Cationic microparticles: A potent delivery system for DNA vaccines

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An approach involving the preparation of biodegradable microparticles with a cationic surface was developed to improve the delivery of adsorbed DNA into antigen-presenting cells after i.m. injection. The microparticles released intact and functional DNA over 2 weeks *in vitro*. In addition, the microparticles induced higher levels of marker gene expression *in vivo*. After i.m. immunization, the microparticles induced significantly enhanced serum antibody responses in comparison to naked DNA. Moreover, the level of antibodies induced by the microparticles was significantly enhanced by the addition of a vaccine adjuvant, aluminum phosphate. In addition, in contrast to naked DNA, the cationic microparticles induced potent cytotoxic T lymphocyte responses at a low dose.

DNA delivery | poly(lactide-*co*-glycolide) microparticles

Following up on reports that direct injection of plasmid DNA resulted in gene expression (1), several groups pursued the possibility that direct injection of plasmid DNA could be exploited as a vaccine strategy. The first peer-reviewed report of protective immunity and cytotoxic T lymphocyte (CTL) induction in mice after i.m. injection of a DNA plasmid appeared in 1993 (2). Subsequently, the use of DNA vaccines in preclinical studies has become well established, with reports of protective immunity in many different independent studies (3). In recent studies, both antibody and CTL responses were induced in nonhuman primates, although 1–2 mg of DNA was immunized on multiple occasions in these studies (4). Antibody and CTL responses also have been induced in human volunteers, but again, high doses of DNA were used (5–7). For example, in one study in naïve subjects, optimal CTL responses were induced with 2.5 mg of DNA from *Plasmodium falciparum* (6). Nevertheless, DNA vaccines have proven very effective in small animal models and are also effective in larger animals, including cattle, horses, and swine (8, 9). However, although the use of DNA vaccines at milligram doses is feasible, it would impose serious limitations on the number of constructs that could be included in a vaccine. In addition, the use of very high doses of DNA is less favorable from a process economics standpoint. Therefore, there is a clear need to induce effective immunity in humans with lower and fewer doses of DNA, as well as to increase the magnitude of the immune responses obtained.

There are a number of strategies available that have the potential to improve the potency of DNA vaccines. These strategies include: (*i*) vector modification to enhance antigen expression, which may involve targeting of the expressed protein to a particular cellular location, the inclusion of immunostimulatory sequences, or the elimination of inhibitory sequences in the plasmid; (*ii*) improvements in DNA delivery; or (*iii*) the inclusion of adjuvants, either as a gene or as a coadministered agent. Our group has focused predominantly on the use of DNA delivery systems to enhance the response to DNA vaccines. To achieve this, we have developed cationic microparticles to enhance delivery of adsorbed DNA to antigen-presenting cells (APCs) after i.m. injection. The polymer we have chosen to prepare microparticles is poly(lactide-*co*-glycolide) (PLG), which is a biodegradable and biocompatible polymer (10), and previously has been used for a range of biomedical purposes, including the preparation of controlled release drug delivery systems (11). Although PLG polymers previously have been used as DNA delivery systems (12, 13), the previous studies described the entrapment of DNA inside the microparticles. Therefore, during microparticle preparation, the DNA was exposed to a range of conditions that have the potential to cause denaturation and degradation, including high shear, an organic/aqueous interface, localized high temperature, and freeze drying. Not surprisingly, a recent study has reported that DNA is significantly degraded during encapsulation in PLG microparticles (14). In addition, once entrapped in microparticles, the rate of release of DNA is slow, limiting the amount of DNA available for transfection of target cells and induction of immune responses.

To overcome the problems of DNA degradation during microencapsulation and to enhance the amount of DNA immediately available to APCs after cellular uptake of microparticles, we have adopted the strategy of presenting DNA on the surface of microparticles. To achieve this, microparticles were prepared that displayed a positive surface charge for DNA adsorption, through the inclusion of cationic surfactants in the preparation process. After preparation and characterization, cationic microparticles with adsorbed DNA were administered to experimental animals and the immune responses induced were compared with immunization with naked DNA. A significant improvement in immunogenicity over naked DNA was achieved for both antibody and CTL induction.

