Increased DNA Vaccine Delivery and Immunogenicity by Electroporation In Vivo

Georg Widera, Melissa Austin, Dietmar Rabussay, Cheryl Goldbeck, Susan W. Barnett, Minchao Chen, Louisa Leung, Gillis R. Otten, Kent Thudium, Mark J. Selby and Jeffrey B. Ulmer

*J Immunol* 2000; 164:4635-4640; doi: 10.4049/jimmunol.164.9.4635
http://www.jimmunol.org/content/164/9/4635

**References**
This article cites 29 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/164/9/4635.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Increased DNA Vaccine Delivery and Immunogenicity by Electroporation In Vivo


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 BamHI fragment of pAM6 (American Type Culture Collection (ATCC), Manassas, VA) was inserted into pEF-BOS, an eukaryotic expression vector containing the human elongation factor 1\(\alpha\) promoter and first intron and the polyadenylation signal from human G-CSF cDNA in a pUC19 prokaryotic backbone (16). pAM6 (ATCC 45020) is a genomic clone of hepatitis B virus (HBV) serotype adw, and the 1.4-kb BamHI 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 \(\mu\)g/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

\(^{a}\) Vaccines Research, Chiron Corporation, Emeryville, CA 94608; and \(^{b}\) Genetronics, Inc., San Diego, CA 92121

Received for publication December 10, 1999. Accepted for publication February 24, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^{1}\) Address correspondence and reprint requests to Dr. Jeffrey B. Ulmer, Vaccines Research, Chiron Corporation, 4560 Horton Street, Mail Stop 4-3, Emeryville, CA 94608. E-mail address: jeffrey-ulmer@cc.chiron.com

\(^{2}\) Abbreviations used in this paper: HBsAg, hepatitis B surface Ag; TA, tibialis anterior; HBV, hepatitis B virus.
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 EcoRI-XbaI fragment encoding a human tissue plasminogen activator (PA) signal sequence and the reading frame for the envelope 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. DNA immunization and in vivo electroporation

Animals
Female BALB/c, BALB/c × C57BL/6 F1 (CB6F1), and nude mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN), Charles River Breeding Laboratories (Wilmington, MA), Tacoins Farms (Germantown, NY), or The Jackson Laboratory (Bar Harbor, ME) used at 6–10 wk of age. Female BALB/c, BALB/c3 genetic background, and housed at Genetronics (San Diego, CA) and Chiron (Emeryville, CA). Rabbits were obtained and housed at Josman LLC (Napa, CA) 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 μg 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-Lipofectin 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 the hair and opening the skin. A total of 300 μl were used for 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 Ab 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 1:250 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 devel- oped using 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% H2O2. The reaction was stopped with 50 μl/well 4% H2O2, and optical density was measured at dual wavelengths 492–690. The reported titters 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 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 a 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 guinea pig sera (30 min at 37°C). The plates were stopped with 2 N H2SO4 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.

Efficacy of Electroporation in Vivo on DNA Vaccines

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 waves of 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
animal after muscle electroproportion. 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. 1A). 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. 1B), 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 electroproportion 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, electroproportion 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 electroproportion

To test whether in vivo electroproportion 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. A level of 10 mIU/ml is considered protective against HBV infection in humans. In electroproportion-treated cohorts that received 3 µg DNA
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, CB6F1 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. 3A). 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. 3B). 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).

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. 5A). 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. 5B). 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.
The potency of 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...
Efficacy of Electroporation in Vivo on DNA Vaccines

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.

Acknowledgments

We thank Dr. Jan zur Megede for providing the HIV DNA vaccine constructs and acknowledge the technical assistance of Susan Bleecher, Diana Atchley, and Pedro Benitez for animal husbandry.

References


Downloaded from http://www.jimmunol.org/ by guest on June 9, 2017