Cationic Microparticles Are an Effective Delivery System for Immune Stimulatory CpG DNA

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Received March 20, 2001; accepted June 26, 2001

KEY WORDS: cationic microparticles; PLG (polylactide-co-lycolide); CpG adjuvant; vaccine delivery.

INTRODUCTION

It has been demonstrated that bacterial DNA, but not vertebrate DNA, has direct immunostimulatory effects on immune cells *in vitro* and *in vivo* (1,2). This is a consequence of the activation of pattern recognition receptors of the innate immune system, which distinguish prokaryotic DNA from vertebrate DNA and respond accordingly to a perceived bacterial infection (2). The immunostimulatory effect of bacterial DNA is mainly due to the presence of unmethylated CpG dinucleotides, which are under-represented in mammalian DNA and are mostly methylated (3). However, the mechanism of cellular uptake and activation for CpG DNA remains to be defined. Nevertheless, it has been reported that CpG are taken up by non-specific endocytosis and that endosomal maturation is necessary for the activation of stress kinase pathways (4). In addition, it has also been reported recently that CpG binds to Toll-like receptor 9, although the localization of the receptor remains to be established (5). Because exposure to CpG brings about conversion of immature DC's to mature APC's (6) and many other immune activation events, CpG oligonucleotides represent a promising new class of vaccine adjuvants. In vivo, phosphorothioate oligonucleotides containing CpG have been shown to be potent adjuvants for the induction of Th1 responses, mainly through stimulating TNFα, IL-1, IL-6, and IL-12, and through the expression of co-stimulatory molecules (2,6–8). In addition, it has been reported that the adjuvant effect of CpG can be further enhanced by covalent conjugation to protein antigens (9). In addition to their potential use as adjuvants in a broad range of vaccines, CpG also have significant potential to modulate existing immune responses, which may be useful for the treatment of allergic diseases (10).

In the current studies, we sought to improve the potency of CpG as a vaccine adjuvant by using a delivery system to promote the uptake and delivery of CpG into APC's. CpG was adsorbed onto the surface of cationic poly lactide-co-glycolide microparticles (PLG/CpG), which have previously been shown to be effective for enhancing immune responses to adsorbed plasmid DNA (11). In addition, recent studies have begun to define the mechanism of enhancement and have confirmed that cationic microparticles enhance the delivery of adsorbed plasmid into DC's (12). In the current studies we evaluated the potential of PLG/CpG to induce enhanced antibody and cytotoxic T lymphocyte (CTL) responses to p55 gag and gp120 env from HIV-1 following intramuscular immunization in mice.

MATERIALS AND METHODS

Materials

Polylactide-co-glycolide (PLG), RG505 which has a copolymer ratio of 50/50 and a molecular weight of 65Kd (manufacturer's data), was obtained from Boehringer Ingelheim, USA. ELISA microtiter plates were obtained from Nunc, Denmark. The CpG oligonucleotide used was, 5′-TCC ATG ACG TTC CTG ACG TT-3′, which had been previously described as the most potent adjuvant active sequence for mice (14). CpG was synthesized with a phosphorothioate backbone by Oligos Etc. (Wilsonville, OR, 99% purity by HPLC) and was ethanol precipitated and then resuspended in 10mM Tris (pH7.0) 1mM EDTA for storage at –80°C. Yeast derived recombinant $p55_{SF2}$ gag protein and CHO cell derived $gp120_{SF2}$ were purified at Chiron Corporation, Emeryville. CTAB (hexadecyl trimethyl ammonium bromide) and DSS (dioctyl sulphosuccinate) were obtained from Sigma Chemicals, St. Louis USA. The emulsion adjuvant MF59 was prepared as previously described (13).

Preparation of Charged Microparticles for Adsorption

Cationic PLG microparticles for CpG adsorption were prepared as described previously (11) using a modified solvent evaporation process. Briefly, the microparticles were prepared by emulsifying 10 mL of a 5% w/v polymer solution in methylene chloride with 1mL of PBS at high speed using an IKA homogenizer. The primary emulsion was then added to 50mL of distilled water containing CTAB (0.5% w/v). This resulted in the formation of a w/o/w emulsion which was stirred at 6000 rpm for 12 h at room temperature, allowing the methylene chloride to evaporate. The resulting microparticles were washed in distilled water by centrifugation at 10,000 g and freeze dried. The size distribution of the microparticles was determined using a particle size analyzer (Master sizer, Malvern Instruments, UK).

