Enhancing B- and T-Cell Immune Response to a Hepatitis C Virus E2 DNA Vaccine by Intramuscular Electrical Gene Transfer

SILVIA ZUCCHELLI,1 STEFANIA CAPONE,1 ELENA FATTORI,1 ANTONELLA FOLGORI,1 ANNALISE DI MARCO,1 DANilo CASIMIRO,2 ADAM J. SIMON,2 RALPH LAUFER,1 NICOLA LA MONICA,1 RICCARDO CORTESE,1 AND ALFREDO NICOSIA1*

Istituto di Ricerche di Biologia Molecolare P. Angeletti, 00040 Pomezia (Rome), Italy,1 and Department of Virus and Cell Biology, Merck Research Laboratories, West Point, Pennsylvania 194862

Received 8 May 2000/Accepted 18 September 2000

We describe an improved genetic immunization strategy for eliciting a full spectrum of anti-hepatitis C virus (HCV) envelope protein E2 (E2) glycoprotein responses in mammals through electrical gene transfer (EGT) of plasmid DNA into muscle fibers. Intramuscular injection of a plasmid encoding a cross-reactive hypervariable region 1 (HVR1) peptide mimic fused at the N terminus of the E2 ectodomain, followed by electrical stimulation treatment in the form of high-frequency, low-voltage electric pulses, induced more than 10-fold-higher expression levels in the transfected mouse tissue. As a result of this substantial increment of in vivo antigen production, the humoral response induced in mice, rats, and rabbits ranged from 10- to 30-fold higher than that induced by conventional naked DNA immunization. Consequently, immune sera from EGT-treated mice displayed a broader cross-reactivity against HVR1 variants from natural isolates than sera from injected animals that were not subjected to electrical stimulation. Cellular response against E2 epitopes specific for helper and cytotoxic T cells was significantly improved by EGT. The EGT-mediated enhancement of humoral and cellular immunity is antigen independent, since comparable increases in antibody response against ciliary neurotrophic factor or specific anti-human immunodeficiency virus type 1 gag CD8+ T cells were obtained in rats and mice. Thus, the method described potentially provides a safe, low-cost treatment that may be scaled up to humans and may hold the key for future development of prophylactic or therapeutic vaccines against HCV and other infectious diseases.

Hepatitis C virus (HCV) is the major etiological agent of both community and posttransfusionally acquired non-A, non-B viral hepatitis. Approximately 70% of patients develop chronic hepatitis, of which 20 to 30% progress onto liver cirrhosis, and all cases of infection carry an increased risk of hepatocellular carcinoma (1). Presently, the only available therapies are alpha interferon (IFN-α) alone or in combination with ribavirin (17, 34, 45). Such treatments are expensive, show low-response rates, and carry the risk of significant side effects. Consequently, the development of a vaccine against hepatitis C remains a high priority goal.

The putative envelope protein E2 of HCV and, in particular, the hypervariable region 1 (HVR1) are the most variable antigenic fragments in the whole viral genome and are the target of neutralizing antibodies (7, 16, 41). Antibodies against a single E2 HVR1 are isolate specific and lead to the emergence of escape mutants during chronic infection (16, 26, 27, 52, 58, 63). Thus, the major task in developing a HCV vaccine would be to generate an immunogen that induces a highly cross-reactive anti-HVR1 response to prevent the outgrowth of escape mutants rather than require the immune system to deal with them after they arise.

A few reports support the notion that cytotoxic-T-lymphocyte (CTL) immunity plays an essential role in limiting HCV infection in humans (38, 48, 51). Similarly, an early, strong, and multispecific CTL response positively correlates with disease resolution in chimpanzees (8), the only other species susceptible to infection by HCV. Thus, an ideal HCV vaccine should have a dual function: to induce a cross-reacting humoral response to block the majority of the infecting viral quasi species and to elicit a strong CTL immunity to limit spreading of those viruses that eventually escaped antibody neutralization.

