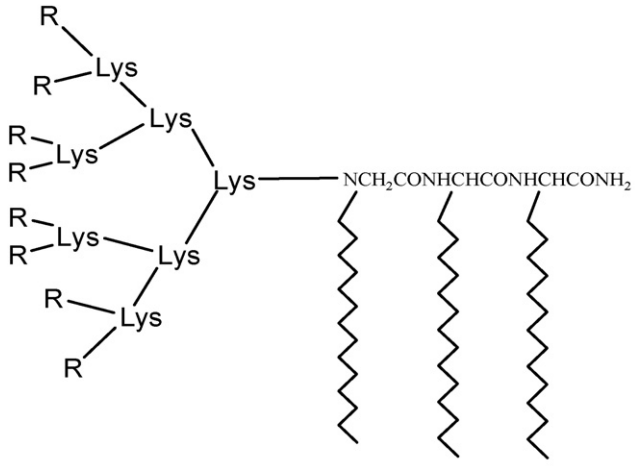
Three systems were prepared for the animal study. These were (1) PLGA C_{18} dendriplexes, (2) PLGA C_0 dendriplexes and (3) PLGA-naked PA DNA particles. Encapsulation efficiency for the 10:1 dendriplex in PLGA was 15.6% (w/v) and for DNA-PLGA 9.92% (w/v) (Ribeiro et al., 2005). These figures were used to calculate the concentration of DNA per dose.

2.3. Characterisation of PLGA-dendriplex particles

The mean sizes of the dendriplex PLGA particles, which are all below 1 μm in diameter, were determined by photon correlation spectroscopy (PCS) using a Zetasizer 3000 (Malvern Instruments, Malvern, UK, He–Ne laser, 90° angle of measurement) after dilution of samples. The average of three



$(C)_0(Lys)_7(NH_2)_8$ MW 1174.91



$(C_{18})_3(Lys)_7(NH_2)_8$ MW 1758.45

Fig. 1. The simplified chemical structure of two cationic (lysine)-based dendrons (L = lysine) $(C)_0(Lys)_7(NH_2)_8$ M_w 1174.91 (C_0) and $(C_{18})_3(Lys)_7(NH_2)_8$ M_w 1758.45 (C_{18}) both containing 16 amino groups and 7 lysine groups attached to the core and having either 0 or 3 hydrocarbon chains.

measurements was used and results expressed as the *Z*-average diameter (nm) ± S.D and zeta potential (mV) ± S.D.

2.4. Scanning electron microscopy

The PLGA-dendriplex particles were characterized using a Philips CM 120 BioTwin transmission electron microscope; samples were stained with 1% uranyl acetate.

2.5. Immunisation of animals

BALB/c mice were split into three groups of five animals. Mice in group 1 received 20 μl each of PA DNA-C_0 dendron/PLGA particle (PLGA PA-C_0), mice in group 2 received 20 μl of PA DNA-C_{18} dendron/PLGA particle (PLGA PA-C_{18}), and mice in group 3 received 20 μl of PA DNA/PLGA particle (PLGA-PA) summarized by Scheme 1. Intramuscular immunizations of 14 μg of DNA were given in the right thigh

15 BALB/c mice split into three groups of five animals

Group 1- Intramuscular injection of 20 μl of PA DNA-C_0 dendriplex PLGA particle in right thigh of each animal.

Group 2- Intramuscular injection of 20 μl of PA DNA-C_{18} dendriplex PLGA particle in right thigh of each animal.

Group 3- Intramuscular injection of 20 μl of PA DNA PLGA particles in right thigh of each animal.

3 week test bleeds (1st boost injection)

6 week test bleeds (2nd boost injection)

9 week Terminal bleeds

Scheme 1. The protocol for the study.

at week 3, 6, and 9. For this purpose lyophilized PLGA samples were re-suspended in PBS 1h prior to administration by Hamilton syringe and then injected intramuscularly into the thigh of each mouse. Approximately 100 μl of blood was collected from the tail vein of each animal and stored at 4 °C. The samples were then centrifuged and serum was stored at −20 °C until further use. Experiments were performed according to the Home Office 1986 guidelines, and the local Ethics Committee approved experimental design and procedures.

2.6. Anti-PA antibody levels in serum using an enzyme linked immunosorbent assay (ELISA)

Sera were analysed for specific anti-PA antibodies by ELISA. Briefly, microtiter plates (MaxiSorp™, Nunc) were each coated with 100 μl, 5 μg/ml of recombinant PA (List Biologicals) in a 0.05 M carbonate buffer at pH 9.5 at 4 °C overnight. The wells were washed and blocked with 200 μl per well of 2% skimmed milk powder (Marvel) in PBST for 2 h. Sera were diluted in blocking buffer and incubated for 2 h. The wells were washed extensively with PBS/Tween and anti-PA antibodies were detected by using goat anti-mouse IgG (Sigma) and bound conjugate was visualized by adding 100 μl of TMBlue (Intergen) to each well. The reaction was stopped after 5 min with 25 μl 2 M H_2SO_4. Optical densities were read at 450 nm. Biotinylated anti-mouse IgG1 and IgG2a (Pharmingen) was used for detection of IgG subclasses followed by incubation with streptavidin peroxidase.