Anionic PLG microparticles for protein adsorption were also prepared as previously described (15) using a solvent evaporation technique. Briefly, microparticles were prepared by homogenizing 10mL 6% w/v polymer solution in methylene chloride, with 40 mL of distilled water containing DSS (2.5%), at high speed using a 10 mm probe. This resulted in an oil in water emulsion, which was stirred at 1000 rpm for 12 h at room temperature, and the methylene chloride was allowed to evaporate. The resulting microparticles were filtered through a 38 μm mesh, washed in distilled water, and freeze-dried. The zeta potential measurement of the microparticles was carried out on a DELSA 440 SX zetasizer equipment (Coulter Instruments).

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0724-8741/01/1000-1476$19.50/0

Adsorption of CpG and HIV Antigens to Charged Microparticles

The CpG was adsorbed onto PLG-CTAB microparticles by incubating 5mg of CpG in 10 mL of 1X TE buffer with 500 mg of microparticles overnight at 4°C with gentle rocking. The microparticles were then pelleted down by centrifugation at 10,000 rpm for 10 minutes and washed with 1X TE buffer by re-suspending the pellet. After washing, the pellet was re-suspended back in 5 mL of deionised water and freeze dried.

Adsorption of antigens to microparticles was performed as previously described (14). Briefly, 50 mg lyophilized PLG/DSS microparticles were incubated with 0.5 mg of p55 gag protein in 10 mL 25 mM Borate buffer pH 9.0 with 6M urea at room temperature, or 50 mg PLG/DSS microparticles were incubated with 0.5mg of gp120 protein in 10 mL PBS at room temperature. Particles were left on a lab rocker, (Aliquot mixer, Miles labs) overnight. The microparticles were separated from the incubation medium by centrifugation, and the pellet was washed three times with distilled water, and lyophilized. The loading level of protein adsorbed to microparticles was determined by dissolving 10 mg of the microparticles in 2 mL of 5% SDS-0.2M sodium hydroxide solution at room temperature. Protein concentration was measured by BCA protein assay (Pierce Rockford, Illinois). The in vitro release and loading efficiency were determined as previously reported (15).

Quantification of the Amount of CpG Adsorbed to Microparticles

Ten mg PLG/CpG were hydrolyzed with 1 mL 0.2N NaOH overnight, and neutralized. The sample was injected on a TSK 3000 column (TOSOHAAS Montgomeryville, PA) and run in an isocratic system at 1 mL/min with PBS as mobile phase using a Waters HPLC system, WATERS Corporation, Milford, MA, and the CpG peak area integrated. 100 μg CpG was added to 10mg blank PLG-CTAB particles, hydrolyzed with NaOH overnight and neutralized. This was used to generate a standard curve by injecting different volumes containing 1–20 μg CpG on the column, integrating the CpG peaks, and plotting peak area against concentration. The amount of CpG adsorbed to particles was calculated from the standard curve.

In Vitro Release of CpG from PLG/CTAB/CpG Formulation

In vitro release of CpG was measured in PBS at 37°C. Briefly, 10 mg of PLG/CTAB/CpG formulation was added to 1 mL of PBS in a glass vial and after each time point, the suspension was centrifuged and the supernatant collected. The supernatant was used to estimate the released CpG from the formulation.

Mouse Studies

For the antibody studies, groups of 10 BALB/c female mice, 6–10 weeks-old, obtained from Charles River Breeding Laboratories, were used. Animals were immunized three times at 4-week intervals in the tibialis anterior muscles in the two hind legs of each animal with 50 μL/leg. Doses were 10μg protein and 25 μg CpG. The antigen and adjuvant formulations were combined in a single dose for immunization. For the CTL studies, groups of 10 CB6F1 black female mice 6–10 weeks old were used. Each animal was immunized twice at 3-week intervals. Doses were the same as the antibody studies except for the protein dose, which was 25 μg of p55 gag. Mice were bled through the retro orbital plexus and sera was separated for the immunoassays. Spleens were harvested and pooled from groups of mice prior to the CTL assay.