Materials and Methods

Materials. The PLG polymers were obtained from Boehringer Ingelheim. The PLG polymer used in this study was RG505, which has a copolymer ratio of 50/50 and a molecular mass of 65 kDa (manufacturer's data). The HIV-1 pCMVkm p55 gag plasmid was obtained by transforming *Escherichia coli* strain HB101 with the plasmid and fermenting under defined growth conditions. The plasmids were purified by using a proprietary Chiron process. The final product was endotoxin free (<2.5 units/ml). The pLUC plasmid was also similarly purified. All other chemicals and reagents were obtained from Sigma and used as shipped. ELISA microtiter plates were obtained from Nunc.

The Preparation of Microparticles. Cationic microparticles were prepared by using a modified solvent evaporation process. Briefly, the microparticles were prepared by emulsifying 10 ml

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Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; PLG, poly(lactide-*co*-glycolide); CTAB, cetyltrimethylammonium bromide; DDA, dimethyl dioctadecyl ammonium bromide; DOTAP, 1,2-dioleoyl-1,3-trimethylammoniopropane; TA, anterior tibialis.

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of a 5% (wt/vol) polymer solution in methylene chloride with 1 ml of PBS at high speed using an Ika homogenizer (Ika-Werk Instruments, Cincinnati). The primary emulsion then was added to 50 ml of distilled water containing cetyltrimethylammonium bromide (CTAB) (0.5% wt/vol). This resulted in the formation of a water/oil/water emulsion that was stirred at 6,000 rpm for 12 hr at room temperature, allowing the methylene chloride to evaporate. The resulting microparticles were washed twice in distilled water by centrifugation at 10,000 *g* and freeze-dried. For preparing PLG-dimethyl dioctadecyl ammonium bromide (DDA) and PLG-1,2-dioleoyl-1,3-trimethylammoniopropane (DOTAP) microparticles, DDA or DOTAP was dissolved in the polymer solution along with PLG polymer, and the primary emulsion then was added to 0.5% polyvinyl alcohol solution to form the water/oil/water emulsion.

After preparation, washing, and collection, DNA was adsorbed onto the microparticles by incubating 100 mg of cationic microparticles in a 1 mg/ml solution of DNA at 4°C for 6 hr. The microparticles then were separated by centrifugation, the pellet was washed with Tris-EDTA buffer, and the microparticles were freeze-dried.

Microparticle Characterization. The size distribution of the microparticles was determined by using a particle size analyzer (Malvern Instruments, Malvern, U.K.) and the value was calculated by volume measurement. The loading level of the DNA on the microparticles was determined by assaying both the supernatant after adsorption and by hydrolyzing the microparticles (0.2 M NaOH) and measuring DNA by absorbance at 260 nm. DNA quantitation was performed by using either Hoechst or picogreen dyes followed by fluorimetric estimation for smaller amounts of DNA. The DNA load on the microparticles also was confirmed by a HPLC approach, which determined the total DNA load on the particles after complete dissolution of the polymer. The zeta potential of the microparticles, which is a measure of net surface charge, was measured on a DELSA 440 SX Zetasizer from Coulter. The amount of CTAB and DDA on the microparticles was estimated by a standard titermetric assay, based on the reaction with potassium iodide (23). Selected batches of microparticles were evaluated by scanning electron microscopy for size and surface uniformity.

Plasmid Stability Evaluation. Ten milligrams of PLG/CTAB-p55 DNA microparticles [0.85% (wt/wt) loading level] was incubated with 1 ml of PBS at 37°C. At each time point (days 1, 3, 7, and 14) the suspension was centrifuged and the supernatant was collected. One milliliter of PBS was added to the vial and the pellet was resuspended. The released DNA in the supernatants was run on a 1% agarose gel to evaluate plasmid integrity.

