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Increased DNA Vaccine Delivery and Immunogenicity by Electroporation In Vivo

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DNA vaccines have been demonstrated to be potent in small animals but are less effective in primates. One limiting factor may be inefficient uptake of DNA by cells in situ. In this study, we evaluated whether cellular uptake of DNA was a significant barrier to efficient transfection in vivo and subsequent induction of immune responses. For this purpose, we used the technique of electroporation to facilitate DNA delivery in vivo. This technology was shown to substantially increase delivery of DNA to cells, resulting in increased expression and elevated immune responses. The potency of a weakly immunogenic hepatitis B surface Ag DNA vaccine was increased in mice, as seen by a more rapid onset and higher magnitude of anti-hepatitis B Abs. In addition, the immunogenicity of a potent HIV gag DNA vaccine was increased in mice, as seen by higher Ab titers, a substantial reduction in the dose of DNA required to induce an Ab response, and an increase in CD8$^+$ T cell responses. Finally, Ab responses were enhanced by electroporation against both components of a combination HIV gag and env DNA vaccine in guinea pigs and rabbits. Therefore, cellular uptake of DNA is a significant barrier to transfection in vivo, and electroporation appears able to overcome this barrier. *The Journal of Immunology,* **2000, 164: 4635–4640.**

The prospect of inducing an immune response to a protein expressed in vivo directly from an administered DNA vaccine represents an attractive alternative to other modes of vaccination. The de novo synthesis of DNA vaccine-encoded proteins mimics expression of Ags after viral infection and may improve processing and presentation to the immune system, thereby providing the advantages of live attenuated vaccines without the safety and stability concerns associated with the administration of infectious agents. Because of these potential advantages, considerable effort has been expended in evaluating this technology (for review, see Ref. 1). Early successes in demonstrating protective efficacy in small animal models have helped to drive the testing of DNA vaccines in larger animals, culminating in several human clinical trials. Thus far, however, in only a few cases have immune responses been demonstrated in humans (2–4), and the magnitude of these responses has been insubstantial. Therefore, for this technology to be effective for human vaccination, more potent forms of DNA vaccines must be identified and developed.

One reason for the lack of efficacy in larger animals may be inefficient uptake of DNA by cells in situ. Hence, we sought to test whether cellular uptake of DNA was a significant limitation to efficient transfection in vivo and subsequent induction of immune responses. To this end, we used the technique of electroporation, which is widely used in vitro to effectively introduce DNA into eukaryotic cells and bacteria. Application of short electrical pulses to the target cells permeabilizes the cell membrane, thereby facilitating DNA uptake. Recently, it has been found that applying an electric field to tissues in vivo significantly increases DNA uptake and gene expression (for review, see Ref. 5). This has been shown for reporter genes and for genes of interest for therapeutic applications, such as erythropoietin (6) and HSV-TK (7). Among the tissues targeted for in vivo electroporation have been skin (8, 9), liver (10, 11), tumors (12–14), and muscle (15). Facilitation of gene expression in vivo by electroporation of plasmid DNA has implications for both vaccine and gene therapy applications. In this study, we show that increased Ag expression after DNA injection into muscle significantly increases the potency of DNA vaccines in mice, guinea pigs, and rabbits. Therefore, this technology may prove useful at increasing the effectiveness of DNA vaccines in larger animals, such as nonhuman primates and humans.

Materials and Methods

DNA plasmids

To generate the hepatitis B surface Ag (HBsAg)[2] expression construct, the 1.4-kb *Bam*HI fragment of pAM6 (American Type Culture Collection (ATCC), Manassas, VA) was inserted into pEF-BOS, an eukaryotic expression vector containing the human elongation factor 1α promoter and first intron and the polyadenylation signal from human G-CSF cDNA in a pUC119 prokaryotic backbone (16). pAM6 (ATCC 45020) is a genomic clone of hepatitis B virus (HBV) serotype adw, and the 1.4-kb *Bam*HI fragment was shown to encode the "small" HBV surface Ag (HBsAg) (17). The luciferase expression plasmid was obtained from Promega (Madison, WI). *Escherichia coli* strain XL-1 Blue (Stratagene, La Jolla, CA), carrying the expression plasmids, was grown in LB; antibiotic selection used 50 μg/ml ampicillin. Plasmids were purified using Qiagen Endo Free Plasmid Maxi Kits (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

The plasmid pCMV HIV gag (18) was grown in *E. coli* strain HB101, purified using a Qiagen Endofree Plasmid Giga kit (Qiagen), and resuspended in 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL). The pCMV vector used contains the immediate early enhancer/promoter of cytomegalovirus and a bovine growth hormone terminator, and is described

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[2] Abbreviations used in this paper: HBsAg, hepatitis B surface Ag; TA, tibialis anterior; HBV, hepatitis B virus.

