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Induction of Potent Immune Responses by Cationic Microparticles with Adsorbed Human Immunodeficiency Virus DNA Vaccines

DEREK O'HAGAN,[1]* MANMOHAN SINGH,[1] MILDRED UGOZZOLI,[1] CARL WILD,[2] SUSAN BARNETT,[1] MINCHAO CHEN,[1] MARY SCHAEFER,[1] BARBARA DOE,[1] GILLIS R. OTTEN,[1] AND JEFFREY B. ULMER[1]

Vaccines Research, Chiron Corporation, Emeryville, California 94608,[1] and Panacos Pharmaceuticals, Gaithersburg, Maryland 20877[2]

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The effectiveness of cationic microparticles with adsorbed DNA at inducing immune responses was investigated in mice, guinea pigs, and rhesus macaques. Plasmid DNA vaccines encoding human immunodeficiency virus (HIV) Gag and Env adsorbed onto the surface of cationic poly(lactide-coglycolide) (PLG) microparticles were shown to be substantially more potent than corresponding naked DNA vaccines. In mice immunized with HIV *gag* DNA, adsorption onto PLG increased CD8$^+$ T-cell and antibody responses by ~100- and ~1,000-fold, respectively. In guinea pigs immunized with HIV *env* DNA adsorbed onto PLG, antibody responses showed a more rapid onset and achieved markedly higher enzyme-linked immunosorbent assay and neutralizing titers than in animals immunized with naked DNA. Further enhancement of antibody responses was observed in animals vaccinated with PLG/DNA microparticles formulated with aluminum phosphate. The magnitude of anti-Env antibody responses induced by PLG/DNA particles was equivalent to that induced by recombinant gp120 protein formulated with a strong adjuvant, MF-59. In guinea pigs immunized with a combination vaccine containing HIV *env* and HIV *gag* DNA plasmids on PLG microparticles, substantially superior antibody responses were induced against both components, as measured by onset, duration, and titer. Furthermore, PLG formulation overcame an apparent hyporesponsiveness of the *env* DNA component in the combination vaccine. Finally, preliminary data in rhesus macaques demonstrated a substantial enhancement of immune responses afforded by PLG/DNA. Therefore, formulation of DNA vaccines by adsorption onto PLG microparticles is a powerful means of increasing vaccine potency.

DNA vaccines have been shown to induce immune responses and protective immunity in many animal models of infectious disease (for a review, see reference 11). In mice, such responses can often be achieved with low doses (<1 μg) of naked DNA. However, the immunogenicity of DNA vaccines in larger animals (e.g., guinea pigs, rabbits, and nonhuman primates) has been much lower than that observed in mice, even at higher doses of DNA. In human clinical trials, certain DNA vaccines have been shown to induce immune responses (5, 19, 30), but multiple immunizations of high doses of DNA were required. Therefore, in order to provide protective efficacy in humans, the potency of DNA vaccines needs to be increased. So far, it appears that DNA vaccines are more effective at priming T-cell responses than antibodies, as exemplified by induction of cytotoxic T lymphocytes (CTL) but no antibodies against malaria circumsporozoite protein in humans (30). Similarly, we show here that in mice, human immunodeficiency virus (HIV) *gag* DNA primed CD8$^+$ T-cell responses at doses of DNA 10- to 100-fold lower than that required for priming of antibody responses. Therefore, technologies aimed at increasing the potency of DNA vaccines need to be especially effective at boosting humoral responses.

The technology described herein, formulation of DNA onto cationic poly(lactide-coglycolide) (PLG) microparticles, has been developed as a means to better target DNA to antigen-presenting cells (APCs). PLG microparticles are an attractive approach for vaccine delivery, since the polymer is biodegradable and biocompatible and has been used to develop several drug delivery systems (21). In addition, PLG microparticles have also been used for a number of years as delivery systems for entrapped vaccine antigens (24). More recently, PLG microparticles have been described as a delivery system for entrapped DNA vaccines (15, 18). Nevertheless, recent observations have shown that DNA is damaged during microencapsulation, leading to a significant reduction in supercoiled DNA (2, 29). Moreover, the encapsulation efficiency is often low. Therefore, we developed a novel approach of adsorbing DNA onto the surface of PLG microparticles to avoid the problems associated with microencapsulation of DNA. This approach, involving adsorption of DNA to cationic PLG, was previously demonstrated to markedly increase the potency of DNA vaccines in mice (25) and was shown here to increase potency in larger animal species, i.e., guinea pigs and rhesus macaques, as well. Particularly striking was the increase in antibody titers: the levels induced by PLG/DNA were equal to or better than with recombinant protein given together with a potent adjuvant. Therefore, this approach facilitates both cellular and humoral immune responses and holds promise as an enabling technology to allow the successful use of DNA vaccines in humans.

MATERIALS AND METHODS

DNA plasmids. The plasmid encoding HIV *gag* driven by the cytomegalovirus (CMV) promoter (pCMV HIV *gag*) was grown in *Escherichia coli* strain HB101, purified using a Qiagen Endofree Plasmid Giga kit (Qiagen, Inc.), and resuspended in 0.9% sodium chloride (Abbott Laboratories, North Chicago, Ill.). The

* Corresponding author. Mailing address: Derek O'Hagan, Chiron Corporation, 4560 Horton St., Mail Stop 4.3, Emeryville, CA 94608-2916. Phone: (510) 923-7662. Fax: (510) 658-0329. E-mail: derek_o'hagan@chiron.com.

pCMV vector used contains the immediate-early enhancer/promoter of CMV and a bovine growth hormone terminator and is described in detail elsewhere (6). The HIV *gag* DNA vaccine (pCMV HIV *gag*) contains a synthetically constructed $p55^{gag}$ gene, with codons reflecting mammalian usage, derived from the HIV-1 SF2 strain as previously described (35). The HIV *env* DNA vaccines (pCMV HIV gp120 and gp140) consist of a human tissue plasminogen activator (tPA) signal sequence and the following gene inserts: gp120 from HIV-1 SF2 strain and gp140 from HIV-1 US4 strain, codon optimized for expression in mammalian cells (33). Expression of HIV Gag and Env proteins was determined as described previously (33).

Recombinant HIV-1 gp120 protein (SF2) was expressed in Chinese hamster ovary cells and purified as previously described (14).