Gene Expression: *In Vitro*. Ten micrograms of luciferase released from PLG-CTAB microparticles *in vitro* at day 1 and unformulated luciferase were suspended in 0.5 ml of Tris-EDTA buffer. On day 1 of the transfection protocol, 6-well plates were plated with HeLa cells at 2.5 × 10 E5 cells/well with DMEM. On day 2, the cells were transfected with the released samples, along with luciferase plasmid control at 5 μg. Each sample was placed with 0.5 ml of DMEM containing 10 μg of DNA. The DNA samples were mixed with a transfection reagent, GenePorter (Gene Therapy Systems, San Diego) and were incubated together at room temperature for 30 min. The DNA + transfection agent were added to the HeLa cells and incubated at 37°C for 5 hr. The media were aspirated after 5 hr and were replaced by DMEM at 37°C for 48 hr. On day 4, the cells were lysed in the wells using 1× reporter lysis buffer (Promega) then rocked at room temperature for 15 min. The cells were scraped off the wells into Eppendorf tubes and were freeze-thawed three times. The tubes were spun at 10,000 rpm for 5 min, and the luciferase assay was performed on the supernatant collected.

Gene Expression: *In Vivo*. Two groups of mice ($n = 5$) were injected with either 50 μg of pCMVLuc DNA or 50 μg of pCMVLuc DNA on PLG/CTAB microparticles. Both groups of mice were injected i.m. in the anterior tibialis (TA) muscle on two legs. Both TA muscles from each mouse in the two groups were harvested either at day 1 or day 14 and stored in a −80°C freezer. The muscles were ground up with a mortar and pestle on dry ice. The powdered muscles were collected in Eppendorf tubes with 0.5 ml of 1× reporter lysis buffer. The samples were vortexed for 15 min at room temperature. After freeze/thawing 3×, the samples were spun at 14,000 rpm for 10 min. The supernatants of the TA muscle of each mouse at each time point were pooled, and 20 μl of the samples was assayed by using the luciferase assay described below and normalized to the total protein content in the sample.

Luciferase Assay. Luciferase determination was performed by using a chemiluminescence assay. The buffer was prepared containing 1 mg/ml of BSA in 1× reporter lysis buffer. The luciferase enzyme stock (Promega) at 10 mg/ml was used as a standard, diluted to a concentration of 500 pg/20 μl. This standard was serially diluted 1:2 down the Microliter 2 plate (Dynatech) to create a standard curve. Twenty microliters of the blank and the samples also was placed on the plate and serially diluted 1:2. The plates were placed in the ML3000 (Dynatech) where 100 μl of the luciferase assay reagent (Promega) was injected per well. The relative light units were measured for each sample.

Immunization. For the antibody studies, BALB/c mice in groups of 10 were immunized with DNA formulations at weeks 0 and 4. The microparticle formulations were suspended in saline, 100 μl per animal. Fifty microliters of the formulations was injected in the TA in the two hind legs of each animal. The immunization protocol for the CTL study involved a single injection ($n = 5$ mice per group) in the TA muscles followed by harvesting of splenocytes at the 3-week time point.

Sera and Tissue Collection. Mice were bled through the retroorbital plexus and the sera were separated for the immunoassays. Spleens were harvested and pooled from groups of mice before the CTL assay.

Immunoassay. HIV-1 p55 gag-specific serum IgG titers were quantified by an ELISA. Briefly, ELISA plates (96-well U bottom by Nunc Maxisorp) were coated with p55 protein at 5 μg/well. After washing with 1× PBS + 0.03% Tween 20 (Sigma), the wells were blocked, then coated with serially diluted samples in an assay diluent made up of 1× PBS + 5% goat serum (GIBCO/BRL) + 0.03% Tween 20 (Sigma). A serum standard was included in each assay for quantitation purposes. The samples then were incubated with 1:40,000 dilution of a goat anti-mouse IgG-horseradish peroxidase (Caltag, South San Francisco, CA). The samples then were developed with tetramethylbenzidine (Kirkegaard & Perry Laboratories) for 15 min and then stopped with 2 M HCl. The OD of each well was measured by using Titertek at 450 nm.