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in detail (18). 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 (19). The HIV env DNA vaccine (pCMV HIV env) contains a 2.1-kb *Eco*RI-*Xba*I fragment encoding a human tissue plasminogen activator (tPA) signal sequence and the reading frame for the ectodomain of the Env protein of the HIV-1 US4 strain codon optimized for expression in mammalian cells. The open reading frame in this construct is truncated before the transmembrane spanning region of the Env allowing high level expression of secreted gp140 protein in transfected cells (S. W. Barnett, L. Leung and H. Legg, unpublished observations).

Expression of the encoded Ags was verified by transient expression studies in B16 cells. For Lipofectin (Life Technologies/BRL) transfection, 1 μg of each plasmid DNA was used following the manufacturer's protocol; 5×10^5 cells were used per 3-cm tissue culture dish; incubation time for DNA-Lipofectin on cells was 4 h. Supernatants were harvested 36 h after removal of the DNA-Lipofectin solution and cells were lysed in 500 μl PBS/0.5% TritonX100 (Mallinckrodt). Expression of HBsAg in cell lysates and supernatants was detected by the AUSZYME enzye immunoassay (Abbott). Luciferase activity in cell lysates was detected by commercial Luciferase Reporter Gene Assay (Roche, Indianapolis, IN). Expression of HIV gag and env protein was determined as described previously (18).

Animals

Female BALB/c, BALB/c $\times$ C57BL/6 F_1 (CB6F_1), and nude mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN), Charles River Breeding Laboratories (Wilmington, MA), Taconic Farms (Germantown, NY), or The Jackson Laboratory (Bar Harbor, ME) used at 6–10 wk of age and housed at Genetronics (San Diego, CA) and Chiron (Emeryville, CA). Rabbits were obtained and housed at Josman LLC (Napa, CA). Female guinea pigs were obtained from Elm Breeding Labs (Chelmsford, MA) and housed at Chiron in an American Association of Laboratory Animal Care-accredited facility.

DNA immunization and in vivo electroporation

Mice were anesthetized using 4 parts ketamine HCl, 100 mg/ml stock solution (Fort Dodge Animal Health, Fort Dodge, IA), to 1 part xylazine, 20 mg/ml (Lloyd Labs, Shenandoah, IA). The mice received 1 μl/g of body weight intramuscularly in the posterior thigh. The skin overlying the tibialis anterior (TA) muscle was shaved, and the animals were injected with amounts of plasmid DNA as described in a volume of 50 μl. To control needle depth, a 0.3-ml insulin syringe was covered with polyethylene tubing (inside diameter, 0.38) to expose only the bevel. Two-needle array electrodes (BTX, San Diego, CA) were inserted into the muscle immediately after DNA delivery for electroporation. The distance between the electrodes was 5 mm, and the array was inserted longitudinally relative to the muscle fibers. In vivo electroporation parameters were: 20 V/mm distance between the electrodes; 50-ms pulse length; 6 pulses with reversal of polarity after 3 pulses, at 1, given by a BTX 820 square wave generator.

In rabbits, a total of 0.5 mg HIV gag DNA and 1 mg HIV env DNA in 900 μl PBS was injected into the gracilis muscles of both hind limbs after shaving and anesthesia. In guinea pigs, a total of 50 or 500 μg each of HIV gag and HIV env DNA was given as above. Electroporation was performed with a six-needle electrode array forming a circle (Genetronics, San Diego, CA). The diameter of the electrode array was 1 cm, with a needle length of 1 cm. Six electroporation pulses of 20 V/mm, 50-ms pulse length, 1 pulse/s were given by a BTX 820 square wave generator, combined with an electronic switch (Genetronics) to rotate the electric field in 60-degree increments after each discharge (20).

Measurement of Ab responses

At various times following immunization, blood was collected from anesthetized animals and serum was recovered by centrifugation. Anti-hepatitis B surface Abs were measured using the AUSAB EIA Diagnostic Kit, and quantification in milli-International Units/milliliter was done in parallel with the AUSAB Quantification Panel following instructions provided by the manufacturer (Abbott).