Preparation of PLG microparticles. The PLG polymer (RG505) was obtained from Boehringer Ingelheim. Cationic microparticles were prepared using a modified solvent evaporation process. Briefly, the microparticles were prepared by emulsifying 10 ml of a 5% (wt/vol) polymer solution in methylene chloride with 1 ml of phosphate-buffered saline (PBS) at high speed using an IKA homogenizer. The primary emulsion was then added to 50 ml of distilled water containing cetyltrimethylammonium bromide (CTAB) (0.5% wt/vol), resulting in the formation of a water-in-oil-in-water emulsion, which was stirred at 6,000 rpm for 12 h 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. 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 h. The microparticles were then separated by centrifugation, the pellet was washed with TE (Tris-EDTA) buffer, and the microparticles were freeze-dried. Physical characteristics were monitored as previously described (25).

Animals. Female CB6 F_1 mice (Jackson Labs) and female guinea pigs (Elm Hill Laboratories) were housed at Chiron in an American Association for Accreditation of Laboratory Animal Care-accredited facility. Male and female rhesus macaques were housed at Southern Research Institute (Frederick, Md.).

Measurement of antibody responses. At various times following immunization, blood was collected from anesthetized animals and serum was recovered by centrifugation. Anti-HIV Gag antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as follows. Wells of Immulon 2 HB U-bottomed microtiter plates (Dynex Technologies, Chantilly, Va.) were coated with HIV p55 protein at 5 μg/ml in PBS, 50 μl per well, and incubated at 4°C overnight. The plates were washed six times with wash buffer (PBS, 0.1% Tween 20 [Sigma, St. Louis, Mo.]) and blocked at 37°C for 1 h with 150 μl of blocking buffer (PBS, 0.1% Tween 20 [Sigma], 1% goat serum)/well. Test sera were diluted 1:25 and then serially diluted threefold in blocking buffer. The block solution was aspirated, and then the plates were incubated at 37°C for 2 h with 50 μl of each serum dilution/well. After being washed six times, the plates were incubated for 1 h at 37°C with appropriate horseradish peroxidase-conjugated antibodies (1:20,000 dilution) in blocking buffer for 30 min. Following a final six washes, the plates were developed with OPD for 30 min. The OPD developer consists of one tablet (10 mg) of *o*-phenylenediamine, 12 ml of buffer (0.1 M citric acid, 0.1 M dibasic sodium phosphate), and 5 μl of 30% H_2O_2. The reaction was stopped with 50 μl of 4 N H_2SO_4 per well, and optical density (OD) was measured at dual wavelengths, 492 and 690 nm. The reported titers correspond to the reciprocal of the serum dilution producing an absorbance value of 1.0.

For measurement of anti-Env antibodies by ELISA, Nunc Immunoplate U96 Maxisorp plates (Nalge Nunc International, Rochester, N.Y.) were coated with 200 ng of recombinant gp120SF2 protein per well and incubated overnight at 4°C. Between steps, the plates were washed in a buffer containing 137 mM NaCl and 0.05% Triton X-100. Serum samples were initially diluted 1:25 or 1:100 (in a buffer containing 100 mM $NaPO_4$, 0.1% casein, 1 mM EDTA, 1% Triton X-100, 0.5 M NaCl, and 0.01% thimerosal [pH 7.5]) and were serially diluted threefold. The plates were incubated for 1 h at 37°C. After being washed in a buffer containing 137 mM NaCl and 0.05% Triton X-100, the samples were reacted with appropriate horseradish peroxidase-conjugated antibodies (1:20,000 dilution) for 30 min at 37°C. The plates were then developed with 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid (ABTS; Sigma) for 30 min at 37°C. The reactions were stopped with 10% sodium dodecyl sulfate (SDS) and read at a wavelength of 415 nm. Anti-Env antibody responses were measured as the dilution at which an OD of 0.6 was achieved.

Neutralizing antibody activity in guinea pig sera was measured against the HIV-1_{SF2} laboratory strain and PM-1 target cells. Test sera (stored at −70°C) were thawed at 37°C and heat inactivated at 56°C for 30 min. Specimens were subsequently diluted 1:5 in tissue culture medium comprised of RPMI supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (BioWhittaker), then filter sterilized through a 0.22-μm-pore-size filter disk (Becton Dickinson, Oxnard, Calif.); 80 μl of each diluted sample was added to triplicate wells of a 96-well plate, with subsequent fourfold dilutions being attained by removing 20 μl of sample from each well and diluting this into 60 μl of growth medium in successive wells. To each well of diluted serum was added an equal volume (60 μl) of virus stock containing 25 50% tissue culture infectious doses ($TCID_{50}$) of the HIV-1_{SF2} virus, and the sample-virus mixture was allowed to incubate at 37°C for 1 h. At the end of this time, 2.5×10^4 PM-1 target cells were added in 80 μl of tissue culture medium, and the cultures were placed in a humidified incubator at 37°C and 5% CO_2. On days 3 and 5, 180 μl of culture medium was removed from each well and replaced with fresh medium. On day 7, 180 μl of supernatant was removed and stored at −70°C for subsequent measurement of virus replication using p24 capture ELISA (Coulter). Values shown represent the reciprocals of the serum dilutions at which a 50% inhibition of virus infection was observed. Statistical comparisons of antibody titers were performed using Student's *t* test.

Measurement of T-cell responses in mice. A recombinant vaccinia virus encoding the HIV-1_{SF2} *gag-pol* genes (rVVgag-pol) has been described previously (10). Four or more weeks following *gag* DNA immunization, mice were challenged with an intraperitoneal injection of 10^7 PFU of rVVgag-pol. Five days later, spleens were harvested and stimulated with the H-$2K^d$-restricted p7g Gag peptide (10) and then stained for intracellular gamma interferon (IFN-γ), as follows. Erythrocyte-depleted single-cell suspensions were prepared by treatment with Tris-buffered NH_4Cl (Sigma), and 1×10^6 to 2×10^6 nucleated spleen cells were cultured in duplicate at 37°C in the presence or absence of 10-μg/ml p7g peptide. Monensin (Pharmingen, San Diego, Calif.) was added to block cytokine secretion. After 3 to 5 h, cells were washed, incubated with anti-CD16/32 (Pharmingen) to block Fcγ receptors, stained with fluorescein isothiocyanate (FITC)-conjugated CD8 monoclonal antibody (Pharmingen), and fixed overnight at 4°C in 2% (wt/vol) paraformaldehyde. The following day, cells were treated with 0.5% (wt/vol) saponin (Sigma) and then incubated with phycoerythrin (PE)-conjugated mouse IFN-γ monoclonal antibody (Pharmingen) in the presence of 0.1% (wt/vol) saponin, washed, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.).

