The Effect of CTAB Concentration in Cationic PLG Microparticles on DNA Adsorption and *in Vivo* Performance

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Purpose. Cationic PLG microparticles with adsorbed DNA have previously been shown to efficiently target antigen presenting cells *in vivo* for generating higher immune responses in comparison to naked DNA. In this study we tried to establish the role of surfactant (CTAB) concentration on the physical behavior of these formulations.

Methods. Cationic PLG microparticle formulations with adsorbed DNA were prepared using a solvent evaporation technique. Formulations with varying CTAB concentrations and a fixed DNA load were prepared. The loading efficiency and 24 h DNA release was evaluated for each formulation. Select formulations were tested *in vivo*.

Results. Higher CTAB concentration correlated with higher DNA binding efficiency on the microparticles and lower *in vitro* release rates. Surprisingly though, the *in vivo* performance of formulations with varying CTAB concentration was comparable to one another.

Conclusions. Cationic PLG microparticles with adsorbed DNA, as described here, offer a robust way of enhancing *in vivo* responses to plasmid DNA.

KEY WORDS: CTAB; cationic microparticles; PLG (polylactide-co-lycolide); DNA delivery; adjuvant; vaccines.

INTRODUCTION

The potential for DNA vaccines as a viable approach for inducing protective immune responses has become well established (1). Several studies have demonstrated that intramuscular injection of naked DNA has resulted in the induction of potent immune responses, including antibody and cytotoxic T lymphocytes (CTL) (1,2). The doses of DNA used in most studies have been high and have required several immunizations on multiple occasions (3–6). Some of the approaches used to improve the potency of DNA vaccines include, vector modification to enhance antigen expression (7), physical delivery methods (8,9), and the use of vaccine adjuvants (10).

We have previously described the development of cationic PLG microparticles with adsorbed plasmids as a new delivery system for DNA vaccines (11). This approach uses the positive charge of cetyltrimethylammonium bromide (CTAB) to make a cationic PLG/CTAB microparticle, which adsorbs negatively charged plasmid DNA onto its surface. Biodegradable PLG polymer was selected as a polymer of choice for this application based on its safety profile in humans. This novel approach allows efficient delivery of DNA into antigen presenting cells and induces strong immune response against the antigen encoded by the plasmid. Although we have previously described the preparation and characterization of cationic PLG microparticles (11,12), the role of the concentration of surfactant, and its effect on plasmid adsorption and release has not been described.

In our previous publications, we reported on the use of a fixed concentration of the cationic surfactant (1% w/w) for preparing PLG/CTAB microparticles. In this article, we describe the effect of CTAB concentration on adsorption efficiency and release of DNA *in vitro*. In addition, we investigated the *in vivo* performance of PLG microparticles with different CTAB concentrations. Moreover, we further challenged our own assumptions and attempted to prove definitively that the DNA was indeed associated with the surface of the microparticles. Overall, we showed that the load of CTAB on the PLG microparticles could be varied, resulting in formulations with different release characteristics. However, surprisingly, the *in vivo* performance of PLG/CTAB microparticles was consistently better than naked DNA, irrespective of the rate of release of DNA *in vitro*. In addition, we showed that DNA remained associated with microparticles, even when they were passed through a density gradient that had the ability to separate free DNA, and that the degree of DNA association was dependent on CTAB content.

MATERIALS AND METHODS

Materials

Polylactide-co-glycolide was obtained from Boehringer Ingelheim, USA. CTAB and the optiprep solution were obtained from Sigma Chemical Co., St. Louis, USA and used as shipped. The HIV-1 pCMVkm p55 gag plasmid was made at Chiron and has been previously described (7). U96-Nunc Maxisorp plates (Nalgene Nunc International, Rochester, NY), Goat anti-Mouse IgG-HRP conjugate (Caltag Laboratories, Burlingame, CA), and TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were used for the ELISA.

The Preparation and Characterization of PLG/CTAB Microparticles

The PLG/CTAB microparticles were prepared using a solvent evaporation technique as described previously (11,12), except that the microparticles were prepared with increasing amounts of CTAB (0.2 to 3.0% w/w) remaining bound to the PLG after washing. Controlling the washing conditions mainly enabled this. For higher CTAB residual content (3.0%), the microparticles were washed once by centrifugation. For obtaining lowest CTAB content, the microparticles were washed four times. The size distribution of the microparticles was measured using a particle size analyzer (Malvern Instruments, Malvern, UK). The zeta potential was measured on a DELSA 440 SX Zetasizer (Coulter Corp. Miami, FL). The plasmid was then adsorbed at a fixed 1% w/w load and lyophilized as previously described (11). The amount of adsorbed DNA for varying CTAB loads was determined by hydrolysis of the PLG formulation.