The efficacy of inducing both humoral and T-cell-mediated immune response by intramuscular or intradermal delivery of plasmids directing the expression of foreign antigens has been proven in a number of mammalian species (6, 9, 10, 15, 23, 24, 29, 53, 55). Genetic vaccination against a wide range of viral, bacterial, or parasitic antigens has been shown to induce protective immunity in several rodent preclinical models (18, 47, 55, 60). However, the paucity of successful immunization in larger animals has spurred a new wave of research activity aimed at improving delivery vehicles and vector backbones (3, 31, 37, 42). The increased efficacy of DNA immunization by plasmid formulation with adjuvants or costimulatory factors has been recently reported (for a review, see reference 54). This notwithstanding, a major limitation to developing DNA-based vaccines for human prophylaxis and therapy is still presented by the relatively low in vivo expression levels of the encoded antigens, primarily due to the progressive loss of DNA molecules along their journey from outside the cell to inside the nucleus. An effective way around this problem is to induce muscle regeneration by a necrotizing agent and then transfact regenerating fibers (59). However, though effective in animal models (13), this method has limited clinical applicability due to massive muscle necrosis.

Gene transfer mediated by electric pulses is a well-established method to achieve high levels of expression in a variety
of mammalian cells (43, 44). Efficient in vivo expression of plasmid encoded genes by electrical permeabilization has been obtained in the skin, corneal endothelium, brain, liver, and muscle (4, 22, 33, 35, 39, 40).

Recently, we and others have shown that high and prolonged expression of biologically active gene products are achieved in vivo by intramuscular injection of plasmid DNA followed by electrical stimulation of the injected fibers (14, 49). In the present work we have applied a similar strategy to enhance anti-HCV B and T-cell-immune responses induced by genetic vaccination. Using a plasmid encoding for the HCV E2 glycoprotein, we measured a substantial increase of antigen expression after electrical gene transfer (EGT) of plasmid DNA in the mouse muscle, with a resulting improvement of antibody response up to 30 times greater than naked DNA injection, and at various DNA concentrations. EGT also enhanced the efficiency of induction of HCV E2-specific CD4\(^+\) and CD8\(^+\) T cells.

### MATERIALS AND METHODS

DNA constructs and animals. Supercopied, endotoxin-free pF78E2 plasmid, encoding the chimeric version of the E2 glycoprotein of HCV (S. Zucchelli, R. Roccasecca, R. Brune, E. Tardi, F. I. S., and R. Cortese, and A. Nicosia, submitted for publication), was supplied by Basolabos (Harahan, La.). The human immunodeficiency virus type 1 (HIV-1) gag gene, from 0.5 to 50 m of plasmid DNA dissolved in 50 \(\mu\)l of PBS. The electric field was applied as previously described (49) with a few modifications. Voltage was measured across a floating current [49], we did not detect E2 expression upon injection of 5 \(\mu\)g of DNA in the quadriceps of BALB/c mice (data not shown). However, due to the increased overall resistance (about 4,500 \(\Omega\) at 1 kHz), a much lower current was measured in the tissue with respect to the previous protocol (20 \(\mu\)A of actual current going through the muscle, 50 mA yielded E2 expression levels equivalent to those obtained from the procedure with surgical intervention (data not shown). In a control experiment, EGT treatment was performed under the same conditions 1 min before plasmid DNA injection.

Rats were electrically stimulated as previously described (33). For scaling up in rabbits we constructed two needle electrodes (5 mm apart) connected with a syringe and with the electric-pulse delivery system. The needle electrodes were inserted through the skin into the quadriceps muscle at 5 cm from the electrical pulses were delivered at the same site (50 V, 100 mA) within 5 s of the injection.

### E2 protein expression in mouse muscle. Mice were injected with 5 \(\mu\)g of the pF78E2 plasmid with or without EGT treatment. Mock-injected mice were used as a control. At different times after injection, animals were sacrificed and quadriceps muscles were removed and kept in liquid nitrogen. Extracts were prepared by homogenization (Polytron Kinematica AG Littau-Luzern) of muscle in 1 ml of lysing buffer (catalog number 9581902; Promega Corporation, Madison, Wis.) and three cycles of freeze-thawing. Soluble proteins were recovered in the supernatant after centrifugation. Protein content was determined by using a Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany).