Measurement of T-cell responses in macaques. A set of 51 Gag peptides 20 residues long, overlapping by 10 amino acids (aa) and spanning residues 1 to 496 of HIV-1_{SF2} $p55^{gag}$, was synthesized (Chiron Mimotopes, Clayton, Australia). Peptides were used as a pool.

Rhesus macaque peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by centrifugation on Ficoll-Paque (Pharmacia Biotech, Piscataway, N.J.) gradients. PBMC were cultured for 8 days in 24-well plates at 3×10^6 per well in 1.5 ml of AIM-V/RPMI 1640 (50:50) culture medium (Gibco-BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum. Gag-specific CTL were stimulated by the addition of the Gag peptide pool (13.3 μg of total peptide/ml) and recombinant human interleukin-7 (IL-7; 15 ng/ml; R&D Systems, Minneapolis, Minn.). Human recombinant IL-2 (20 IU/ml; Proleukin; Chiron) was added on days 1, 3, and 6.

Stable rhesus B-lymphoblastoid cell lines (B-LCL) were derived by exposing PBMC to herpesvirus papio-containing culture supernatant from the S594 cell line (13, 23) in the presence of 0.5-μg/ml cyclosporin A (Sigma, St. Louis, Mo.).

Autologous B-LCL were infected with rVVgag-pol (PFU:cell ratio of 10) and concurrently labeled with $^{51}Cr_2O_4$ (NEN, Boston, Mass.) at 25 μCi per 10^6 B-LCL. After overnight culture at 37°C, rVV-infected, ^{51}Cr-labeled B-LCL were washed and then added (2,500 per round-bottomed well) to duplicate wells containing threefold serial dilutions of cultured PBMC. Then 10^5 unlabeled, uninfected B-LCL were added per well to inhibit nonspecific cytolysis. After 4 h at 37°C, 50 μl of culture supernatant was harvested and added to LumaPlates (Packard, Meriden, Conn.), and radioactivity was counted with a Microbeta 1450 liquid scintillation counter (Wallac, Gaithersburg, Md.). ^{51}Cr released from lysed targets was normalized by using the formula % specific ^{51}Cr release = 100 × (mean experimental cpm − SR)/(MR − SR), where SR is mean cpm from targets alone and MR is mean cpm from targets exposed to Triton X-100.

RESULTS

Priming of antibody and T-cell responses in mice by formulated DNA vaccines. To assess the relative ability of a promising DNA vaccine technology to prime antibody and T-cell responses, mice were immunized with HIV *gag* DNA with and without formulation onto cationic PLG microparticles. To quantify T-cell responses, mice were challenged at 28 days after a single immunization with a recombinant vaccinia virus expressing Gag. Then 5 days later, Gag-specific $CD4^+$ and

TABLE 1. Priming of $CD8^+$ T cells and antibodies in mice by HIV *gag* DNA[a]

Formulation	Dose (ng)	% $CD8^+$ CTL	ELISA GMT	
			Before	After
Control	0	0.2	8	8
Saline	10,000	19.5	207	19,531
	1,000	9.3	8	23
	100	2.8	8	8
	10	0.1	8	8
	1	0.1	8	8
PLG/CTAB	10,000	16.3	316	12,255
	1,000	13.2	131	7,691
	100	9.7	22	2,124
	10	9.2	8	1,278
	1	0.8	8	476

[a] Groups of 10 CB6 F_1 mice were immunized a single time with the indicated doses of DNA in saline or PLG/CTAB or were not immunized (control). At 28 days, mice were challenged with recombinant vaccinia virus encoding HIV *gag*, and 5 days later spleens were harvested for analysis of IFN-γ production, as measured by flow cytometry. Data are presented as mean percentages of $CD8^+$ T cells specific for *gag* for two separate experiments, each involving pools of five spleens. Serum samples were collected prior to vaccinia virus challenge and at time of sacrifice. Antibody data are presented as geometric mean ELISA titers ($n = 10$) pre- and postchallenge.

$CD8^+$ T cells were measured in vitro by determining IFN-γ production in spleen cells in response to brief restimulation with an H-2^d-restricted CTL epitope (10), as measured by flow cytometry. In an extensive dose-response titration, naked DNA primed Gag-specific $CD8^+$ T cells after a single dose of DNA as low as 100 ng (Table 1). In contrast, PLG/DNA was effective at 1 ng, indicating a ~100-fold increase in DNA vaccine potency, as judged by reduction of DNA vaccine dose. Anti-Gag antibodies were measured in serum before and after recombinant vaccinia virus challenge. In mice immunized with naked DNA, measurable anti-Gag antibodies were observed in unchallenged mice only at the highest DNA dose of 10 μg, whereas PLG/DNA induced antibodies at a dose as low as 100 ng. To determine the level of antibody priming by Gag DNA, anti-Gag antibodies were also measured at 5 days after recombinant vaccinia virus challenge. In naive challenged mice, no measurable antibody (or T-cell) responses were seen at this time. In challenged mice previously primed with naked DNA, substantial anti-Gag antibodies were seen only in the high-dose group, with only one of five mice showing a measurable titer at the 1-μg DNA dose. However, significant antibody priming was observed in mice immunized with as little as 1 ng of PLG/DNA (lowest dose tested). Therefore, the PLG/DNA formulation increased antibody priming by 1,000- to 10,000-fold. These data also illustrate that naked DNA primed $CD8^+$ T-cell responses much more effectively than antibody responses (~100-fold, based on the lowest dose of DNA required to prime a measurable response). However, formulation of DNA onto PLG microparticles substantially enhanced immune responses, with a particularly marked effect on humoral responses, such that T-cell and antibody priming appeared equally potent. In a separate study, PLG/DNA was similarly potent, priming $CD8^+$ and $CD4^+$ T-cell responses at 1- and 10-ng doses of *gag* DNA given a single time (Table 2).

The rationale for using the rVV virus for in vivo restimulation was twofold. First, because CD8 T-cell responses are relatively easy to induce in mice, which can be given high doses of DNA (0.01 to 0.1 mg per 20 g of body weight) relative to DNA doses in nonhuman primates or humans (1 to 5 mg per 5 to 70 kg), we needed a means of detecting T-cell priming in mice at suboptimal DNA doses (i.e., 1 to 100 ng). We have shown that the magnitude of the post-rVV challenge Gag CD8 T-cell response is directly proportional to the magnitude of Gag T-cell priming, and this was demonstrated using adoptive transfer of increasing numbers of Gag-specific splenocytes in naive mice (unpublished observations). The utility of the rVVgag challenge model is exemplified in a previous paper (35) that demonstrated the superior potency of a codon-optimized *gag* DNA versus wild type. Second, the rVV challenge serves to demonstrate that a strong DNA vaccine prime can result in a very strong booster response to the transgene in a viral vector. This has implications for a rapid, strong anamnestic response upon exposure to a live pathogenic virus such as HIV and for the use of this DNA vaccine technology in a prime-boost scenario with a viral vector boost.