Anti-HIV gag Abs in mice were measured by ELISA as follows. Wells of Immulon 2 HB U-bottom microtiter plates (Dynex Technologies, Chantilly, VA) were coated with HIV p55 protein at 5 μg/ml in PBS, 50 μl/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/well blocking buffer (PBS, 0.1% Tween 20 (Sigma), 1% goat serum). Test sera were diluted 1/25 followed by serial 3-fold dilutions in blocking buffer. The block solution was aspirated; then the plates were incubated at 37°C for 2 h with 50 μl/well of each serum dilution. After six washings, the plates were incubated for 1 h at 37°C with 50 μl/well goat anti-mouse IgG-HRP (Caltag, Burlingame, CA) diluted 1/40,000 in block buffer. After a final six washes, the plates were developed with OPD for 30 min. The OPD developer consists of 1 tablet (10 mg) *o*-phenylenediamine, 12 ml buffer (0.1 M citric acid, 0.1 M dibasic sodium phosphate), and 5 μl 30% H_2O_2. The reaction was stopped with 50 μl/well 4 N H_2SO_4, and optical density was measured at dual wavelengths 492–690. The reported titers correspond to the reciprocal of the serum dilution, producing an absorbance value of 1.0. For rabbits, the ELISA procedure was as for mice with the following changes. The blocking buffer was PBS, 0.5% casein, and 5% goat serum; the dilution buffer was blocking buffer plus 0.3% Tween 20; the secondary Ab was goat anti-rabbit IgG used at 1/20,000; and the OD cutoff used was 0.6. For guinea pigs, the ELISA procedure was as for mouse except that the secondary Ab was goat anti-pig IgG used at 1/20,000.

For measurement of anti-env Abs in rabbits and guinea pigs, Nunc Immunoplate U96 Maxisorp plates (Nalge Nunc International, Rochester, NY) were coated with 200 ng/well recombinant gp120SF2 protein and incubated for at least 14 h 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 sodium phosphate, 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 3-fold. The plates were incubated for 50 min for rabbit sera or 1 h for guinea pig sera, at 37°C. After a washing in buffer containing 137 mM NaCl, 0.05% Triton X-100, the samples were then reacted with an HRP-conjugated Ab against the appropriate animal (50 min for rabbit sera; 30 min for guinea pig sera; at 37°C.) The plates were then developed using either a TMB substrate kit (Pierce, Rockford, IL) for rabbit sera (50 min at 37°C) or ABTS (Sigma), for the guinea pig sera (30 min at 37°C). The plates were stopped with either 2 N H_2SO_4 or 10% SDS, respectively, and read at wavelengths of 450 or 415 nm, respectively. Anti-env Ab responses were measured as the dilution at which an OD of 0.6 was achieved.

Measurement of T cell responses

A recombinant vaccinia virus encoding the HIV-1_{SF2} gag-pol genes (rVVgag-pol) has been described previously (21). Four weeks or more after gag DNA immunization, mice were challenged with an i.p.injection of 1×10^7 PFU rVVgag-pol. Five days later, spleens were harvested and stimulated with the H-2^d-restricted p7g gag peptide (21) and then stained for intracellular IFN-γ, as follows. Erythrocyte-depleted single-cell suspensions were prepared by treatment with Tris-buffered NH_4Cl (Sigma), and $1–2 \times 10^6$ 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, CA) was added to block cytokine secretion. After 3–5 h cells were washed, incubated with anti-CD16/32 (PharMingen) to block Fcγ receptors, stained with FITC-conjugated CD8 mAb (PharMingen), and fixed overnight at 4°C in 2% (w/v) paraformaldehyde. The following day, cells were treated with 0.5% (w/v) saponin (Sigma) and then incubated with PE-conjugated mouse IFN-γ mAb (PharMingen) in the presence of 0.1% (w/v) saponin, washed, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Results

Enhancement of DNA delivery in vivo by electroporation

It has previously been shown that in vivo electroporation can substantially increase gene expression in muscle up to 100-fold. To test whether DNA vaccine potency could be improved by this method, we used plasmid vectors encoding HBsAg, HIV env and HIV gag. A plasmid expressing HBsAg driven by the human elongation factor 1α promoter (E1-sAg) was injected into the tibialis anterior muscles of nude mice, and in one cohort pulses of electric current were applied after DNA injection. Because low voltage, long duration pulses have been found to be most efficacious for increase of DNA uptake in tissues in vivo (15, 22), we applied 6 square wave pulses of 100 V, 50-ms pulse duration. For this purpose, a two-needle electrode array with a gap of 5 mm between the needles was positioned so that the needles were centered over the DNA injection site and was inserted directly into the muscle without any surgical removal of skin. After 3 pulses, the polarity of the electric field was reversed by switching the connectors to the pulse generator. No signs of adverse reactions were observed in any