CTL Assay. Spleens from immunized mice were harvested 3 weeks after a single immunization and used as pools of five. Spleen cells were cultured in a 24-well dish at 5×10^6 cells per well. Of these cells, 1×10^6 were sensitized with synthetic p7 g peptide (amino acids 194–213) at a concentration of 10 mM for 1 hr at 37°C and then washed and cocultured with the remaining 4×10^6 untreated cells. The cells were stimulated as a bulk culture in 2 ml

Table 1. Cationic microparticles with adsorbed DNA: Particle size, net surface charge, loading efficiency, and DNA loading levels based on hydrolysis of PLG-DNA formulation

Formulation	Mean size, μm	Zeta potential, mV	Loading level, % wt/wt	Mean loading efficiency, %
PLG/CTAB-p55 DNA	1.54 ± 0.20	+36 ± 6	0.92 ± 0.12	92.0
PLG/DDA-p55 DNA	2.20 ± 0.12	+30 ± 10	0.68 ± 0.08	68.0
PLG/DOTAP-p55 DNA	0.98 ± 0.11	+24 ± 8	0.62 ± 0.14	62.0

PLG miroparticles prepared with polyvinyl alcohol as a particle stabilizer, but without cationic surfactants have a zeta potential of −13 ± 3 mV. Values are mean ± SE of triplicate measurements.

of Splenocyte culture medium: RPMI medium 1640 with 100 mM L-glutamine (GIBCO)/MEM (minimum essential medium alpha medium with L-glutamine, deoxyribonucleosides, or ribonucleosides) (1:1) supplemented with 10% heat-inactivated FCS (HyClone), 100 units/ml of penicillin, 100 g/ml of streptomycin, 10 ml/liter of 100 mM sodium pyruvate, and 50 M 2-mercaptoethanol. In addition, 5% rat T-Stim IL-2 (Collaborative Biomedical Products, Bedford, MA) was used as a source of IL-2 and added to the culture media just before the cells were to be cultured.

After a stimulation period of 6–7 days, the cells were collected and used as effectors in a chromium ^{51}Chromium release assay. Approximately 1×10^6 SV/BALB target cells were incubated in 200 μl of medium containing 50 Ci of ^{51}Cr and with the correct peptide p7 g or a mismatched cell-target pair as the negative control at a concentration of 1 M for 60 min and washed. Effector cells were cultured with 5×10^3 target cells at various effector-to-target ratios in 200 μl of culture medium in 96-well tissue culture plates (round or v-bottom) for 4 hr. The average cpm from duplicate wells was used to calculate percent specific release as presented here.

Results

Microparticle Preparation and Characterization. The microparticles were prepared with a mean size of about 1 μm and showed a surface positive charge, because of the inclusion of cationic surfactants in the preparation process (Table 1). The cationic surfactants used in the current studies were CTAB, DDA, and DOTAP. The surfactant or stabilizer most commonly used during PLG microparticle preparation, polyvinyl alcohol, imparts a negative surface charge on the microparticles, because of physical entrapment within the surface layer of the polymer (15). The positive charge on the surface of the cationic microparticles allowed efficient adsorption of plasmid DNA (Table 1).

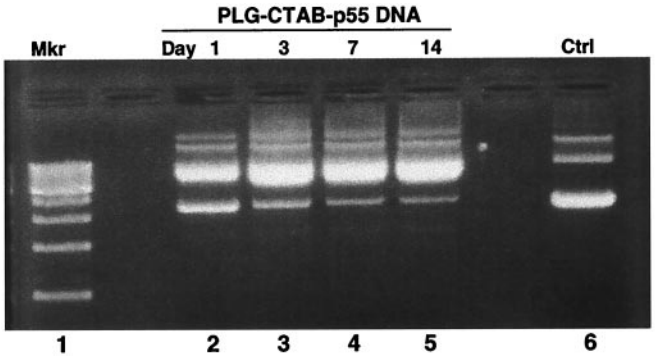

Fig. 1. Agarose gel of DNA after release from the surface of PLG/CTAB microparticles at various time points, after incubation *in vitro* at 37°C. Lane 1 is a marker lane, lanes 2–5 are DNA released at days 1, 3, 7, and 14, respectively, and lane 6 is control unformulated p55 gag DNA.