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Determination of CTAB Content in the Microparticles and It's Distribution

Because the previously described colorimetric CTAB detection method (11) was not sufficiently sensitive to determine low levels of CTAB, we developed a more sensitive HPLC approach. The total amount of CTAB in PLG/CTAB microparticles was estimated after hydrolysis by HPLC. Briefly, 10 mg of PLG/CTAB particles were dissolved in 1 ml 1N NH_4OH-1% SDS solution overnight at room temperature and 100 μl of this was injected on an IonPac NS1(10 μM) 4 × 250 mm column (Dionex, USA) with an IonPac NG1 guard column (4 × 35 mm). The column was run at room temperature with a water-acetonitrile gradient containing 2 mM nanafluoropentanionic acid, using a Waters Alliance System (Waters, USA) at a flow rate of 1ml/min, and an Alltech 2000 Evaporative Light Scattering detector (Altech, Inc. USA) using 2.8 L/min ultra high purity 5.0 nitrogen gas and drift tube temperature at 93°C. A standard curve was generated by dissolving 5 mg of RG504 polymer in 1 ml of 1N NH_4OH-1% SDS with varying amounts of CTAB and from it unknown samples were calculated. The limit of detection (LOD) of this assay was around 500 ng. The assay had a linear range from 0.5 μg to 500 μg. The extraction efficiency of CTAB from the PLG by hydrolysis by >99%.

The amount of CTAB bound to the microparticles and the amount released over time was calculated by resuspending 10 mg of freeze dried microparticles in 1 ml of PBS and separating the pellet from the supernatant after one hour at room temperature.

DNA Adsorption Efficiency and Release Rate

The adsorption efficiency of DNA was estimated for batches of PLG/CTAB microparticles with varying amounts of CTAB. After incubation with 1% w/w of target DNA, 1 ml of the suspension was centrifuged at 5,000 rpm on a bench-top eppendorf centrifuge. The supernatant was tested for free DNA. Similarly, the 24 h release was estimated by incubating 10 mg of freeze dried PLG/CTAB/DNA microparticles in PBS at 37°C and estimating the released DNA by measuring the supernatant at A260 nm.

Evaluation of the Effect of CTAB Concentration on DNA Adsorption to PLG/CTAB

To confirm DNA association with microparticles and to evaluate the effect of CTAB concentration on the extent of association, we used a density gradient method. This method was designed to determine if the microparticles and the DNA remained associated following ultracentrifugation, using a one-step density gradient made with OPTIPREP solution. Because the density of the OPTIPREP solution is higher than the DNA, and much lower than the PLG microparticles, it permits separation between free DNA (unbound DNA, Top layer), and DNA adsorbed to the microparticles (bound DNA, bottom layer) after ultracentrifugation. Five milligrams of PLG/CTAB/DNA microparticles were resuspended in 500 μl of PBS and incubated for 1 h, before 250 μl samples were carefully laid on top of a 4 mL OPTIPREP solution (60% [w/v] iodixanol in water) contained in ultraclear centrifuge tubes (13 × 51 mm Beckman Cat# 344057). Two controls were used for comparison, one tube loaded with DNA which had been incubated with CTAB, and a second tube containing DNA in PBS. The clear tubes were centrifuged at 30,000 RPM for 1 h. To identify the location of the DNA in the gradient, nine identical parts (~500 μl each) were carefully collected starting from the top. Then, the amount of DNA in each fraction was quantified by fluorimetry.

Fluorimetry Assay

The concentration of DNA for each fraction of the gradient (250 μl) was determined by adding 5 μL of Ethidium bromide solution (10 μg/ml), followed by analysis with an F-2000 Fluorescence spectrophotometer (HITACHI) with excitation 360 nm and emission 580 nm. A standard curve ranging form 0 to 100 μg/ml was generated diluting DNA with OPTIPREP and phosphate buffer and the concentration of DNA was determined using the slope of the curve**.** The fluorimeter results were confirmed by agarose gel electrophoresis. The results were summarized as percent of total DNA load on the top and bottom layer of the gradient.

Evaluation of Immune Responses

We have previously shown that PLG/CTAB/DNA microparticles prepared with varying load of DNA but fixed CTAB concentration worked similarly *in vivo* (11,12). In the this study, we evaluated microparticles prepared with varying CTAB concentration, but constant DNA load (1% w/w). Groups of 10 female Balb/C mice aged 6 to 8 weeks and weighing about 20–25 g were immunized with a 10 μg dose of DNA adsorbed to microparticles at days 0 and 28. One hundred microliters of the formulations in saline was injected by the TA route in the two hind legs (50 μl per site) of each animal. Mice were bled on day 42 through the retro-orbital plexus and the sera were separated. HIV-1 p55 gag specific serum IgG titers were quantified by ELISA, as previously described and were compared to immunization with the same dose of naked DNA (11,12).