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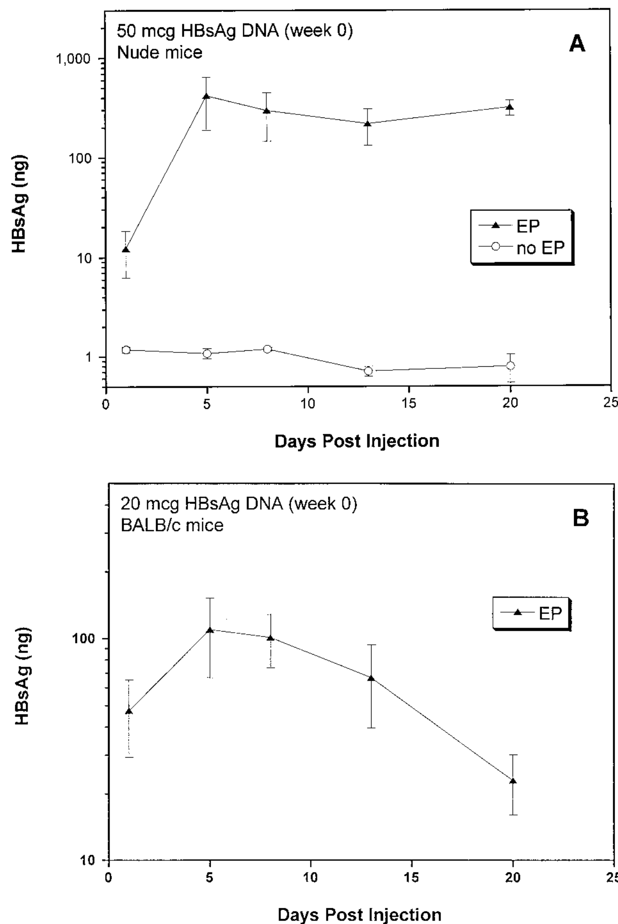

FIGURE 1. Increase in expression of HBsAg after electroporation (EP) of mouse muscle. HBsAg DNA was injected into TA muscles of nude mice (*A*) or BALB/c mice (*B*), and tissues were harvested at the indicated times after injection. Expression was measured by the AUSZYME assay and plotted as mean HBsAg (nanograms per 200 μl), where error bars represent SEM and $n = 6-8$.

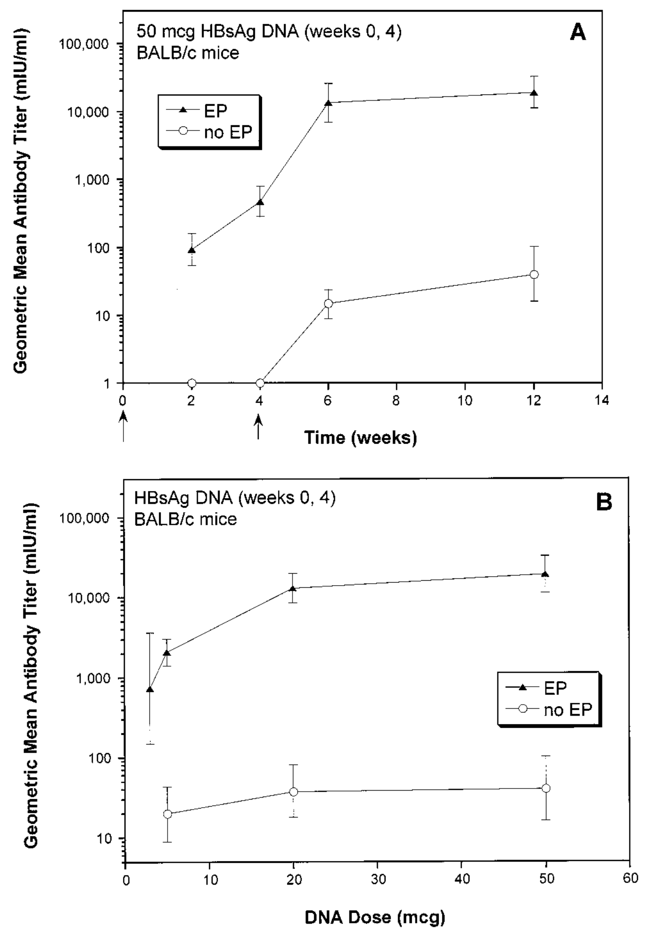

FIGURE 2. Increase in anti-HBsAg Ab titers after electroporation (EP) of mouse muscle. *A*, HBsAg DNA (50 μg) was injected into TA muscles of BALB/c mice at 0 and 4 wk (arrows), and sera were collected at the indicated times. *B*, HBsAg DNA at the indicated doses was injected into TA muscles at 0 and 4 wk, and sera were collected at 12 wk. Ab titers were determined by the AUSAB assay and plotted as geometric mean titer (milli-international units per milliliter), where error bars represent SEM and $n = 4$.