Potency of DNA vaccines in guinea pigs and rhesus macaques. To test the efficacy of PLG formulation of DNA in a larger animal species, HIV DNA vaccines were evaluated in guinea pigs and rhesus macaques. First, the potency of DNA encoding HIV gp120 derived from the HIV-1 SF2 strain was assessed at 25- and 250-μg DNA doses in guinea pigs and compared to that induced by a very immunogenic recombinant gp120 protein (SF2) administered with the powerful adjuvant MF-59. Two immunizations of naked DNA at the 25-μg dose induced very modest levels of antibody responses, as measured by ELISA (geometric mean titer [GMT] = ~100) (Fig. 1). In contrast, substantially higher levels of response were observed in animals that received PLG/DNA (GMT = ~1,000) ($P < 0.0001$). Further enhancement was achieved by formulation of PLG/DNA with aluminum phosphate ($AlPO_4$) (GMT = ~4,000) ($P < 0.001$). This combination of PLG/DNA/$AlPO_4$ induced levels of anti-Env antibodies equivalent to that induced by recombinant protein with MF-59 ($P =$ 0.881). The levels of neutralizing antibody titers correlated well with ELISA, as very similar results were seen with naked DNA $<$ PLG/DNA $<$ PLG/DNA/$AlPO_4$ $\approx$ rgp120/MF-59 ($P = 0.003$, 0.09, and 0.266, respectively) (Table 3). In general, higher levels of antibody responses (both ELISA and neutralizing titers) were

TABLE 2. Priming of $CD8^+$ and $CD4^+$ T cells and antibodies in mice by HIV *gag* DNA[a]

DNA dose (ng)	% of cells specific for Gag							
	$CD8^+$				$CD4^+$			
	Saline		PLG		Saline		PLG	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
0 (control)	0.1	0.0	nd	nd	0.1	0.3	nd	nd
1	0.1	0.1	7.2	13.9	0.1	0.2	0.9	1.1
10	0.2	0.2	8.4	13.5	0.1	0.3	1.1	0.7
10,000	21.6	24.1	nd	nd	1.9	1.9	nd	nd

[a] Groups of 10 CB6 F_1 mice were immunized a single time with the indicated doses of DNA in saline or PLG/CTAB or unimmunized (control). At 28 days, mice were challenged with recombinant vaccinia virus encoding HIV *gag*, and 5 days later spleens were harvested for analysis of IFN-γ production, as measured by flow cytometry. Data are presented as mean percentages of $CD4^+$ or $CD8^+$ T cells specific for Gag for two separate experiments, each involving pools of five spleens. nd, not done.

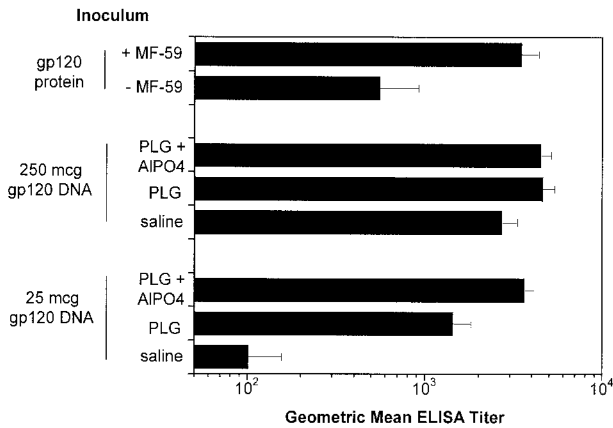


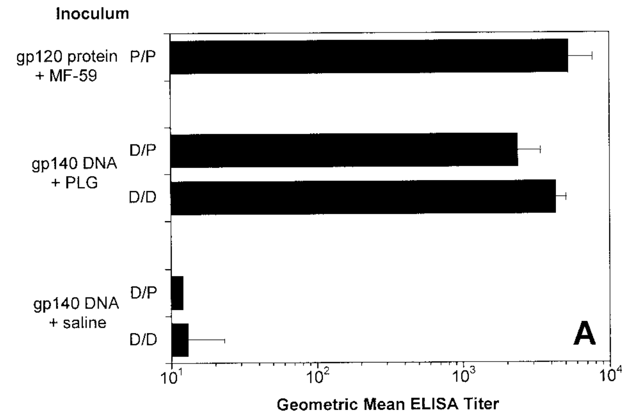
FIG. 1. Groups of 10 guinea pigs were immunized with HIV gp120 DNA in saline, PLG, or PLG plus $AlPO_4$ at doses of 25 or 250 μg of DNA. As positive controls, animals were immunized with 50 μg of recombinant gp120 protein with or without MF59 adjuvant. All animals were immunized at 0 and 4 weeks, and sera were collected at 2 weeks post-second immunization. Data are presented as geometric mean ELISA titer ± standard error of the mean (SEM) for $n = 10$.

induced by the 250-μg dose of DNA than by 25 μg. However, formulation of 25 μg of DNA (with either PLG or PLG/$AlPO_4$) increased potency to levels equivalent to that induced by 250 μg of naked DNA. This was true for both ELISA and neutralizing antibody titers. Therefore, by two separate criteria, increase in titer and decrease in DNA dose, PLG formulation of DNA increased HIV gp120 DNA vaccine potency in guinea pigs by ~10-fold.