### Difference in the optical density at 405 nm (OD\(_{405}\)) and the OD\(_{620}\) by an automated ELISA reader (Labsystems Multiskan Bichromatic, Helsinki, Finland).

ELISAs. ELISAs with recombinant E2 were performed by aliquoting 1 \(\mu\)g of GNA (catalog number A8025; Sigma) per well in PBS into 96-well Nunc ELISA plates and incubating them overnight at 4°C. Plates were washed with washing buffer and incubated for 1 h at 37°C with 250 \(\mu\)l of blocking buffer (2% BSA, 1\% PBS, 0.05% Tween 20) per well at 50 mA yielded E2 expression levels equivalent to those obtained from the procedure with surgical intervention (data not shown). In a control experiment, EGT treatment was performed under the same conditions 1 min before plasmid DNA injection.

Rats were electrically stimulated as previously described (33). For scaling up in rabbits we constructed two needle electrodes (5 mm apart) connected with a syringe and with the electric-pulse delivery system. The needle electrodes were inserted through the skin into the quadriceps muscle at 5 cm from the

### ELECTRONIC GENE TRANSFER OF PLASMID DNA VACCINES

VOL. 74, 2000

ELECTRONIC GENE TRANSFER OF PLASMID DNA VACCINES

11599
anti-mouse IFN-γ antibody (catalog number 18181D; Pharmingen, San Diego, Calif.). After overnight incubation, plates were washed with 1× PBS-0.005% Tween and blocked with 250 μl of R10 medium per well. Spleenocytes from immunized mice were prepared and incubated for 24 h in the presence or absence of 10 μM peptide at a density of 2.5 × 10^5 or 5 × 10^5/well. After extensive washing (1× PBS, 0.005% Tween), biotinylated rat anti-mouse IFN-γ antibody (catalog number 18112D; Pharmingen) was added and incubated overnight at 4°C. For development, streptavidin-alkaline phosphatase (catalog number 13043E; Pharmingen) and 1-step NBT-BCIP Development Solution (catalog number 34042; Pierce, Rockford, Ill.) were added. A pool of 20mer overlapping peptides encompassing the entire sequence of an HCV E2 protein from a viral genotype strain H (E2 pool; from amino acids [aa] 317 to 700) was used to reveal HCV-specific IFN-γ-secreting T cells. Synthetic peptides containing a CD4+ (pep1303; from aa 391 to 410 of the F78 minitope sequence) and a CD8+ epitope (pep1323, from aa 591 to 610 of the HCV strain H polyprotein) were previously identified (S. Capone and A. Folgori, unpublished data). The HIV-1 gag peptide (from aa 197 to 205) (32) was used to detect specific IFN-γ-secreting CD8+ T cells.

**Cytotoxicity assay.** p815 cells (ATCC TIB64) expressing the H-2b class I molecule were maintained in culture in R10 medium at 37°C and 5% CO₂ and then used as target cells for cytotoxicity assay. Spleen cells from immunized animals were cultured to amplify effector CTLs. A total of 4 × 10^4 spleen cells were resuspended in R10 supplemented with 5 μg of CD³⁺-specific peptide (pep1323) and 10 μg of recombinant human interleukin-2 (catalog number 799068; Boehringer Mannheim GmbH, Mannheim, Germany) per ml and plated in quadruplicate in 24-well flat-bottom plates (Multiwell; Costar, Cambridge, Mass.). After 7 days of in vitro restimulation, cultures were assayed for cytotoxic activity in a standard 51Cr release assay. Briefly, target cells were labeled with Na⁵¹CrO₄ (catalog number CSJ1; Amersham Pharmacia Biotech, Buckinghamshire, England) and pulsed with either pep1323 or medium alone for 2 h at 37°C and 5% CO₂. After an extensive washing with R10 medium, target cells were mixed with CTLs at designed effector/target ratios in 96-well round bottom plates (Cell Wells, catalog number 25850; Corning Glass Works, Corning, N.Y.) and incubated for 4 h at 37°C and 5% CO₂. Next, 50 μl samples of supernatants were transferred to a LuminoPlate-96 (catalog number 6005164; Packard Instrument Company, Meriden, Conn.) allowed to dry overnight, and then counted (Top Count, Microplate Scintillation Counter; Packard Instrument Company) to determine the amount of ⁵¹Cr released in each well. The percentage of specific lysis was calculated by using the following formula: percent specific lysis = (experimental release - spontaneous release)/(maximum release - spontaneous release × 100), where the spontaneous release represents the number of counts in the presence of medium only and the maximum release represents the number of counts measured in the 1% Triton X-100 target cell lysate.