animal after muscle electroporation. Mice were sacrificed at different time points after DNA administration, as indicated in Fig. 1, and HBsAg expression was measured in serum and muscle tissues, using the AUSZYME MONOCLONAL assay (Abbott), which is an enzyme immunoassay for the detection of HBsAg in human serum or plasma. No significant HBsAg levels were detected in the sera or muscle tissues of immunodeficient nude mice receiving DNA only. In contrast, in the in vivo electroporation-treated cohort, HBsAg was detected within 1 day after DNA injection in muscle tissue. Peak levels of HBsAg were reached by day 5 and remained practically unchanged at least 20 days after DNA injection (Fig. 1*A*). No HBsAg was detected in sera of any animal, unlike observations previously reported by others (23), likely as a consequence of relatively low level expression of HBsAg by the E1-sAg plasmid compared with vectors containing the CMV promoter. In immunocompetent BALB/c mice, peak expression of HBsAg was similarly found in electroporated muscle tissues at day 5, but expression began to wane by day 13 (Fig. 1*B*), possibly as a consequence of CTL activity directed toward transfected cells or clearance of Ag by the formation of Ag-Ab complexes.

A similar increase in reporter gene expression was observed after electroporation of DNA encoding luciferase and β-galactosidase. For luciferase, expression levels were >100-fold higher in electroporated muscle tissue (~3000 relative light units vs ~500,000 relative light units). In the case of β-galactosidase, staining of muscle tissue sections revealed a substantial increase in the number of muscle fibers detectably transfected, as well as an apparent increase in the distribution of expression within the tissue (M. Dupuis, K. Denis-Mize, C. Woo, C. Goldbeck, M. J. Selby, J. B. Ulmer, J. J. Donnelly, G. Ott, and D. M. McDonald et al., unpublished observations). These results correlated with an increase in the distribution and cellular uptake of plasmid, as judged by fluorescence using a rhodamine-tagged plasmid. Therefore, electroporation of DNA-injected muscle tissues resulted in more efficient transfection of muscle cells in situ, leading to higher levels of protein production.

Enhancement of DNA vaccine potency by electroporation

To test whether in vivo electroporation could increase the magnitude of immune responses induced by DNA vaccination, several lines of experimentation were undertaken. First, BALB/c mice were immunized with the low expressing HBsAg DNA vaccine at doses ranging from 0.5 to 50 μg. Anti-hepatitis B surface Ab titers were measured using the ABBOTT AUSAB assay and expressed in standard milli-international units per milliliter serum. A level of 10 mIU/ml is considered protective against HBV infection in humans. In electroporation-treated cohorts that received 3 μg DNA

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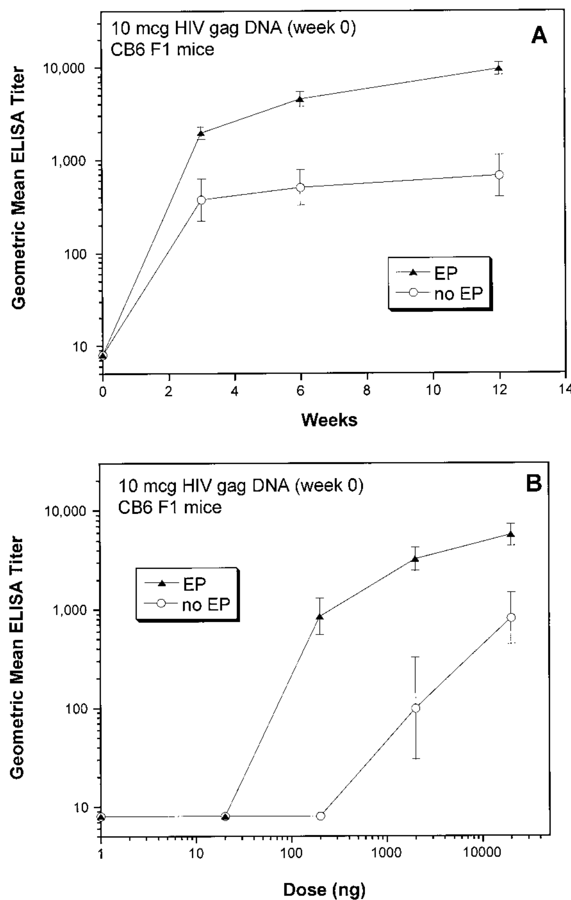

FIGURE 3. Increase in anti-HIV gag Ab titers after electroporation (EP) of mouse muscle. *A*, HIV gag DNA (10 μg) was injected into TA muscles of $CB6F_1$ mice, and sera were collected at 3, 6, and 12 wk after a single immunization. *B*, Mice were immunized with the indicated DNA doses, and sera were collected at 12 wk after a single immunization. Ab titers were determined by ELISA and plotted as geometric mean titer, where error bars represent SEM and $n = 10$.