Immunoassays

Serum anti-gp120 and p55 gag titers was measured by an ELISA as previously reported with a cutoff OD of 0.5 (11,15). CTL assays on pooled spleen cells were also performed as previously described (11,15). Intraperitoneal infection with a recombinant vaccinia virus encoding the HIV-1_{SF2} gag-pol genes (rVVgag-pol) was used as a positive control group in the CTL studies, as previously described (11,15).

RESULTS AND DISCUSSION

Characterization of CpG and Antigen Formulations

Prior to adsorption of CpG or antigens respectively, the cationic microparticles had a zeta potential of +42 ±10 mV and the anionic microparticles had a zeta potential of –32 ± 8mV (Table I). The zeta potential was reduced following adsorption. All the formulations had a high loading efficiency for the targeted load of 1% w/w (70–92% adsorption efficiency). Following 24 h in vitro incubation, protein release was <30%, while the CpG release was around 78% (Table I). The release of CpG at 3 h was 52%.

Induction of Antibody Responses to p55 gag with PLG/CpG

CpG alone did not appear to be a potent adjuvant for p55 gag, since the responses induced were only comparable to

Table I. Characterization of Charged Microparticle Formulations - Prior to and Post Adsorption

Microparticles	Mean size (μm)	Zeta potential (mV)pre-adsorption	Zeta potential (mV)post-adsorption	Percent loading efficiency	Percent released after 24 h in vitro
PLG-CTAB	1.50	42 ± 10	—	—	—
PLG-DSS	1.10	–32 ± 8	—	—	—
PLG/p55	1.10	—	–24 ± 4	70.0	5.0
PLG/gp 120	1.10	—	–30 ± 6	90.0	25.0
PLG/CpG	1.50	—	23 ± 4	92.0	78.0

those induced by the protein adsorbed to PLG (Fig. 1). However, CpG + p55 gag induced an enhanced response in comparison to immunization with a control inactive oligonucleotide (data not shown). Nevertheless, when the CpG was used in combination with PLG microparticles, there was a clear and significant adjuvant effect at 2 weeks post second immunization (2wp2) and at 2 weeks post third immunization (2wp3), in comparison to immunization with PLG/p55, or with CpG + p55. Hence, the optimal mouse CpG sequence was only potent as an adjuvant for p55 gag, when it was used in combination with PLG microparticles.

Induction of CTL Responses to p55 gag with PLG/CpG

The observations on antibody induction with CpG were reproduced in the study to evaluate CTL responses to p55 gag. CpG was effective for CTL induction against p55 gag only when adsorbed onto PLG microparticles (Fig. 2). As previously described, PLG/p55 alone was effective for CTL induction (14). In contrast, p55 + CpG was not effective for CTL induction. However, the CTL response to p55 gag adsorbed to PLG, appeared to be enhanced by the addition of CpG. Remarkably, the PLG/p55 + PLG/CpG group was more potent than the positive control, involving infection with vaccinia virus. In this study, we also evaluated the antibody responses to p55 gag and the results obtained were consistent with those described in the previous study (data not shown).

Induction of Antibody Responses in Mice to env gp120 with PLG/CpG

Our studies on antibody induction with CpG and env gp120 showed very similar responses to those described above for p55 gag. CpG alone was an ineffective adjuvant for gp120, but was very effective when used in combination with PLG microparticles. In comparison to MF59 + gp120, CpG + gp120 induced minimal responses (Fig. 3). In addition, no enhancement of response over that achieved with MF59 was induced by using PLG/gp120. However, PLG/gp120 in combination with PLG/CpG induced significantly enhanced antibody responses in comparison to MF59 + gp120. Nevertheless, in contrast to the observations for p55 gag, PLG/CpG in combination with soluble gp120 was not effective for the induction of potent antibody responses. No measurement of CTL activity with gp120 was performed. Hence for the induction of

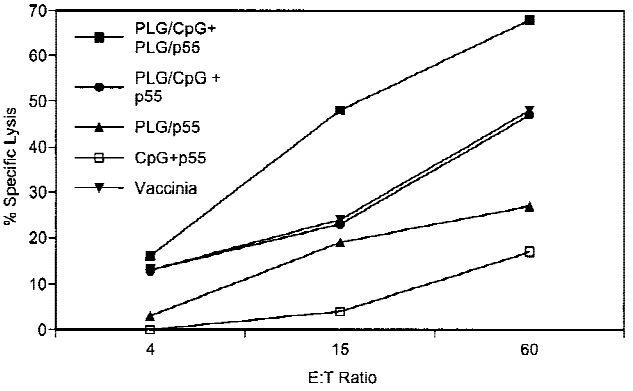

Fig. 2. The adjuvant effect of CpG for the induction of cytotoxic T lymphocyte responses against p55 gag. Both CpG and antigen were evaluated as soluble components, and adsorbed to PLG microparticles. Infection with vaccinia gag-pol was used as a positive control.

enhanced responses to env gp120, it appeared that both the antigen and the CpG adjuvant needed to be presented to the immune system on the surface of PLG microparticles (Fig. 3).

