The Preparation, Characterization, and Evaluation of Cationic Microparticles for DNA Vaccine Delivery

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INTRODUCTION

The use of DNA vaccines has become well established (1), and intramuscular injection has resulted in the induction of potent immune responses, including antibody and cytotoxic T lymphocytes (CTL) (1,2). However, although immune responses have been induced in primates, including humans, high doses of DNA on multiple occasions have been required (3–6).

Therefore, several approaches are currently under evaluation to improve the potency of DNA vaccines, including vector modification to enhance antigen expression (7), physical delivery methods (8,9), and the use of vaccine adjuvants (10). As an alternative, we have developed cationic microparticles with adsorbed plasmids as a delivery system for DNA vaccines (11), using the biodegradable and biocompatible polymer, polylactide-co-glycolide (PLG) (12). PLG has previously been used for a wide range of biomedical purposes, including the preparation of drug delivery systems (13). Although PLG microparticles (14–20) have previously been described as a delivery system for DNA vaccines, these formulations had some inherent disadvantages over our proposed concept. The entrappment of DNA within the microparticles (14–16), results in significant degradation during encapsulation and release (16,18,20). An additional problem with DNA microencapsulation is that following administration, the DNA is released very slowly, limiting the amount of DNA available to transfect target cells and induce immune responses.

In the current paper, we describe in detail the preparation and characterization of microparticles with adsorbed DNA, and show *in vivo* data to support this approach for the development of improved DNA vaccines. We show that the load of DNA on microparticles can be varied, and that more than one plasmid can be delivered simultaneously. In addition, we demonstrate the requirement for the preparation of cationic PLG microparticles with adsorbed DNA, to achieve potent immune responses.

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MATERIALS AND METHODS

Materials

Polylactide-co-glycolide was obtained from Boehringer Ingelheim, U.S.A. All other chemicals were obtained from Sigma Chemical Co., St. Louis, U.S.A. and used as shipped. The HIV-1 pCMVkm p55 gag and gp-140 plasmids were made at Chiron. 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 described in our previous publication (11). The size distribution of the microparticles was determined using a particle size analyzer (Malvern Instruments, Malvern, U.K.). The zeta potential was measured on a DELSA 440 SX Zetasizer (Coulter Corp. Miami, FL). The HIV-1 p55 or gp140 plasmids were adsorbed onto the microparticles by incubating 100 mg of microparticles with a 200 μg/ml solution of DNA in 1X TE buffer under gentle stirring at 4°C for 12 hours. The microparticles were then separated by centrifugation, followed by lyophilization.

To adsorb two plasmids simultaneously onto the microparticles, the p55 was adsorbed first, as described above, followed by a second identical adsorption of gp140. The microparticles were then separated by centrifugation, and the microparticles were lyophilized. The loading level of DNA and the CTAB content of the microparticles were determined as previously described (11). Recently we were able to confirm the CTAB content of the microparticles using a newly developed sensitive HPLC assay (unpublished). The HPLC assay confirmed that the CTAB content of the microparticles ranged from 0.5–2.0% w/w.

To confirm that CTAB was required as a component of the microparticle to allow DNA adsorption, DNA was combined with PLG/CTAB, with PLG/PVA without CTAB, and with PLG/PVA with free CTAB. The following samples were prepared in 1X PBS and were incubated at 37°C; (i) 10 mg of PLG/CTAB and 100 μg of p55 DNA; (ii) 10 mg of PLG/PVA and 100 μg of p55 DNA, (iii) 10 mg of PLG/PVA, and 100 μg of p55 DNA, combined with 50 μg of CTAB; and (iv) 100 μg of p55 DNA combined with 50 μg of CTAB. After one hour, the samples were centrifuged and the supernatants were collected, and evaluated on a 1% agarose gel for the presence of DNA. Sample (i) containing PLG/CTAB would be expected to adsorb DNA and therefore, DNA would not be present in the gel. However, the alternative samples (ii–iv) would not be expected to adsorb DNA under these conditions.