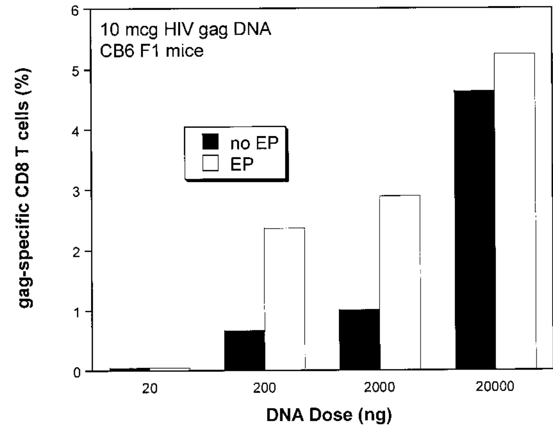

FIGURE 4. Increase in anti-gag T cell responses after electroporation (EP) of mouse muscle. HIV gag DNA was injected into TA muscles of $CB6F_1$ mice at the indicated DNA doses, and at 4 wk mice were challenged with a recombinant vaccinia virus expressing HIV gag. Five days later spleens were collected, pooled ($n = 5$) and T cell responses were measured as detailed in *Materials and Methods*. Data are plotted as percent gag-specific $CD8^+$ T cells for untreated mice (■) and electroporated mice (□).

or more, strong and consistent Ab responses were found 2 wk after the first immunization (Fig. 2). These responses were boosted to titers of >10,000 mIU/ml 2 wk after the second immunization for the high dose groups and remained stable for at least 3 mo. One microgram or less of DNA was found not be sufficient to induce reliable immune responses, whether or not animals were treated with electroporation (not shown). In contrast, mice that received 5, 20, or 50 μg HBsAg DNA without electroporation showed only weak to moderate anti-HBsAg Ab titers (up to ~30 mIU/ml), with not all animals responding even after the boost. Therefore, electroporation substantially increased the potency of a weakly immunogenic DNA vaccine.

Second, $CB6F_1$ mice were immunized with a DNA vaccine expressing high levels of HIV-1 gag, due to a potent CMV promoter with intron A and a codon-optimized gag coding region (19). This construct has previously been shown to be potent at inducing immune responses in mice and monkeys and, as shown in Figs. 3 and 4, readily primes Ab and T cell responses in mice after a single immunization of 0.2 to 2 μg of DNA. Yet, electroporation substantially enhanced these responses even further. After a single immunization of 10 μg, anti-gag Ab titers were increased up to 20-fold by electroporation, and this was maintained for at least 12 wk (Fig. 3*A*). An even greater enhancement in Ab responses was observed in BALB/c mice (>100-fold), possibly related to an overall lower Ab response to HIV gag in this mouse strain. In addition to increasing the magnitude of Ab responses, electroporation significantly lowered the dose of DNA required to induce immune responses. Strong Ab responses were seen in electroporation-treated mice after a single dose of 20–200 ng of DNA, whereas similar titers were achieved without electroporation only at a 100-fold higher DNA dose (Fig. 3*B*). The variability of the titers from animal to animal within a group appeared to be less in the electroporation-treated mice. This observation is consistent with previous reports on the consistency of expression levels after electroporation (24). Anti-gag T cell responses were also increased by electroporation. Immunized mice were challenged with a recombinant vaccinia virus expressing gag, and 5 days later spleens were removed and restimulated briefly in vitro with a known MHC class I-restricted CTL peptide. IFN-γ production by $CD8^+$ T cells, as measured by flow cytometry, indicated that up to ~5% of total $CD8^+$ T cells were gag specific (Fig. 4). At doses of gag DNA ranging from 200 ng to 20 μg, electroporation increased the frequency of gag-specific CTL. Therefore, electroporation increased the immunogenicity of DNA vaccines in mice for both Ab and T cell responses.