**Statistical analysis.** Throughout this study, a two-sample t test assuming equal variances was employed from the data analysis “plug-in” of Microsoft Excel 97. This enabled not only determination of the statistical significance of the cohort data (by analyzing the natural log of individual antibody titers) but also definition of the 95% confidence interval of the ratio of geometric means of differing cohorts of animals (e.g., comparing the fold enhancement after one immunization with the secreted alkaline phosphatase gene cloned in the same plasmid vector used in the present study measured the ratio between the level of protein expression with EGT and the level of protein expression without EGT to be in the range of 20 to 100 in mice and rats (50). These findings support the minimum estimate of a 10-fold increase in E2 gene expression induced by EGT shown in Fig. 1. The enhanced gene expression is probably due to improved vector transfection, since no E2 expression could be observed in animals treated with EGT and injected immediately afterward with the plasmid DNA solution (data not shown).

**EGT increases the humoral response in pF78E2-immunized mice.** We reasoned that the large increase in EGT-driven gene expression could result in a stronger stimulus to the immune system, leading to a more efficient response. Therefore, we determined the seroconversion rates in mice injected with 0.5, 5, and 50 μg of pF78E2, followed by EGT. Control mice received the same amount of DNA but were not treated. At the highest dose, anti-E2 antibodies were measured in both groups of mice, but all EGT-treated animals seroconverted, while only 7 of 10 sera from the untreated group showed a positive reaction (Fig. 2A, lower panel). At the intermediate dose, only two mice (animals 34 and 39) developed a measurable though very weak response in the untreated group. In contrast, all mice receiving EGT showed strong anti-E2 reactivity, as indicated by ELISA (Fig. 2A, upper panel). At the lowest dose, only two mice (animals 5 and 11) developed a detectable antibody response (data not shown). Based on these findings, we chose to inject 50 μg of the same plasmid to confirm peak levels observed in the 5-μg time course. The horizontal axis indicates the number of days postinjection (p.i.). For each time point, extracts from three injected mice were prepared and E2 expression levels were determined by ELISA with an anti-E2 MAb. Average values (k⁺/k⁻) from two replications were determined and normalized over the total amount of extracted proteins as reported on the vertical axis (OD/milligram of total proteins). Error bars represent the standard deviation values. E2 expression was measured in mice injected with 50 μg of pF78E2 plasmid with EGT (5 μg + EGT), 50 μg of pF78E2 plasmid without EGT (5 μg − EGT), 5 μg of pF78E2 plasmid with EGT (5 μg + EGT), 5 μg of pF78E2 plasmid without EGT (5 μg − EGT), or PBS alone (C, mock).
contrast, EGT led to a significant increase in seroconversion frequency, with 90% of the sera of this immunization group displaying a good reactivity to E2 (Fig. 2A, middle panel). None of the animals injected with the 0.5-μg dose developed antibodies against the E2 protein (Fig. 2A, upper panel). All mice were given a booster immunization 3 weeks after priming. The analysis of post-dose 2 sera confirmed the efficacy of EGT in increasing humoral response. The major difference was observed between the 5-μg DNA-EGT immunization group, which displayed 100% seroconversion and strong reactivity against E2 in all sera, and the corresponding control group, among which only 4 of 10 mice developed a weak response (Fig. 2B, middle panel). A less-pronounced difference was observed at the higher dosage, since untreated mice showed an increased frequency of seroconversion (80%) after boosting, thus approaching the efficiency observed in EGT-treated mice who developed an antibody response in 100% of the cases (Fig. 2B, lower panel). Finally, one mouse in the 0.5-μg DNA-EGT immunization group developed a response after the second injection (Fig. 2B, upper panel). Further experiments performed by immunizing mice (in groups of 10) with two doses of 1 and 2 μg of DNA led to 70 and 90% seroconversion, respectively, in mice treated with EGT, while only 10 and 40% of the animals in the corresponding untreated groups developed anti-E2 antibodies (data not shown).