Evaluation of Immune Responses

Groups of 10 female Balb/C mice aged 6–8 weeks and weighing about 20–25g were immunized with DNA formulations at days 0 and 28. 50 μl of the formulations in saline were injected by the TA route in the two hind legs of each animal. Mice were bled on day 42 through the retro-orbital plexus and

the sera was separated. HIV-1 p55 gag and gp120 specific serum IgG titers were quantified by ELISA, as previously described (11).

RESULTS AND DISCUSSION

The Characterization of PLG/CTAB Microparticles

The PLG/CTAB microparticles were prepared with a mean size of 1.45 ± 0.8 μm, using CTAB at 0.5% w/v. The size of the microparticles remained relatively unchanged with increasing input of CTAB, but there was some increase in zeta potential. The zeta potential of the PLG/CTAB microparticles prepared with 0.5% w/v CTAB was +53 ± 22 mV due to the incorporation of the cationic surfactant. The zeta potential following adsorption of 1% w/w DNA was +25 ± 14 mV, showing a reduction in positive charge due to the adsorption of the negatively charged DNA. In comparison, the zeta potential of PLG/PVA microparticles was –15 ± 8 mV. Unless otherwise stated, all PLG/CTAB microparticles were prepared with 0.5% w/v CTAB and 1% w/w DNA was adsorbed.

The mean loading efficiency for a 1.0% w/w p55 gag DNA theoretical load was 96%. In the study which involved consecutive adsorption of two different plasmids on the same microparticles, the loading efficiency for p55 gag DNA was 80%, and the loading efficiency for gp140 DNA was 68%. The loading efficiency of gp140 DNA on the microparticles when used as a single plasmid was 74%. With increasing CTAB concentration in the preparation process, the loading efficiency for DNA at higher loads was increased. The release rate of DNA was comparable in PBS and TE buffer, with about 20–30 % release ocurring at day 1 (data not shown). Adsorption of the plasmid onto the PLG/CTAB microparticles offered significant protection against degradation by DNase in vitro (data not shown).

DNA Does Not Adsorb to PLG Microparticles Unless CTAB Is Present

After one hour incubation at 37°C, the supernatants from samples (i)–(iv) described above were evaluated on a 1% agarose gel to determine the presence of unbound DNA. (Fig. 1). Lane 3 from sample (i) shows very little DNA remaining in the supernatant, demonstrating that the DNA has efficiently adsorbed onto the PLG/CTAB microparticles. In contrast, the other lanes, from samples (ii)–(iv), show the presence of DNA, indicating that under the conditions described DNA adsorption does not occur. Hence, the gel showed that the plasmid did not adsorb to PLG microparticles unless CTAB was incorporated into the microparticles during the preparation process. Incubation of DNA with PLG/PVA microparticles, even in the presence of CTAB, did not allow DNA adsorption to the microparticles. In a separate experiment, about 80–90% of the DNA was adsorbed in 1 hour, showing that adsorption is rapid and efficient, unlike microencapsulation of DNA.

The Immunogenicity of Adsorbed DNA on PLG/CTAB Microparticles

The PLG/CTAB microparticles with adsorbed DNA induced significantly enhanced antibody titers in mice in comparison to naked DNA. In contrast, no other combination of the formulation components resulted in enhanced responses over naked DNA (Fig. 2). In addition, PLG/CTAB microparticles with adsorbed DNA were effective for CTL induction following a single IM immunization at 1 μg dose of DNA. In contrast, all other formulations shown in Figure 1 were ineffective (data not shown). Hence, the PLG/CTAB microparticles with adsorbed DNA must be prepared as described, to allow the induction of enhanced immune responses.