Neutralizing assay antibody titers were determined with RAW 264.7 cells as described by Hering et al. (2004). Briefly lethal toxin (LT) was prepared in DMEM HEPES by mixing 80 ng ml^{-1} of lethal factor and 100 ng PA ml^{-1} (List Biologicals). LT was then added to an equal volume of titrated serum and incubated for 3 h at 37 °C. The suspension was added to adhered RAW 264.7 cells and viability was measured after 3 h with water-soluble tetrazolium salt (WST-1) (Roche). The data were analysed by standard statistical methods, analysis of variance and unpaired Student's *t*-test. In comparing groups, a P value of ≤ 0.05 was considered to indicate a significant difference.

3. Results and discussion

The hydrodynamic size of the PLGA-dendriplex particles was strongly related to the efficiency of DNA condensation. Generally an increase in PLGA-dendriplex particle size was observed at N/P (number of dendron nitrogen residues per DNA phosphate) ratios >2, in comparison to the primary dendriplexes where an increase in size may reveal greater condensation efficiency. There was little disparity in the size and size distribution of PLGA nanoparticles containing dendriplexes before and after lyophilization (data not shown).

The particles used in the study were all approximately 500 nm in diameter (Fig. 2). The relationship between structural properties of these PLGA formulations and the immunological outcome could provide an insight into the mechanism of action in stimulating immune responses. It has been shown

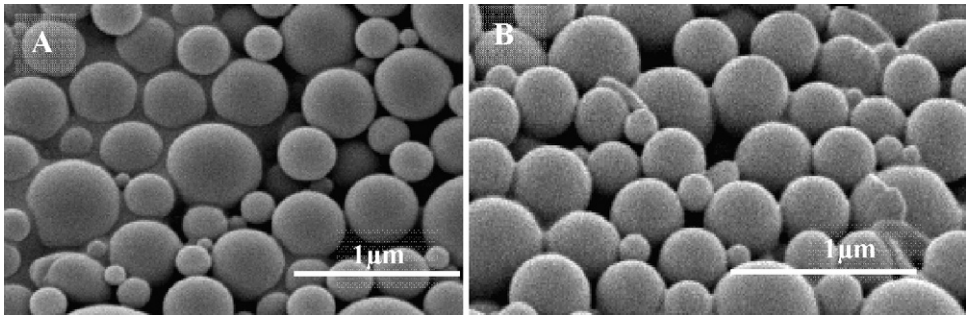


Fig. 2. Scanning electron microscopy (SEM) was performed for lyophilised PLGA-dendriplex particles illustrated by panel A: encapsulated with C_{18} dendriplexes (14 μg of PA DNA); and B: encapsulated with C_0 dendriplexes with equivalent DNA concentration.

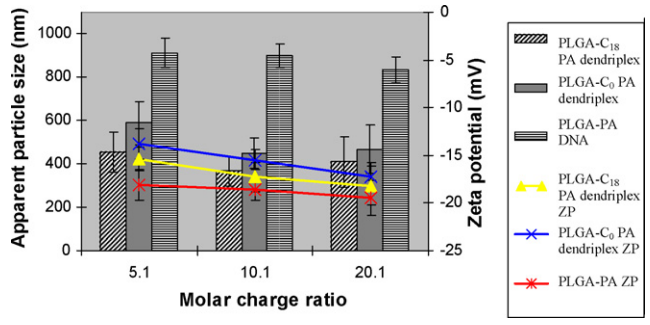


Fig. 3. The overall effect molar charge ratio of dendriplexes, on the mean particle size of the resulting PLGA particles. The zeta potential of these PLGA particles plotted here. The characterisation of the dendriplexes was evaluated in order to select the formulation with optimal colloidal properties for *in vivo* and further *in vitro* studies.

that PLGA particles greater than 30 μm induce no greater antibody titer than DNA alone (Singh et al., 2001). The aim of the characterisation was to select potential particles for the animal study (Fig. 3). PLGA particles were formulated only with the 10:1 dendron:DNA ratio complexes for assessment of antibody production, primarily as these have the smallest diameters concomitant with a low concentration of dendron, which minimizes also toxicity.