Stability of DNA Released from Microparticles. The *in vitro* release rate of p55 DNA from PLG/CTAB microparticles was initially rapid, with about 35% released at day 1. Subsequently, the rate of release was slower, but by day 14 about 75% of the adsorbed DNA had been released. p55 gag DNA released *in vitro* from the surface of PLG/CTAB microparticles at various time points was evaluated for integrity on a 1% agarose gel (Fig. 1). The DNA released at days 1, 3, 7, and 14 was largely intact and comparable to the native material. However, there was some evidence of a gradual reduction in the percentage of super-coiled DNA at later time points.

Gene Expression After Release of Adsorbed DNA from Microparticles. Although a significant proportion of the adsorbed DNA was released relatively rapidly from the microparticles *in vitro* (≈35% released on day 1), the remainder of the adsorbed DNA appeared more strongly bound and was released relatively slowly. *In vitro* gene expression studies were performed with pLuc released from PLG/CTAB microparticles at day 1, to confirm that the DNA released from the microparticles was intact and able to be expressed in cells. The pLuc released from the microparticles *in vitro* produced similar expression levels in HeLa cells to control unformulated plasmid (Table 2). DNA adsorbed to the PLG/CTAB microparticles also showed enhanced resistance to DNase I degradation *in vitro* in comparison to naked DNA (data not shown).

Gene Expression After Intramuscular Injection. The PLG-CTAB-luciferase DNA microparticles induced expression of luciferase enzyme *in vivo* after injection into the TA muscle in BALB/c mice. The level of *in vivo* expression of luciferase was higher for

Table 2. *In vitro* and *in vivo* gene expression of luciferase enzyme from PLG-CTAB-luciferase DNA formulations

Formulation	DNA dose	*In vitro* luciferase expression in HeLa cells, pg	*In vivo* luciferase expression in muscle (pg) day 1	*In vivo* luciferase expression in muscle (pg) day 14
PLG/CTAB-Luc DNA	5 μg	1,248 ± 122	—	—
Luc DNA alone	5 μg	2,250 ± 240	—	—
PLG/CTAB-Luc DNA	50 μg	—	9.51	44.95
Luc DNA alone	50 μg	—	6.78	9.29

In vitro gene expression was carried out in HeLa cells with pLuc DNA released from PLG/CTAB formulation and unformulated DNA at day 1. The luciferase values have been normalized to total protein content. Mean ± SE of triplicate readings are represented. *In vivo* gene expression of luciferase enzyme was performed at days 1 and 14 with either the PLG/CTAB formulation or unformulated pLuc DNA.

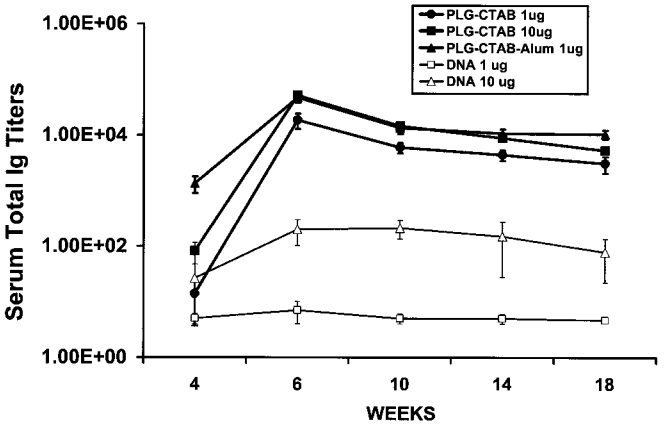

Fig. 2. Serum IgG titers in groups ($n = 10$) of BALB/c mice immunized with either PLG/CTAB p55 gag DNA microparticles at 1 and 10 μg, PLG-CTAB DNA microparticles combined with aluminum phosphate at 1 μg, or naked DNA alone at 1 and 10 μg. Antibody titers are geometric mean titers ± SE at each time point. The responses from formulated DNA at both doses was significantly higher ($P < 0.05$) at all time points.

the PLG-CTAB microparticles than for naked DNA injection at the day 14 time point (Table 2).