Second, DNA encoding HIV gp140 derived from the HIV-1 US4 strain was assessed at a dose of 50 μg and compared to rgp120/MF-59 in a matrix of prime-boost scenarios in guinea pigs. After two immunizations, naked DNA was ineffective, with only a minority of animals responding by ELISA (Fig. 2A). This was true whether the animals received rgp120/MF-59 or DNA at the second immunization. In contrast, animals that received PLG/DNA had substantially higher levels of antibodies (GMT = ~4,000) ($P < 0.001$). Equivalent titers were achieved with two doses of PLG/DNA/$AlPO_4$ (data not shown) or when the second immunization was rgp120/MF-59 (Fig. 2A). These titers were similar to those observed in animals immunized twice with rgp120/MF-59. After three immunizations, all vaccines, or combinations thereof, showed a booster

TABLE 3. Induction of neutralizing antibodies in guinea pigs by PLG/DNA formulation

Inoculum	Dose (μg)	50% Neutralization		90% Neutralization	
		GMT	% Seropositive	GMT	% Seropositive
DNA	250	35	100	14.6	100
DNA/PLG	250	74.5	100	24.9	100
DNA/PLG/$AlPO_4$	250	101.1	90	39.3	90
DNA	25	10.5	10	<10	0
DNA/PLG	25	29.3	70	13.9	40
DNA/PLG/$AlPO_4$	25	49.8	100	16.7	75
Protein/MF59	50	89.9	100	43.3	71.4



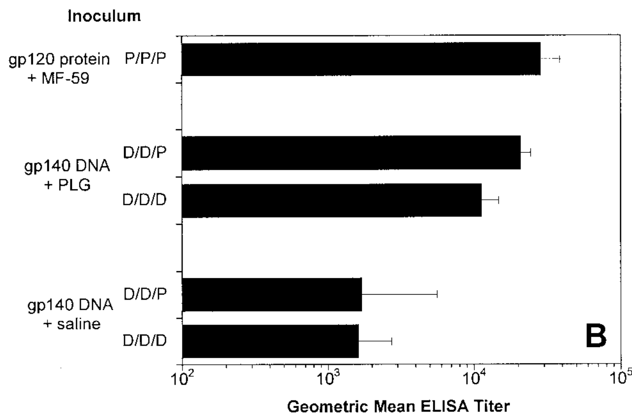


FIG. 2. Groups of 10 guinea pigs were immunized with HIV gp140 DNA in saline or PLG at a dose of 50 μg of DNA. As positive controls, animals were immunized with 25 μg of recombinant gp120 protein with MF59 adjuvant. All animals were immunized at 0, 4, and 8 weeks, and sera were collected at 2 weeks post-second (A) and -third (B) immunization. Some groups were immunized with DNA only (D/D/D) or protein plus MF-59 only (P/P/P) or primed with DNA and boosted with protein plus MF-59 (D/P or D/D/P). Data are presented as geometric mean ELISA titer ± SEM for $n = 10$.

effect (Fig. 2B). That is, animals vaccinated with DNA twice (D/D), protein twice (P/P), or DNA once and protein once (D/P) had significantly higher anti-Env antibodies after a boost with DNA or protein ($P < 0.001$ in all cases). Three immunizations of naked DNA induced consistent but moderate levels of anti-Env antibodies (GMT = ~2,000) (Fig. 2B), whereas three injections of PLG/DNA induced significantly higher antibodies (GMT = ~20,000) ($P < 0.001$). This was true for animals receiving PLG/DNA or rgp120/MF-59 at dose three, reaching levels of anti-Env antibodies similar to those after three injections of rgp120/MF-59 ($P = 0.518$). Therefore, as with gp120 DNA, formulation of gp140 DNA with PLG significantly increased antibody responses, as judged by earlier onset of responses (equivalent titers were seen post-dose two with PLG/DNA versus post-dose three with naked DNA) and a 10-fold increase in titer post-dose three.

Third, a combination DNA vaccine containing separate plasmids encoding HIV gp140 (US4) and HIV *gag* (SF2) was tested with and without PLG formulation in guinea pigs at doses of 1 and 0.5 mg, respectively. Anti-Gag (Fig. 3A) and anti-Env antibodies (Fig. 3B) were measured by ELISA at 3

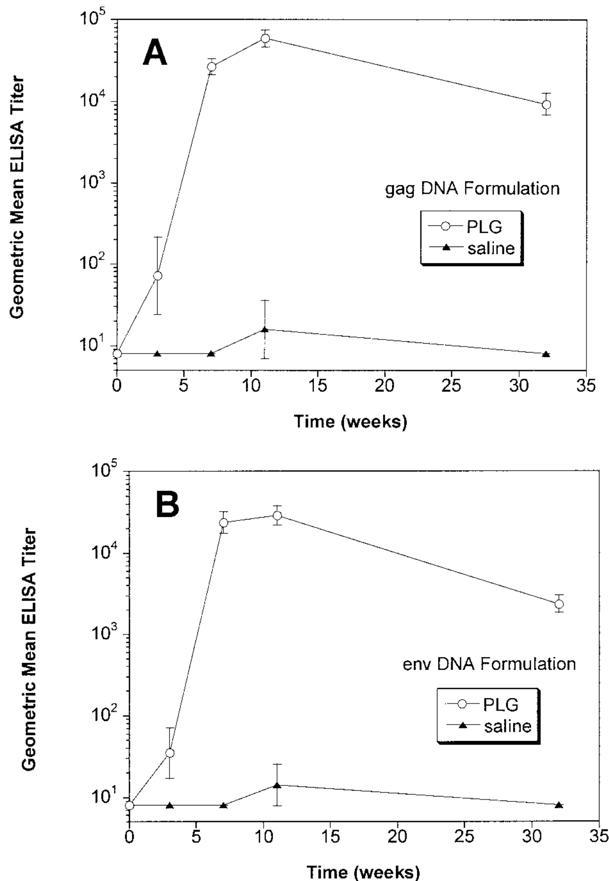


FIG. 3. Groups of five guinea pigs were immunized with a combination of HIV gp140 DNA and HIV *gag* DNA in saline or PLG at doses of 1 and 0.5 mg of DNA, respectively. Animals were immunized at 0, 4, and 8 weeks, and sera were collected at 3, 7, 11, and 32 weeks. Data are presented as geometric mean ELISA titer ± SEM ($n = 5$) for anti-Gag (A) and anti-Env (B) antibodies.

weeks post-dose one, 3 weeks post-dose two, and 3 and 24 weeks post-dose three. The combination of PLG-formulated *gag* and *env* DNA induced significant anti-Gag and anti-Env antibodies as early as 3 weeks post-dose one. The titers were increased substantially (~100- to 1,000-fold) after the second immunization and modestly (~2-fold) after the third. Significant anti-Gag and anti-Env antibody titers were maintained for at least 24 weeks after the last immunization (GMT = ~10,000 and 3,000, respectively). In contrast, the unformulated DNA combination did not induce measurable antibodies against either component until post-dose three and then with only very low titers (GMT = ~20). Therefore, PLG formulation was very potent and obligatory for effectiveness of this combination DNA vaccine. The lack of effectiveness of 1 mg of naked gp140 DNA given three times in combination with naked *gag* DNA was surprising, since less naked gp140 DNA (50 μg) induced significant antibody responses post-dose three when administered alone (see Fig. 2B). Therefore, addition of *gag* DNA to *env* DNA appears to have resulted in decreased immunogenicity of the *env* DNA component. However, this interference was only manifest when the combination was given as naked DNA, as a combination of PLG-formulated plasmids was very potent and overcame this interference.