RESULTS AND DISCUSSION

Characterization of PLG/CTAB Microparticles

To allow adsorption of DNA onto the microparticles, it is assumed that the CTAB needs to be present on the microparticle surface. Following microparticle preparation, the presence of CTAB on the particle is indicated by the positive zeta potential. However, we were concerned with the stability of the surface and wanted to confirm that the CTAB remains on the particle over a reasonable time period to allow adsorption. To investigate this, we determined the CTAB distribution between microparticles and supernatant for a batch of microparticles prepared with 1% w/w CTAB. Essentially, this represented an *in vitro* release study for CTAB from the microparticles. The conclusion was that CTAB remained predominantly bound to the PLG surface (99%) and was stable for at least 24 h. This finding implies that the CTAB remains associated to the microparticle and is available for DNA adsorption.

To investigate the effects of CTAB content on DNA adsorption and release, we prepared various batches of PLG/CTAB microparticles, containing various CTAB contents (0.2–3.0% w/w). The size of the microparticles remained un-

changed with increasing input of CTAB (between 800 nm to 1.1 um), but there was a slight increase in the zeta potential with increasing CTAB load.

Adsorption Efficiency and Release Rate of DNA

Figure 1 shows the loading efficiencies and release rates over 24 h for DNA adsorbed to PLG microparticles prepared with increasing concentrations of CTAB (0.2–3.0% w/w). It is clear that with lower CTAB concentrations, the loading efficiency is poor and consequently, release is high following reconstitution. However, as the CTAB concentration is increased above 0.6% (w/w), the loading efficiency increases and *in vitro* release decreases. At around 1.0% (w/w) CTAB or higher, the loading efficiency is almost 100% and *in vitro* release in 24 h is minimal. Therefore, we have demonstrated that there is a clear connection between CTAB content of PLG and adsorption efficiency and release rate for DNA.

Confirmation of the Association of DNA with PLG/CTAB Using a Density Gradient

Table I shows the DNA distribution within the gradient for PLG/CTAB microparticles containing 0.2 to 1.2% w/w CTAB, with a fixed load of 1% w/w DNA. The experiment showed that the percentage of DNA at the bottom layer, where the PLG are found, increased as the concentration of CTAB in the microparticle increased. This would appear to confirm that a higher CTAB content results in a higher degree of association of the DNA to the PLG microparticle. In contrast, with lower DNA load, more of the DNA was found at the top of the gradient, where naked DNA and CTAB/DNA complexes were found. This study indicated that to achieve a higher association of DNA with the PLG microparticles, >0.6% (w/w) of CTAB needed to be present in the formulation. This data appeared to be very supportive and consistent with the data obtained in the DNA adsorption and release work.

Table I. Localization of DNA Adsorbed to Cationic PLG/CTAB Microparticles following Their Movement through a Density Gradient, for Microparticles Prepared with Increasing Concentrations of CTAB (0.2 to 1.2% w/w) and a Fixed DNA Load (1% w/w). Higher CTAB Content Increased the Amount of DNA Found Localized with the PLG Microparticles

Location of DNA in the density gradient	% w/w CTAB in PLG microparticles					
	0.0	0.2	0.3	0.4	0.6	1.2
% of DNA in top fraction	100	37	36	21	32	0
% of DNA in lower fraction with PLG	0	63	64	73	68	100

Immunogenicity of PLG/CTAB/DNA Microparticles with Varying CTAB Content

Surprisingly, PLG/CTAB microparticles with 1% w/w DNA load, but prepared with varying CTAB content (0.2, 0.5, 1.0, 1.8 w/w) induced comparable antibody responses and were all significantly higher than naked DNA in mice (Fig. 2). The fold increase over naked DNA was lower in these studies than those published earlier (11–13). This is mainly because of the use of a newer lot of plasmid DNA than the one used earlier. But the PLG formulation consistently outperformed naked DNA by generating higher antibody responses. As expected, all groups (naked DNA and PLG/DNA) induced potent CTL responses in mice (data not shown). Although, PLG microparticles with different CTAB contents had different adsorption efficiencies and release rates for DNA, the *in vivo* performance did not appear to be impaired by high rates of DNA release.