Enhanced Antibody Responses with p55 Gag DNA Adsorbed to Microparticles. The PLG/CTAB microparticles induced significantly enhanced antibody responses over naked DNA at all time points and at both doses evaluated, until the termination of the study at week 18 (Fig. 2). In addition, the inclusion of aluminum phosphate into the PLG/CTAB formulation induced a significant enhancement over the responses observed with PLG/CTAB alone (Fig. 2). In contrast, PLG microparticles without cationic detergents are ineffective for the induction of potent immune responses to DNA (Fig. 3). The combination of blank PLG microparticles immunized with free DNA induced the same level of response as naked DNA (Fig. 3).

To demonstrate that the enhancement observed was not exclusively caused by the presence of CTAB, but was a general consequence of the adsorption of DNA onto cationic microparticles, we also prepared and evaluated PLG/DOTAP and PLG/DDA microparticles. All three cationic microparticle formulations (CTAB, DDA, and DOTAP) induced significantly enhanced ($P < 0.05$) antibody responses in comparison to immunization with naked DNA alone (Fig. 4). This observation suggests that surface presentation of DNA on the microparticles appears to be more important than the actual surfactant chosen to prepare the microparticles.

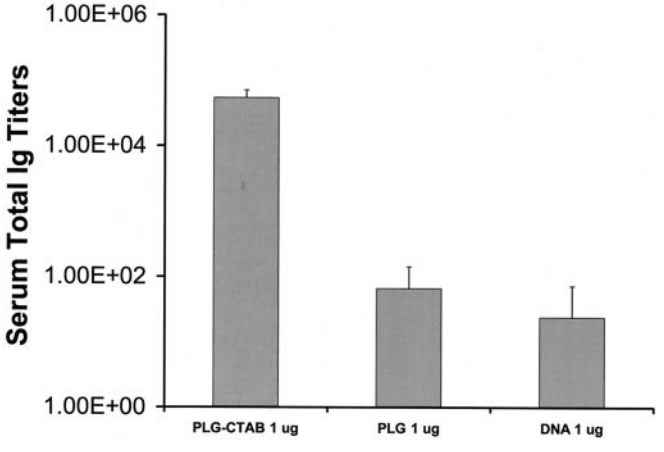

Fig. 3. Serum IgG titers in groups ($n = 10$) of BALB/c mice immunized with either PLG/CTAB-p55 gag DNA, blank PLG microparticles + DNA, or DNA alone at 1 μg dose. Antibody titers are geometric mean titers ± SE at 2 weeks post-second immunization (week 6) time point. The response from the PLG/CTAB-p55 DNA was significantly higher ($P < 0.05$) than other groups.

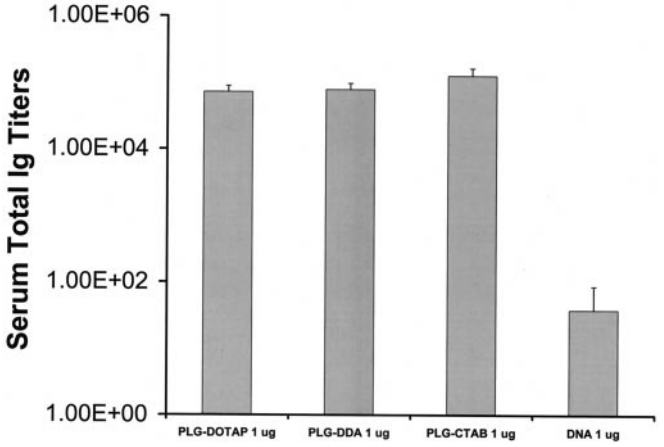

Fig. 4. Serum IgG titers in groups ($n = 10$) of BALB/c mice immunized with either PLG/DOTAP, PLG/DDA, or PLG/CTAB p55 DNA microparticles or naked DNA alone at 1 μg dose. Antibody titers are geometric mean titers ± SE at 2 weeks post-second immunization (week 6) time point. The response from all three formulations was significantly higher ($P < 0.05$) than the naked DNA group.