Finally, the potency of PLG-formulated HIV *gag* DNA was evaluated in a pilot study in rhesus macaques. The animals were immunized at 0 and 4 weeks with 0.5 mg of *gag* DNA given as either naked DNA or PLG/DNA, and anti-Gag antibody and CD8 T-cell responses were measured. As shown in Fig. 4, naked *gag* DNA was ineffective at priming anti-Gag antibodies, as measured by ELISA. This lack of potency for naked *gag* DNA was also observed in guinea pigs (see Fig. 3A). In contrast, PLG/DNA primed strong anti-Gag antibodies as early as 2 weeks after the first immunization, which was the earliest time point tested. CD8 T-cell responses were assessed by Gag-specific CTL activity in cultured PBL, and data from representative animals in the PLG/DNA (Fig. 5A) and naked DNA (Fig. 5B) groups are shown. In the PLG/DNA group, three of the five animals had Gag CTL at 2 weeks post-first immunization (first time point tested), whereas no Gag CTL were seen at that time in the group of five that received naked DNA. Thereafter, approximately the same frequency of CTL responders was seen over time in the two groups ($n = 13$ positive samples at five separate times for naked DNA, $n = 10$ for PLG/DNA). Therefore, PLG formulation of DNA is an effective means of increasing DNA vaccine potency (particularly for antibody responses) in several animal species, including primates.

DISCUSSION

DNA vaccines were initially investigated because of their potential to induce CTL, as a consequence of antigen expression within cells of the vaccinated animals. In fact, this technology has been demonstrated to effectively induce CTL in mice (28), monkeys (31), and humans (30). Antibody and, to a lesser extent, helper T-cell responses, however, have proven more difficult to elicit by DNA vaccines. This relative deficiency is particularly true for larger animals, such as primates, but is also apparent in mice. For example, Table 1 demonstrates that CTL are primed by naked DNA at much lower doses (10 to

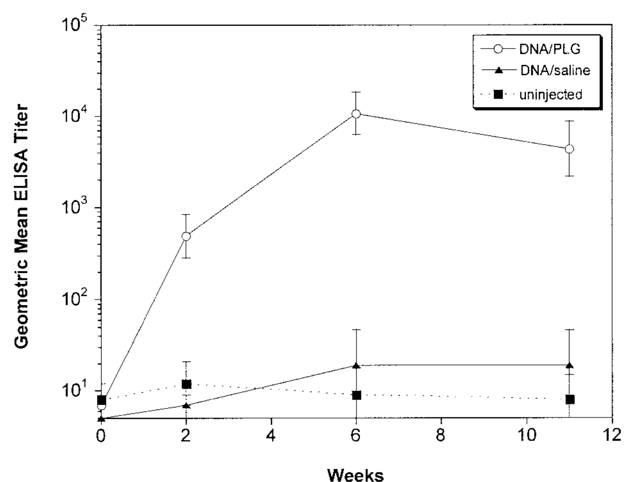


FIG. 4. Groups of five rhesus macaques were immunized with HIV *gag* DNA in saline (solid triangles) or PLG (open circles) at a DNA dose of 0.5 mg. Animals were immunized at 0 and 4 weeks, and sera were collected at 2, 6, and 11 weeks. Data are presented as geometric mean ELISA titer ± SEM for $n = 5$. For comparison, anti-Gag antibody titers are shown for unimmunized animals (solid squares) ($n = 4$).

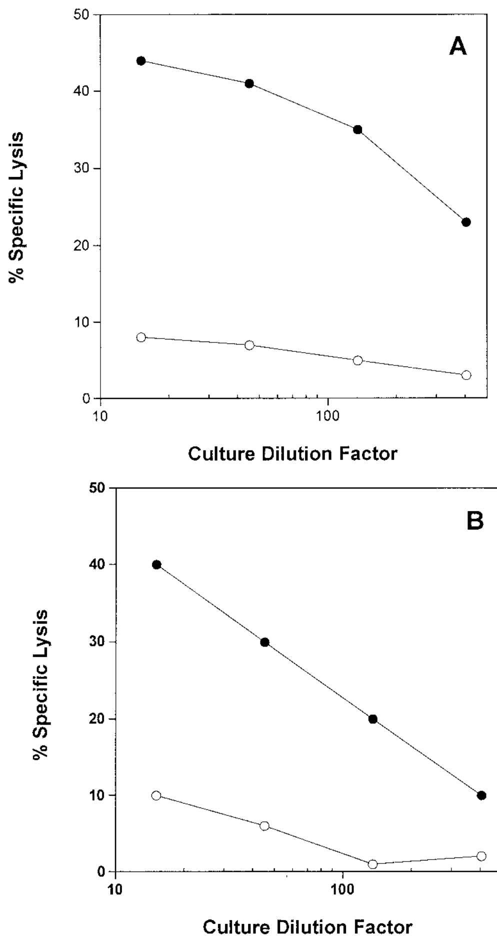


FIG. 5. Gag-specific cytolytic activity of PBMC from individual rhesus macaques given two doses of PLG/CTAB-formulated pCMV-gag DNA (A) or unformulated pCMV-gag DNA (B). Two weeks after the second DNA immunization, PBMC were stimulated with a pool of overlapping Gag peptides and cultured in the presence of IL-2 for 8 days. Serial dilutions of cultures (1:15, 1:45, 1:135, and 1:405) were added to ^{51}Cr-labeled rVVgagpol$_{SF2}$-infected autologous B-LCL (●) or rVVgp160env$_{SF162}$-infected autologous B-LCL (○). ^{51}Cr release was determined 4 h after addition of cultured PBMC to B-LCL.

100 ng) than are antibodies (1 to 10 μg). The reasons for low immunogenicity in larger animals are unclear but may be related to relative body mass (32), poor delivery of DNA to APCs, and/or less efficient distribution of DNA within the tissue. In support of the latter hypothesis, we observed that administration to mice of potent DNA vaccines in low volume (i.e., 5 μl) limited the distribution of DNA and uptake by cells within injected tissue, resulting in very poor immunogenicity (12). Moreover, increased distribution and delivery of DNA within the tissue through the use of electroporation (12, 33) overcame this limitation in potency of DNA given in low volume. Therefore, the low volume of inoculum relative to muscle mass, which is particularly true in larger animals, may be an important factor in limiting DNA delivery and immunogenicity.