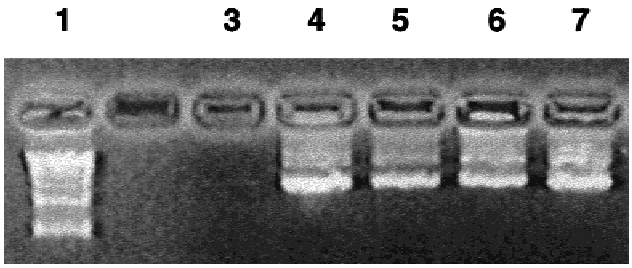

Fig. 1. DNA does not adsorb to PLG microparticles unless CTAB is present. Full explanation of all samples is provided in the text. Lane 1 is the marker lane. Lane 3 from sample (i) shows very little DNA remaining in the supernatant, demonstrating that the DNA has efficiently adsorbed onto the PLG/CTAB microparticles. In contrast, the other lanes, from samples (ii)–(iv), show the presence of DNA, indicating that under the conditions described DNA adsorption does not occur. Lane 4 is PLG/PVA particles mixed with DNA, sample (ii). Lane 5 is PLG/PVA particles mixed with DNA and CTAB, sample (iii). Lane 6 is DNA is mixed with CTAB, sample (iv) and Lane 7 is unformulated p55 DNA control incubated at 37°C.

The Effect of DNA Load on PLG/CTAB Microparticles on Antibody Induction

The antibody responses were not significantly different between animals immunized with microparticles with adsorbed DNA at 1.0%, 2.5%, and 5.0% load at the 1 μg dose level (Fig. 3). However, for the 10μg dose level there appeared to be some reduction in potency at the 5% w/w DNA loading level. Since it is clear that DNA vaccines will need to be used in humans at high doses, at low DNA load, the dose of PLG that could be administered might become a limiting

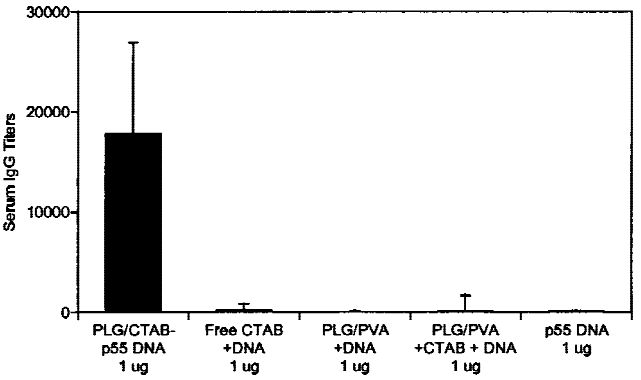

Fig. 2. Serum IgG responses in mice following immunization with p55 gag DNA. The formulations evaluated included PLG/CTAB with adsorbed DNA, CTAB with DNA, PLG/PVA particles with DNA, PLG/PVA particles with DNA and CTAB, and naked DNA alone. Antibody responses are shown as geometric mean titers ± SE (n = 10) at day 42. The responses from PLG/CTAB with adsorbed DNA was significantly higher than all other groups.

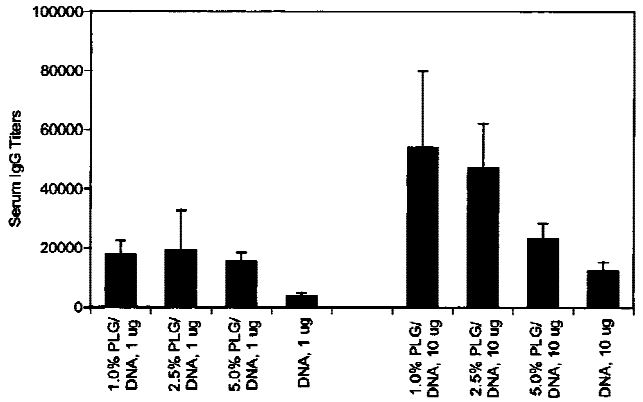

Fig. 3. The effect of DNA load on the antibody responses to PLG/CTAB microparticles with adsorbed DNA. Serum IgG titers in mice immunized with PLG/CTAB with p55 gag DNA adsorbed at 1.0%, 2.5%, and 5.0% w/w loading levels, for 1 and 10 μg doses. Antibody responses are shown as geometric mean titers ± SE (n = 10) at day 42. The responses from the PLG/CTAB microparticles with different loading levels are not significantly different. All PLG/CTAB formulations induced significantly higher antibody responses than naked DNA.

factor. This is much less likely to be a problem if higher loads of DNA are used on the microparticles.