The studies described here demonstrate that the potency of CpG as a vaccine adjuvant for HIV-1 antigens can be significantly enhanced by presentation on the surface of cationic microparticles. In previous studies, cationic microparticles were shown to be potent delivery systems for adsorbed DNA (11,16), probably due to enhanced uptake of the plasmids into APC's, following phagocytosis of the particles (12). It is postulated that the CpG adjuvant is also delivered in the same way into the APCs. Although adsorption of DNA onto cationic microparticles makes it more resistant to nucleases (16) this was not evaluated for CpG. The studies with the freely soluble antigen, env gp120, were clear and consistent on the need for microparticle formulations to be present for an adjuvant effect to be achieved. This observation provided support for the hypothesis that the adjuvant effect of PLG/CpG microparticles was mainly due to enhanced uptake of CpG into APC's.

Hence, although it was clear that CpG DNA was capable of performing as a vaccine adjuvant for the HIV-1 proteins evaluated, it was ineffective when used as an adjuvant in the absence of microparticles. The cationic microparticles described in the current studies may offer an attractive, practical and potent approach for the delivery of adjuvant active DNA

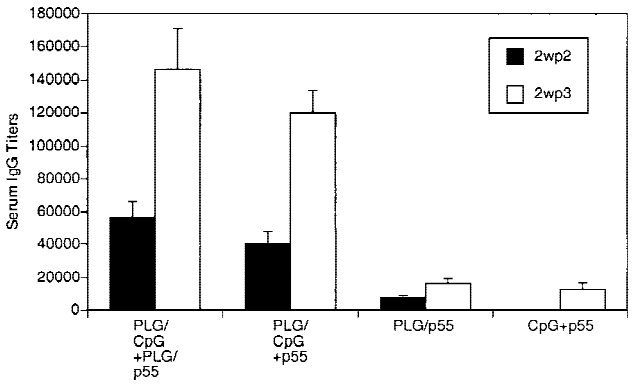

Fig. 1. The adjuvant effect of CpG for the induction of antibody responses against p55 gag. Both CpG and antigen were evaluated as soluble components, and adsorbed to PLG microparticles.

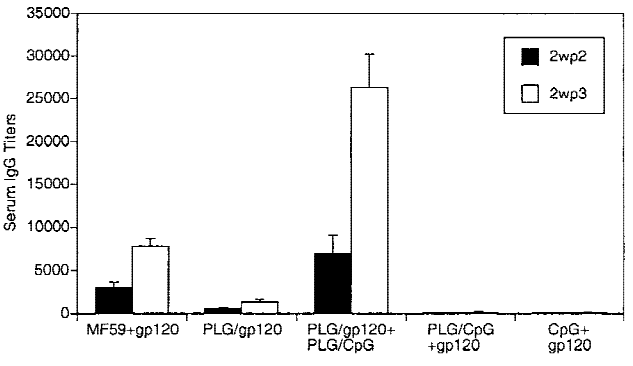

Fig. 3. The adjuvant effect of CpG for the induction of antibody responses against env gp120. Both CpG and antigen were evaluated as soluble components, and adsorbed to PLG microparticles. MF59 adjuvant was used as a positive control.

sequences. The range of antigens for which microparticle formulations are necessary to ensure the potency of CpG adjuvants remains to be determined.

CONCLUSIONS

The current studies indicated that cationic PLG microparticles may represent an enabling technology for CpG DNA adjuvants to be used in combination with HIV-1 p55 gag and env gp120 antigens. Moreover, the need for effective delivery systems for CpG DNA adjuvants may prove to be a common observation for a wide range of antigens. However, this needs to be further evaluated.

ACKNOWLEDGMENT

We are grateful to Indresh Srivastava for purification and provision of HIV-1 gp120.

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