A second possible limitation in larger animals is poor DNA uptake by and transfection of APCs. In small animals, such as mice, intramuscular injection of DNA results primarily in the transfection of myocytes (34). However, APCs may also be transfected, albeit at a very low frequency (1, 4, 7). It is well known that expression of antigens in APCs by DNA vaccines is a potent means of priming immune responses (20, 26), including those in nonhuman primates (3). Hence, targeting DNA to APCs is likely to increase DNA vaccine potency. To this end, we have developed a microparticle-based PLG formulation of DNA (25), based on the hypothesis that particles ~1 μm in diameter would be readily internalized by phagocytic cells, such as immature dendritic cells (16), thereby facilitating nonspecific targeting. Indeed, this formulation is very potent in mice (25) and appears to function, at least in part, by facilitating DNA uptake by APCs (9). PLG has been used previously for delivery of small-molecule drugs (17), proteins (21, 22), and DNA (8, 15, 18, 27). However, in these cases the delivered cargo was encapsulated inside the particles and, hence, may have functioned in a slow-release depot manner. In contrast, the present formulation, consisting of surface-adsorbed DNA, has a twofold rationale: (i) adsorption onto preformed PLG particles avoids the harsh emulsion conditions, which are known to affect the integrity of DNA (2, 27, 29); and (ii) it provides a means of delivery to and rapid release of DNA in APCs.

Previously, we have shown a marked enhancement of DNA vaccine potency using PLG in mice, based on antibody and CTL responses (25). We have now extended those observations in mice to include the enhancement of CD8$^+$ and CD4$^+$ T-cell responses, based on a quantitative assay measuring intracellular IFN-γ production, across a wide dose range of DNA (1 ng to 10 μg). In terms of dose reduction, PLG/DNA was 100- to 1,000-fold more potent than naked DNA for CD8$^+$ T-cell and antibody responses, respectively. Similarly, in much more limited dose-range studies, PLG/DNA was also able to reduce the dose of DNA in guinea pigs (at least 10-fold). More importantly, though, PLG/DNA was able to elicit antibody responses to levels equivalent to those induced by recombinant HIV gp120 administered in a strong adjuvant, which is currently the best means short of live virus infection of inducing anti-Env antibody responses. This was true for both ELISA and neutralization assays. The ability of a DNA vaccine technology to reduce the dose of DNA is likely to be an important parameter. First, at high doses of DNA in mice (~10 μg), an apparent ceiling of T-cell responsiveness was achieved, with ~20% of CD8$^+$ T cells responding to Gag. At these doses, naked and formulated DNA showed equivalent responses, and further enhancement is unlikely. Therefore, enhancement at low doses may be a more relevant indicator of potency. Second, to date CTL responses in humans have only been reliably seen with high doses of DNA (>1 mg), and antibody responses are not induced (30). Hence, for reasons of potency, ability to manufacture, cost of goods, and safety, it is imperative that the DNA dose be reduced.

During the course of these studies, an apparent interference between the two components of a combination vaccine consisting of unformulated HIV *gag* and *env* DNA was observed. Specifically, anti-Env antibody responses were considerably lower in guinea pigs given the combination vaccine containing 1 mg of naked *env* DNA than in those animals given as little as 50 μg of naked *env* DNA alone (compare Fig. 2B and 3B). This hyporesponsiveness to the *env* component may be related to antigenic competition between *gag* and *env* or to differential uptake of the DNA plasmids by cells in vivo. Whatever the cause, this interference was not observed in other studies with mice or rabbits (C. Goldbeck, M. Selby, and J. Ulmer, unpub-

lished observations) and, hence, appears to be specific to this animal species. Moreover, formulation of the DNA components with PLG overcame this hyporesponsiveness to the *env* component. Therefore, the use of PLG microparticles for DNA delivery may facilitate the development of combination vaccines.

The ability of vaccines to induce potent immune responses in small animals does not ensure that the vaccines will be effective in primates. This has been shown to be true for several naked DNA vaccines. We show here that naked HIV *gag* DNA did not induce significant anti-Gag antibodies in rhesus macaques unless it was formulated on PLG microparticles. In summary, we have demonstrated markedly increased potency of HIV *gag* and/or *env* DNA vaccines in mice, guinea pigs, and rhesus macaques by formulation with PLG. Based on dose reduction and the ability to enhance both cellular and humoral immune responses (particularly the latter), this technology holds promise for use in humans.