To quantify the effect of EGT on the B-cell response to the pF78E2 vaccine, anti-E2 antibody titers from individual animals were determined by fitting the OD405 measurements of threefold serial dilutions of sera to the Michaelis-Menten equation as described in Materials and Methods. Titers of individual animals and cohort geometric mean titers (GMTs) for the animals injected with 50 or 5 μg are reported in Fig. 3. After a single DNA injection of 50 μg, no statistically significant difference was observed between treated and untreated animals, which developed anti-E2 antibody titers of 590 and 260, respectively. After boosting, a potent enhancement of the humoral response was observed in 1EGT mice with a cohort GMT of 6500, whereas the 2EGT cohort measured a GMT of 650. This boosting of the 1EGT animals corresponds to anti-E2 titers 11-fold higher than those measured in the post-dose 1 sera (with an enhancement factor ranging from 3.8 to 32.7 at the 95% confidence level). Moreover, EGT resulted in a 10-fold increase (3.4- to 29.6-fold, 95% confidence interval) in specific antibody titers compared to conventional intramuscular needle injection.

Significant enhancement of the B-cell response by EGT was
also observed at the 5 μg DNA dose. In this case, pF78E2 injection without electrical treatment was poorly immunogenic, as shown by seroconversion rates. After EGT, antibody titers were significantly elevated (GMT = 370) above the non-EGT levels (GMT = 60) after the first injection, showing an initial enhancement of roughly sixfold (2.0- to 17-fold, 95% confidence interval). After boosting, the EGT cohort GMT increased significantly (GMT = 3,700), whereas the non-EGT-treated cohort did not (GMT = 130). Thus, EGT-treated mice developed anti-E2 titers nearly 28-fold higher (9.7- to 83.1-fold, 95% confidence interval) than untreated animals (Fig. 3). In fact, only 6 HVR1 peptides were recognized by the −EGT sera, while 15 different HVR1 sequences reacted with the pool of sera from electrically stimulated mice. In most cases, HVR1

FIG. 3. EGT increases antibody titers of pF78E2 injected mice. Individual titers against recombinant E2 were measured by ELISA on each serum from EGT-treated (+ EGT) and untreated (−EGT) animals after one (pd1, △) or two (pd2, ○) intramuscular injections of 5 or 50 μg of pF78E2 plasmid. The doses were administered 3 weeks apart. Individual titers (all open symbols) and cohort GMTs (■) from EGT-treated and untreated animals are reported. The P values are shown between groups which gave statistically significant differences. The lowest serum dilution tested was 1:100. One animal from the +EGT group given 5 μg and one animal from the −EGT group given 50 μg were not given the booster injection (indicated by arrows).

Similar responses were obtained in a follow-up experiment, where groups of two rabbits were immunized with 0.3 mg of pF78E2 plasmid with or without EGT (data not shown).