Effect of Particle Size on Immune Responses to Cationic Microparticles. To evaluate the effect of microparticle size on the immune response and to support our theory that delivery into APCs is an important contributor to the response, PLG/CTAB microparticles of three different mean sizes were prepared and evaluated with adsorbed DNA (300 nm, 1 μm, and 30 μm). Fig. 5 shows that smaller microparticles, which have the capacity to be taken up by APCs, are effective for the induction of potent antibody responses. In contrast, no enhancement was observed with the 30-μm microparticles, which are too large to be taken up by APCs.

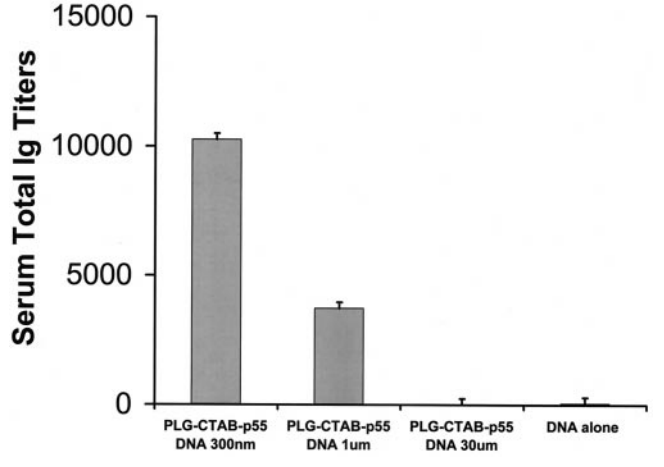

Fig. 5. Serum IgG titers in groups ($n = 10$) of BALB/c mice immunized with PLG/CTAB microparticles of three different mean sizes (300 nm, 1 μm, and 30 μm) with 1 μg of adsorbed p55 gag DNA. Antibody titers are geometric mean titers ± SE at 2 weeks post-second immunization (week 6) time point. The responses from PLG/CTAB (300 nm and 1 μm) were significantly higher ($P < 0.05$) than 30 μm and naked DNA group.

Table 3. Percent bulk cytolysis from splenocytes at various effector-to-target cell ratios induced by single immunizations with PLG-CTAB-p55 gag DNA, PLG-DDA-p55 gag DNA, and naked p55 gag DNA

Formulation	DNA dose	Target/effect or cell ratio	% Cytolysis, Sv/B p7g
PLG/CTAB-p55 DNA	1 μg	60	71
		15	55
		4	31
PLG/DDA-p55 DNA	1 μg	60	70
		15	54
		4	17
DNA alone	1 μg	60	3
		15	1
		4	0
Vaccinia-gag-pol	2×10^7 pfu	60	64
		15	35
		4	11

Vaccinia-gag-pol-injected mice served as a positive control in the assay. pfu, plaque-forming unit.

Induction of CTL Responses with Cationic Microparticles. Both PLG/CTAB and PLG/DDA microparticles were capable of inducing potent CTL responses with 1 μg of adsorbed p55 gag DNA, whereas naked DNA at the same dose failed to induce CTL activity (Table 3). Subsequent studies have confirmed that PLG/DOTAP microparticles also induce CTL responses at a similar dose of DNA.

Discussion

These studies demonstrated that the cationic microparticles were potent delivery systems for DNA vaccines and induced significantly enhanced antibody and CTL responses, after i.m. immunization with p55 gag plasmid. Furthermore, the levels of immune enhancement achieved in the current studies appears much greater than previously reported with alternative PLG formulations. In a previous study involving DNA entrapped in PLG microparticles, no enhancement of antibody responses was reported (13). In addition, in the previous study, the microparticles were immunized by a different route to naked DNA, preventing a direct comparison of efficacy for CTL induction (13). In the current studies, the cationic microparticles were immunized by the i.m. route, the route that is optimal for immunization with naked DNA.