Two Different Plasmids Can Be Adsorbed onto the Same, or Different, Microparticles

Two different plasmids, p55 gag and gp140 env, were adsorbed on the same, or different PLG/CTAB microparticles and still showed statistically enhanced antibody responses in comparison to naked DNA (Fig. 4). This observation shows that the enhancement achieved with PLG/CTAB microparticles is not unique to the p55 plasmid, and suggests that this approach may be applicable to a wide range of plasmids. In addition, it shows that the PLG/CTAB formulations have significant flexibility in how they can be used for DNA delivery. This is important since more than one plasmid are

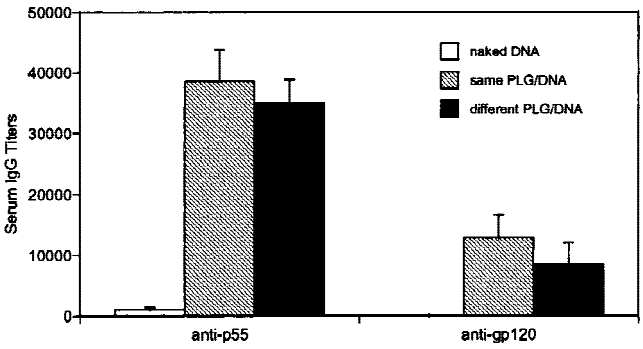

Fig. 4. The effect of delivering two plasmids simultaneously on the same or different PLG/CTAB microparticles. Serum IgG titers in mice immunized with p55 gag DNA and gp140 env DNA adsorbed on separate PLG/CTAB microparticles, or on the same PLG/CTAB particles, in comparison to immunization with naked DNA. Antibody responses are shown as geometric mean titers ± SE (n = 10) at day 42. The responses to p55 gag and gp140 env were not statistically different adsorbed either on the same or on different particles. However, the responses to both p55 and gp140 were significantly better than naked DNA on the same or different particles.

likely to be needed to allow the development of vaccines against HIV and other important pathogens.

We believe that the microparticle preparation process described in the current studies, involving surface adsorption of DNA, has a number of advantages over microencapsulation of DNA (14–20). Our approach represents a relatively simple process, which allows efficient adsorption and release of intact DNA, for more than one plasmid, at different loading levels. This will allow for delivery of multiple antigens on the same formulation. In contrast, recent reports describing the microencapsulation of DNA have reported low loading levels (0.1–0.4% w/w) at low efficiency (20–50%) for a single plasmid, which had been significantly damaged by the encapsulation process (14–20). Similar anionic microparticles have also been used to induce potent immune responses against surface adsorbed protein antigens (21). Hence, surface presentation of antigens represents a novel way to use PLG microparticles as an effective vaccine delivery system.

Recent studies performed 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). However, the ability of the microparticles to protect DNA against degradation and to increase gene expression *in vivo* (11) may also contribute to the potency of the formulations.

CONCLUSIONS

Overall, the current studies have shown that PLG/CTAB microparticles can efficiently adsorb more than one plasmid, at a range of loading levels, offer protection from degradation by DNAse, and induce enhanced immune responses over naked DNA. In addition, we have shown that it is necessary to prepare the PLG/CTAB formulation as described and to adsorb the DNA, to achieve enhanced responses. Studies that are currently underway in non-human primates suggest that the PLG/CTAB technology may prove to be a broadly enabling approach for a range of DNA vaccines.

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