Third, the efficacy of the electroporation technology for DNA vaccines was tested in larger animals. Rabbits and guinea pigs, which are up to 100-fold larger than mice, were immunized with a combination of DNA vaccines encoding HIV gag and HIV env and monitored for Ab responses specific to both Ags. Rabbits were immunized with a mixture of 0.5 mg HIV gag and 1 mg HIV env DNA. Anti-env Ab responses were observed after the first DNA immunization in the electroporation-treated group, whereas such responses were detected only after two DNA immunizations without treatment (Fig. 5*A*). Thereafter, anti-env Ab titers were consistently 5- to 10-fold higher in the treated group. In those same rabbits, significant anti-gag Ab responses were seen only in animals that had received electroporation treatment (Fig. 5*B*). In the untreated DNA-vaccinated animals, little or no titers were detected at any time during the study, even after three immunizations. In guinea pigs, similar enhancement of Ab responses was seen in electroporation-treated groups. Animals were immunized with a combination of 50 or 500 μg each of HIV gag and HIV env DNA, and Ab responses were monitored after one and two immunizations. No responses were seen in any animal, treated or untreated,

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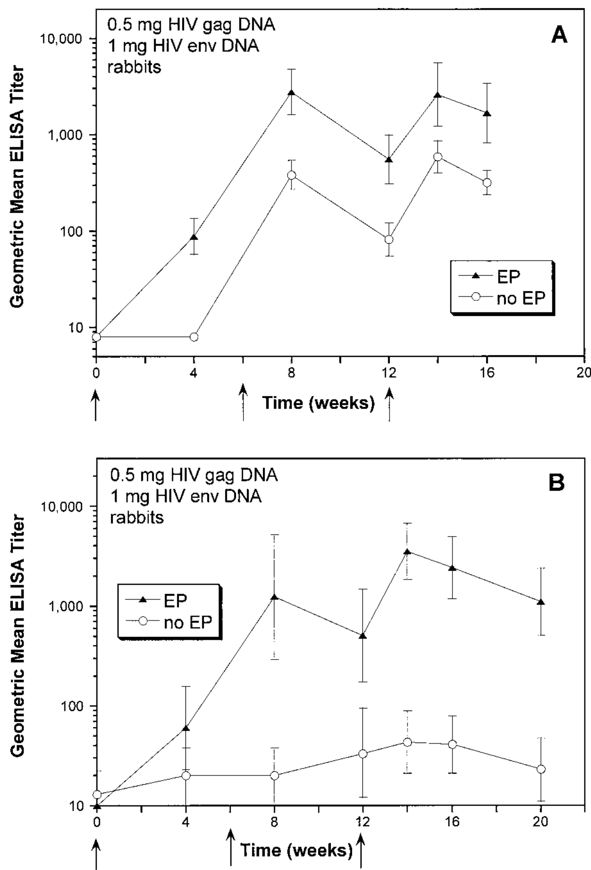

FIGURE 5. Increase in anti-HIV Ab titers after electroporation (EP) of rabbit muscle. Animals were immunized with a combination of HIV gag DNA (0.5 mg) and HIV env DNA (1 mg) at 0, 6, and 12 wk (arrows). Sera were collected at the indicated times, and Ab titers were measured by ELISA. Data are plotted as geometric mean ELISA titer for anti-env (*A*) and anti-gag (*B*) Abs, where error bars represent SEM and $n = 6$.

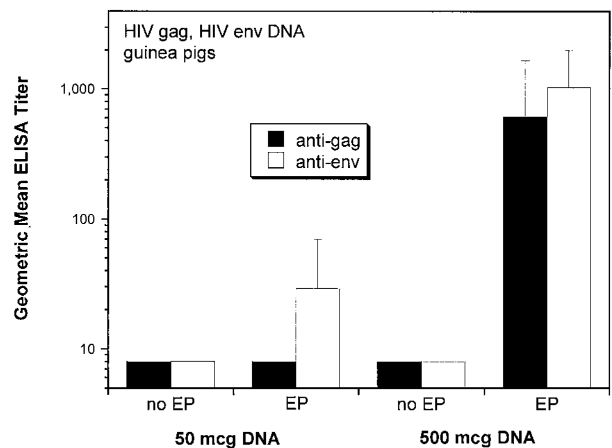

FIGURE 6. Increase in anti-HIV Ab titers after electroporation (EP) of guinea pig muscle. Animals were immunized with a combination of HIV gag DNA and HIV env DNA at doses of 50 and 500 μg each at 0 and 6 wk. Sera were collected at 9 wk, and Ab titers were measured by ELISA. Data are plotted as geometric mean ELISA titer for anti-env (□) and anti-gag (■) Abs, where error bars represent SEM and $n = 6$.

after a single immunization (data not shown). In the untreated DNA-vaccinated animals, no measurable anti-gag or anti-env Abs were detected even after two immunizations at the high DNA dose (Fig. 6). However, Ab responses against both Ags were seen in all guinea pigs immunized with the high DNA dose and treated with electroporation. Therefore, electroporation substantially enhanced the potency of DNA vaccines in mice and larger animals.