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REFERENCES

1. **Akbari, O., N. Panjwani, S. Garcia, R. Tascon, D. Lowrie, and B. Stockinger.** 1999. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. J. Exp. Med. **189:**169–178.
2. **Ando, S., D. Putnam, D. W. Pack, and R. Langer.** 1999. PLGA microspheres containing plasmid DNA: preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. J. Pharm. Sci. **88:**126–130.
3. **Barratt-Boyes, S. M., M. I. Zimmer, L. A. Harshyne, E. M. Meyer, S. C. Watkins, S. Capuano, M. Murphey-Corb, L. D. Falo, Jr., and A. D. Donneberg.** 2000. Maturation and trafficking of monocyte-derived dendritic cells in monkeys: implications for dendritic cell-based vaccines. J. Immunol. **164:**2487–2495.
4. **Bouloc, A., P. Walker, J.-C. Grivel, J. C. Vogel, and S. I. Katz.** 1999. Immunization through dermal delivery of protein-encoding DNA: a role for migratory dendritic cells. Eur. J. Immunol. **29:**446–454.
5. **Calarota, S., G. Bratt, S. Nordlund, J. Hinkula, A. C. Leandersson, E. Sandstrom, and B. Wahren.** 1998. Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. Lancet **351:**1320–1325.
6. **Chapman, B. S., R. M. Thayer, K. A. Vincent, and N. L. Haigwood.** 1991. Effect of intron A from human cytomegalovirus (Towne) immediate early gene on heterologous expression in mammalian cells. Nucleic Acids Res. **19:**3979–3986.
7. **Chattergoon, M. A., T. M. Robinson, J. D. Boyer, and D. B. Weiner.** 1998. Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages/antigen-presenting cells. J. Immunol. **160:**5707–5718.
8. **Chen, S. C., D. H. Jones, E. F. Fynan, G. H. Farrar, J. C. S. Clegg, H. B. Greenberg, and J. E. Herrmann.** 1998. Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. J. Virol. **72:**5757–5761.
9. **Denis-Mize, K. S., M. Dupuis, M. L. MacKichan, M. Singh, D. O'Hagan, J. B. Ulmer, J. Donnelly, D. MacDonald, and G. Ott.** 2000. Plasmid DNA adsorbed onto PLG-CTAB microparticles mediates target gene expression and antigen presentation by dendritic cells. Gene Ther. **7:**2105–2112.
10. **Doe, B., and C. M. Walker.** 1996. HIV-1 p24 Gag-specific cytotoxic T-lymphocyte responses in mice. AIDS **10:**793–794.
11. **Donnelly, J., J. Ulmer, J. Shiver, and M. Liu.** 1997. DNA vaccines. Annu. Rev. Immunol. **15:**617–648.
12. **Dupuis, M., K. Denis-Mize, C. Woo, C. Goldbeck, M. J. Selby, J. B. Ulmer, J. J. Donnelly, G. Ott, and D. M. MacDonald.** 2000. Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice. J. Immunol. **165:**2850–2858.
13. **Falk, L., F. Deinhardt, M. Nonoyama, L. G. Wolfe, and C. Bergholz.** 1976. Properties of a baboon lymphotropic herpesvirus related to Epstein-Barr virus. Int. J. Cancer **18:**798–807.
14. **Haigwood, N. L., P. L. Nara, E. Brooks, G. A. Van Nest, G. Ott, K. W. Higgins, N. Dunlop, C. J. Scandella, J. W. Eichberg, and K. S. Steimer.** 1992. Native but not denatured recombinant human immunodeficiency virus type 1 gp120 generates broad-spectrum neutralizing antibodies in baboons. J. Virol. **66:**172–182.
15. **Hedley, M., J. Curley, and R. Urban.** 1998. Microspheres containing plasmid encoded antigens elicit cytotoxic T cell responses. Nat. Med. **4:**365–368.
16. **Inaba, K., M. Inaba, M. Naito, and R. M. Steinman.** 1993. Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. J. Exp. Med. **178:**479–488.
17. **Jain, R. A., C. T. Rhodes, A. M. Railkar, A. W. Malick, and N. H. Shah.** 2000. Controlled delivery of drugs from a novel injectable in situ formed biodegradable PLGA microsphere system. J. Microencapsul. **17:**343–362.
18. **Jones, D. H., S. Corris, S. McDonald, J. C. Clegg, and G. H. Farrar.** 1997. Poly (DL-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses after oral administration. Vaccine **15:**814–817.
19. **MacGregor, R., J. Boyer, K. Ugen, K. Lacy, S. Gluckman, M. Bagarazzi, M. Chattergoon, Y. Baine, T. Higgins, R. Ciccarelli, L. Coney, R. Ginsberg, and D. Weiner.** 1998. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. J. Infect. Dis. **178:**92–100.
20. **Manickan, E., S. Kanangat, R. J. Rouse, Z. Yu, and B. T. Rouse.** 1997. Enhancement of immune response to naked DNA vaccine by immunization with transfected dendritic cells. J. Leukoc. Biol. **61:**125–132.
21. **Okada H.** 1997. One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate. Adv. Drug Deliv. Rev. **28:**43–70.
22. **Oldham, J. B., L. Lu, X. Zhu, B. D. Porter, T. E. Hefferan, D. R. Larson, B. L. Currier, A. G. Mikos, and M. J. Yaszemski.** 2000. Biological activity of rhBMP-2 released from PLGA microspheres. J. Biomech. Eng. **122:**289–292.
23. **Rabin, H., R. H. Neubauer, N. J. Woodside, J. L. Cicmanec, W. C. Wallen, B. A. Lapin, V. A. Agrba, L. A. Yakoleva, and G. N. Chuvirov.** 1976. Virological studies of baboon (Papio hamadryas) lymphoma: isolation and characterization of foamyviruses. J. Med. Primatol. **5:**13–22.
24. **Singh, M., and D. T. O'Hagan.** 1999. Advances in vaccine adjuvants. Nat. Biotechnol. **17:**1075–1081.
25. **Singh, M., M. Briones, G. Ott, and D. O'Hagan.** 2000. Cationic microparticles: a potent delivery system for DNA vaccines. Proc. Natl. Acad. Sci. USA **97:**811–816.
26. **Timares, L., A. Takashima, and S. A. Johnston.** 1998. Quantitative analysis of the immunopotency of genetically transfected dendritic cells. Proc. Natl. Acad. Sci. USA **95:**13147–13152.
27. **Tinsley-Brown, A. M., R. Fretwell, A. B. Dowsett, S. L. Davis, and G. H. Farrar.** 2000. Formulation of poly(lactide-co-glycolide) microparticles for rapid plasmid DNA delivery. J. Control. Release **66:**229–241.
28. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. Dewitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Martinez, H. C. Perry, J. W. Shiver, D. L. Montgomery, and M. A. Liu.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science **259:**1745–1749.
29. **Walter, E., K. Moelling, J. Pavlovic, and H. P. Merkle.** 1999. Microencapsulation of DNA using poly(DL-lactide-co-glycolide): stability issues and release characteristics. J. Control. Release **61:**361–374.
30. **Wang, R., D. L. Doolan, T. P. Le, R. C. Hedstrom, K. M. Coonan, Y. Charoenvit, T. R. Jones, P. Hobart, M. Margalith, J. Ng, W. R. Weiss, M. Sedegah, C. de Taisne, J. A. Norman, and S. L. Hoffman.** 1998. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science **282:**476–480.
31. **Wang, R., D. L. Doolan, Y. Charoenvit, R. C. Hedstrom, M. J. Gardner, P. Hobart, J. Tine, M. Sedegah, V. Fallarme, J. B. Sacci, Jr., M. Kaur, D. M. Klinman, S. L. Hoffman, and W. R. Weiss.** 1998. Simultaneous induction of multiple antigen-specific cytotoxic T lymphocytes in nonhuman primates by immunization with a mixture of four *Plasmodium falciparum* DNA plasmids. Infect. Immun. **66:**4193–4202.
32. **West, G. B., J. H. Brown, and B. J. Enquist.** 1997. A general model for the origin of allometric scaling laws in biology. Science **276:**122–126.
33. **Widera, G., M. Austin, D. Rabussay, C. Goldbeck, S. Barnett, M. Chen, L. Leung, G. R. Otten, K. Thudium, M. J. Selby, and J. B. Ulmer.** 2000. Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. J. Immunol. **164:**4635–4640.
34. **Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner.** 1990. Direct gene transfer into mouse muscle in vivo. Science **247:**1465–1468.
35. **zur Megede, J., M.-C. Chen, B. Doe, M. Schaefer, C. E. Greer, M. Selby, G. R. Otten, and S. W. Barnett.** 2000. Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 *gag* gene. J. Virol. **74:**2628–2635.