Mice which received PLGA particles containing naked PA DNA were only able to generate a weak anti-PA response (Fig. 4). Animals in both groups which were immunized with PLGA PA-C_0 and PLGA PA-C_{18} had higher IgG anti-PA titres than those immunized with PLGA-PA. This was particularly clear after the boosters (Fig. 4). Within the PLGA treatment group, immunization with PLGA PA-C_{18} resulted in a more marked elevation of anti-PA IgG in sera after the second boost at week 9 ($P \leq 0.05$). IgG subtype analysis demonstrated that the IgG response elicited by the dendriplex formulations was predominantly of IgG1. Other studies by Jilek et al. (2004) found that IgG1 and IgG2a production was dependent on the surface charge of the carrier microparticles. Comparison of anionic microparticles with cationic particles showed that the anionic particles resulted in reduced titers, which confirms the importance of particle design before conducting *in vivo* studies.

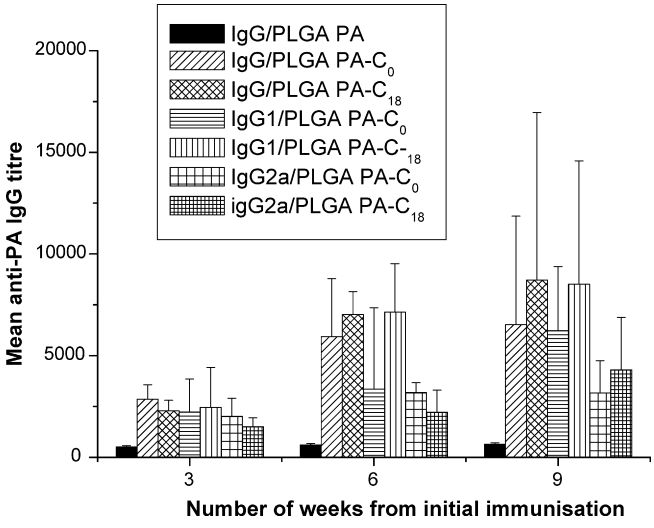


Fig. 4. Mean anti-PA IgG antibody levels with the specific subgroups IgG1 and IgG2a is given for three treatment groups: PLGA PA-C_{18}, PLGA PA-C_0 and PLGA PA. Two booster immunisations were given 3 weeks apart.

The increase in IgG anti-PA titre after booster immunization is consistent with a T cell dependent response. The dominant presence of IgG1 points towards a Th2 type response, however the presence of IgG2a in sera and the presence of bacterial DNA, itself a stimulator of Th1 type response (Halpern et al., 1996; Klinman, 2004), in the particles makes a balanced Th1/Th2 type response more likely.

A gradual increase in antibody response was observed for both PLGA PA-C_{18} and PLGA PA-C_0 particles over the 9-week period. This was likely to be due to the booster effect in addition to the sustained release of the DNA from the PLGA particles, and hence transfection of muscle cells aiding antigen presentation. Gupta et al. (1998) suggests that PLG particles used in their vaccine formulations provide a depot of vaccine with controlled release properties. Mize et al. (2003) has used fluorescence-labelled DNA and PLG particles to assess cell trafficking after intramuscular administration and observed the presence of a depot of PLG-DNA particles at the injection site. Gene expression of the target antigen was reported in the draining lymph nodes following intramuscular injection. Mize et al. (2003) has proposed that the persistence of plasmid enables the recruitment of mononuclear cells, and following activation these express the plasmid coded antigen to elicit humoral and cell-mediated immune responses.

Toxin neutralisation antibodies were not found in sera from animals immunized with PA-C_{18}, PA-C_0, and PA-PLGA (data not shown). Challenge studies conducted by Hahn et al. (2005a,b) in both mice and sheep had limited toxin neutralizing antibodies when pDNA was administrated via the intramuscular route, which was not the case with gene gun delivery (Hahn et al., 2004). Protection against anthrax is believed to depend upon the production of an effective humoral immune response (serum antibodies), against the protective antigen produced by the infecting microorganism (Fellows et al., 2001). However additional factors may be involved in order to develop total immunity against this pathogen, but the exact mechanism required for full protection still remains unclear.

Optimisation of the PLGA particulate system by increasing the DNA encapsulated in the particles may be required, as other groups such as Xie et al. (2005) demonstrated that mice immunized with conventional US anthrax vaccine co-administered with PLG particle enhanced the efficacy of the vaccine.

4. Conclusion

PLGA nanoparticles can be designed in such a way that encapsulated DNA can be released in a well-defined and prolonged manner. These nanoparticulate systems offer the opportunity to increase the DNA payload to induce T cell-dependent immune responses. In further studies, by increasing the DNA content, we may also enhance the level of anti-PA antibodies and thus provide protection against anthrax challenge. PLGA particles may be useful as adjuvants for a broad range

of novel vaccines, and evidence of its progress has allowed a number of human clinical trials to be performed (Sheets et al., 2003).

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