The accumulated evidence from the current and previous studies firmly establish PLG/CTAB microparticles as a robust and reliable means to deliver DNA for enhanced immune responses in a variety of species, to include non-human primates (11–13). In this study we have established for the first time that CTAB is stably bound to the microparticle surface

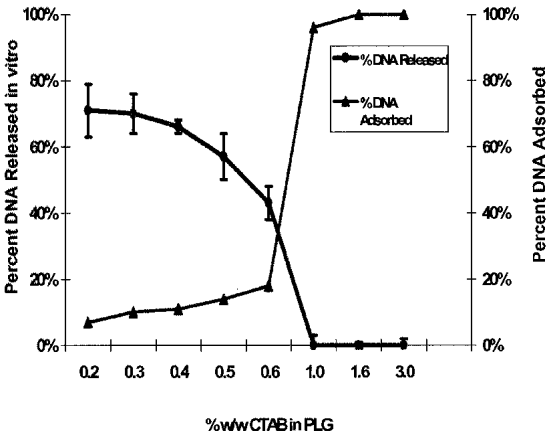

Fig. 1. Percentage of DNA adsorbed and the percentage released in 24 h from 1% w/w DNA loaded microparticles prepared with varying CTAB concentrations (0.2–3.0% w/w). Both loading efficiency and the amount of DNA released is inversely proportional to the CTAB content. Each value represents the geometric mean of three measurements ± SE.

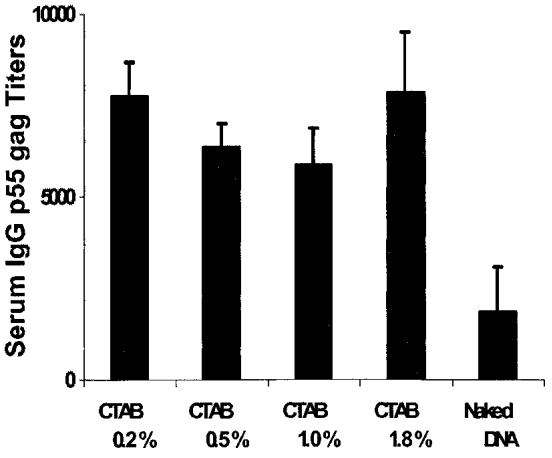

Fig. 2. Serum IgG p55 gag titers for mice immunized with PLG/CTAB/DNA microparticles prepared with 0.2, 0.5, 1.0 and 1.8% w/w CTAB content, with a fixed DNA load of 1% w/w. Antibody responses are shown as geometric mean titers +/– SE ($n = 10$) at day 42. Although the titers for the PLG/CTAB microparticles with different CTAB content are not significantly different from one another, they are all significantly higher than naked DNA ($p < 0.05$).

during preparation, establishing a surface to which DNA can be adsorbed. Moreover, we have further challenged the assumptions about DNA adsorption and have shown that DNA remains associated with microparticles during their passage through a density gradient. In contrast, naked DNA and DNA/CTAB complexes did not enter the gradient. In addition, we have shown for the first time that the degree of DNA association and the rate of release *in vitro* are dependent on the CTAB content of the microparticles. However, we also showed that the *in vivo* performance of PLG/DNA microparticles was not impaired by their preparation with low levels of CTAB, which had a high *in vitro* release of DNA. Although the *in vivo* performance of microparticles with low CTAB content is surprising, the data is highly encouraging. Previously, we have demonstrated that microparticles with fixed CTAB content (1% w/w) can deliver high loads of DNA (up to 5% w/w), without any evidence of impaired *in vivo* performance (12). Here we extend these observations and demonstrate that we can prepare microparticles with low CTAB content, which still perform equally well to particles with higher CTAB content, for a fixed load of DNA. This is an encouraging observation, because it is attractive to lower the amount of CTAB in the microparticles relative to the DNA load to minimize toxicological concerns in relation to the CTAB. Nevertheless, it was somewhat surprising that the microparticles with low CTAB content and high DNA release performed equally well as the particles with lower DNA release. However, the *in vitro* and *in vivo* performance of delivery systems is often different and correlations are notoriously difficult to establish.

Overall, we believe that the novel approach of presenting DNA on cationic PLG microparticles has several advantages over an alternative approach, involving microencapsulation of DNA (14–20). Our approach is simple, scaleable and robust, allows efficient adsorption and release of intact DNA, and induces significantly enhanced responses in comparison to naked DNA (11–13). The approach of presenting antigens on the surface of PLG microparticles has also been used recently to induce potent immune responses against protein antigens (21). Hence, surface presentation of antigens represents a novel way to use PLG microparticles as an effective vaccine delivery system. Recent studies have indicated that the microparticles are effective for the induction of enhanced immune responses largely as a consequence of the delivery of the adsorbed DNA into antigen presenting cells (22).

CONCLUSION

Overall, the current studies have shown that PLG/CTAB microparticles can be prepared with varying CTAB content. Although these formulations showed variable release and loading efficiencies, which depended on the CTAB content, their *in vivo* performance was not significantly different from each other, although they were all significantly better than naked DNA. In addition, we have shown that CTAB remains stably bound to the PLG microparticles, providing a surface for DNA adsorption. In addition, we have shown that DNA remains associated with PLG/CTAB microparticles during their movement through a density gradient and that the degree of association is dependent on CTAB content.

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