Several previous reports have described the use of a range of vaccine adjuvants for DNA immunization, with some adjuvants inducing enhanced immune responses (16–18). In concurrent studies, we evaluated the potential of adjuvants (including MPL, QS21, and cationic liposomes) to induce enhanced immune responses to the p55 gag plasmid. In marked contrast to the observations with cationic microparticles, only marginal levels of enhancement (about 10-fold) were obtained with these adjuvants (unpublished data). The optimal enhancement achieved with the PLG/CTAB microparticles at the 10-μg DNA dose, which gave 100% seroconversion for both the microparticle and the naked DNA groups, was >250-fold. It is also important to note that at the lower DNA dose (1 μg), all the animals immunized with PLG/CTAB microparticles seroconverted 4 weeks after a single immunization. In contrast, after immunization with naked DNA at 1 μg, only four of 10 animals had seroconverted 2 weeks after the second dose. Hence, the PLG microparticles enhanced the total antibody response at the higher DNA dose, but also induced more rapid and complete seroconversion at the lower dose.

It is important to highlight that we consider that the cationic microparticles currently represent a platform technology, which has significant potential to be modified and improved. For example, more recent studies have involved the preparation of additional microparticles with entrapped and adsorbed adjuvants, which are designed to further enhance the levels of immunity. In the current studies, this approach has been illustrated by the addition of aluminum phosphate to the PLG/CTAB microparticles, which resulted in a significantly enhanced response over that achieved with microparticles alone. An important advantage of the microparticle DNA delivery approach described here is flexibility, allowing additional components, e.g., adjuvants, to be entrapped in the particles carrying DNA, entrapped into separate particles and mixed with DNA particles, adsorbed to the surface of additional particles, or any combination of the above.

The mechanism of the adjuvant effect achieved with cationic microparticles is not currently known, but we believe that efficient delivery of the adsorbed DNA to APCs is an important contributing factor. DNA coadministered with microparticles, but not adsorbed, does not induce a similar effect to adsorbed DNA. The theory that delivery of DNA to APCs is an important component of the mechanism of enhancement is further supported by the observation that large microparticles (>30 μm) failed to elicit a potent immune response, whereas smaller particles were effective. Previous studies have shown that bone marrow-derived APCs are essential to present antigen encoded by DNA vaccines to naïve CTL precursors, possibly by transfer of antigen from muscle cells to APCs, termed cross priming. Although the mechanism of induction of immunity after DNA vaccination has not been fully elucidated, recent studies suggest an important role for directly transfected dendritic cells after immunization with the gene gun (8). Furthermore, it is already well established that particles are efficiently taken up by dendritic cells *in vivo* (24). The presence of the cationic surfactants, CTAB, DDA, and DOTAP, on the surface of PLG microparticles also may make an additional contribution to the mechanism, because they may contribute to disruption of endosomes and the release of DNA into the cytoplasm. However, this hypothesis remains to be proven and further studies are necessary.

An important long-term consideration is the safety of cationic microparticles for use in human vaccines. Although PLG polymers previously have been widely used in biomedical applications, including the preparation of several drug delivery systems (8), the effects of the inclusion of CTAB, DDA, or DOTAP in the microparticles needs to be evaluated. Nevertheless, CTAB previously has been used for a range of biomedical applications, including use as an antibacterial agent in eye drops (19). Hence, although the toxicity of cationic microparticles needs to be evaluated, the toxicity of CTAB is well defined and its levels can be kept to a minimum (4 μg of CTAB per mg polymer in the current microparticles). Moreover, although CTAB was chosen as the initial surfactant, the work with DOTAP and DDA clearly shows that CTAB can be replaced if necessary, without reducing the potency of the delivery system. Preliminary studies in guinea pigs indicate no acute toxicity problems when cationic microparticles were used at a dose of 1 mg DNA per animal (unpublished data).

These studies have served to highlight the exciting potential of cationic microparticles for the induction of enhanced immune responses to DNA vaccines. In previous studies, microparticles with entrapped DNA also have been used for mucosal immunization (12, 20, 21), and PLG polymer implants recently have been reported for delivery of plasmid DNA for tissue engineering (22). In preliminary studies, we have observed enhanced immunity with DNA adsorbed to cationic microparticles after mucosal delivery. In addition, the level of enhancement observed was significantly greater than the responses achieved by DNA entrapped in PLG microparticles (unpublished data).

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