Discussion

DNA vaccination has become established as a new methodology in the prophylaxis and therapy of infectious diseases in animal models. However, although robust immune responses can readily be induced in small animals, such as mice, multiple immunizations of high DNA doses are often required to achieve modest responses in primates. To better understand what some of the potential limitations to effective transfection in vivo may be and to explore one technology for its potential to facilitate DNA vaccine delivery, we have evaluated electroporation in several animal models. Our results show that electroporation of DNA vaccines in vivo is an effective method to increase expression in muscle tissue leading to marked improvement in immune responses. Therefore, cellular uptake of DNA is a significant barrier to transfection in vivo, and electroporation appears able, at least in part, to overcome this barrier. This improvement was demonstrated in several ways. First, the potency of a weakly immunogenic HBsAg DNA vaccine was increased in mice, as seen by a more rapid onset and higher magnitude of anti-hepatitis B surface Abs. Second, the immunogenicity of a potent HIV gag DNA vaccine was increased in mice, as seen by higher Ab titers, a substantial reduction in the dose of DNA required to induce an Ab response, and an increase in $CD8^+$ T cell responses. In one previous report, induction of an immune response was detected in mice after electroporation in vivo with DNA encoding a fusion protein containing a CTL epitope from influenza nucleoprotein (9). In this case, however, DNA was applied intradermally, and no comparison was made with inoculation of DNA without electroporation. Finally, Ab responses were enhanced by electroporation against both components of a combination HIV gag and env DNA vaccine in guinea pigs and rabbits.

In this study, in vivo electroporation of muscle tissue was performed by six monopolar electric pulses using low voltage (nominal electric field of 200 V/cm), long pulse duration (50 ms) conditions. In addition to these conditions, trains of low voltage, high frequency bipolar pulses have been found to increase gene expression in muscle (6), demonstrating that the electrical stimuli can be delivered in different ways to the tissue. In vivo electroporation does differ, however, from conditions used in vitro, where high voltage, short pulse duration conditions are typically used. In vitro, electroporation pulses modify biological membranes and facilitate penetration of cells by DNA molecules, with only minor damage to these membranes (25). It is also conceivable that these conditions can affect the integrity of the nuclear membrane, thereby allowing freer passage of plasmid DNA into the nucleus. This may be particularly relevant for delivery of DNA into the nuclei of nondividing cells, such as muscle cells.

The means by which electroporation increases DNA vaccine potency is not yet known, but is likely to be related to increased expression of encoded Ag. This could simply provide more mass of Ag available for priming of immune responses. However, other factors may also be involved. For instance, whereas transfection of muscle cells is increased by electroporation, transfection of other cells, such as APCs, may also be facilitated. However, we have not detected any transfected APCs in muscle or draining lymph node after injection of DNA with or without electroporation (M. Dupuis, K. Denis-Mize, C. Woo, C. Goldbeck, M. J. Selby, J. B. Ulmer, J. J. Donnelly, G. Ott, and D. M. McDonald, unpublished observations). Another possibility is that the application of an electric field directly in the tissue could result in an inflammatory response that

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aids in the priming of immune responses against the DNA-encoded Ags. However, electroporation treatment after vaccination with protein-based vaccines did not result in an increase in immune responses (M. J. Selby, C. Goldbeck, and J. B. Ulmer, unpublished observations). Therefore, any inflammatory responses that may have been elicited by the conditions of electroporation treatment were not sufficient to alter immune priming. Hence, the observed increase in Ag expression in muscle cells likely plays a predominant role in the enhancement of DNA vaccine potency by electroporation. This hypothesis is consistent with previous observations that production of Ag by non-APCs, such as tumor cells (26), fibroblasts (27), and muscle cells (28, 29), is sufficient for priming immune responses.

DNA vaccines hold promise for use in humans. However, there are significant limitations with current technologies that have prevented the full effectiveness of DNA vaccines in larger animals. We have shown here that uptake of DNA and delivery to the cytoplasm is a barrier to efficient transfection of cells in vivo and that electroporation can circumvent this barrier. In vivo electroporation substantially increases DNA delivery and DNA vaccine potency, appears to be well tolerated by the animals, and is a simple technique that takes only a few seconds after inoculation. Electroporation has already been demonstrated to substantially increase the effectiveness of nonviral gene therapy in vivo, and our results extend the usefulness of this methodology to the field of DNA vaccination.

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