EGT-treated mice display broader cross-reactivity against different natural HVR1 variants. To overcome the problem of HVR1 variability, we previously selected cross-reactive antigenic and immunogenic HVR1 peptide mimics (mimotopes [46]). Plasmids encoding some of these mimotopes fused at the N terminus of the E2 ectodomain (including the pF78E2 construct used in this study) elicited cross-reacting anti-HVR1 antibodies in mice and rabbits (Zucchelli et al., submitted). However, the level of cross-reactivity induced by DNA immunization was lower than that obtained by peptide immunization, possibly reflecting the lower efficiency of DNA immunization. We reasoned that the higher efficiency of humoral response induction by EGT could lead to an increase in anti-HVR1 cross-reactivity by the pF78E2 DNA. The cross-reactivity of post-dose 2 sera from mice immunized with 50 μg of pF78E2 with or without EGT was tested by ELISA using a panel of 43 synthetic peptides reproducing the HVR1 sequences of natural isolates. These peptides are a representative set of the known viral variability in this region and were derived from a survey of more than 200 unique HVR1 sequences available in the database (46). Sera from responding animals of each immunization group were pooled and used for this analysis. The level of cross-reactivity induced by pF78E2 injection in EGT-treated mice was significantly higher than that obtained in the control animals (Fig. 5).

FIG. 4. Enhanced antibody response in EGT-treated rabbits immunized with pF78E2. Individual animal anti-E2 antibody titers in sera from rabbits immunized with the 3-mg dose of the pF78E2 plasmid were determined by ELISA on serum samples collected 3 weeks after the first (pd1, △) or the second (pd2, ○) injection. The doses were administered 4 weeks apart. Cohort GMTs are shown (■), and P values are shown above the bars connecting the correlates. The lowest serum dilution tested was 1:100. Samples that did not seroconvert were assigned an endpoint titer of 50. One animal from each immunization group was not given the booster injection (indicated by arrows).
peptides that were recognized by sera from both immunization groups displayed a stronger signal with the +EGT pool of sera (Fig. 5 and data not shown). A similar difference in cross-reactivity patterns was also observed between the post-dose 2 sera from EGT-treated and untreated animals injected with 5 μg of DNA (data not shown).

Enhancement of cell-mediated immunity by muscle EGT. We investigated the ability of EGT to enhance cell-mediated immunity. New groups of BALB/c mice were injected with 5 or 50 μg of pF78E2, and the induction of E2-specific T-cell response was analyzed by the quantitative ELISPOT assay measuring the number of IFN-γ-secreting T cells in response to a pool of overlapping peptides encompassing the E2 ectodomain coding sequence. Only modest T-cell responses were measured by this assay in mice receiving the lower DNA dose (data not shown), while more significant reactivity to the HCV E2 peptide pool was observed in animals injected with 50 μg of pF78E2 (Fig. 6A). In this case, the number of IFN-γ-secreting E2-specific T cells was significantly higher (ca. threefold) in the +EGT immunization group \( (P < 10^{-5}) \). To verify whether the EGT enhancement of cell-mediated immunity was effective on different T-cell subsets, the same groups of mice were analyzed by ELISPOT using two synthetic peptides reproducing previously identified E2-specific CD4+ and CD8+ epitopes for BALB/c mice (pep1303 and pep1323; S. Capone and A. Folgori, unpublished data). Data obtained with the CD4+ -specific peptide closely mirrored those observed when the complete pool was used. Two of six animals in the −EGT group showed a weak response to the CD8+ peptide (about 2 times the background level observed in the absence of the specific peptide), while four mice in the +EGT immunization group developed a CD8+ T-cell reactivity. In two of the latter animals (mice 9 and 13) the number of E2-specific CD8+ precursors was 1 order of magnitude higher than that of the responding animals in the untreated group (Fig. 6A), E2-specific CD8+ T cells were tested for effector function, and a corresponding
increase in specific cytotoxic activity was measured in EGT-treated animals (Fig. 6B and data not shown).

We then tested whether the EGT-mediated enhancement of cellular immunity is antigen independent. To this end, a plasmid encoding HIV-1 gag was used to immunize BALB/c mice with or without EGT. Induction of HIV-1 gag-specific CD8+ T cells was evaluated by ELISPOT using a peptide reproducing a characterized CTL epitope for BALB/c (32). A single injection of 5 μg of plasmid DNA was able to induce HIV-1 gag-specific CD8+ T cells (70 ± 32 spot-forming cells [SFC]/10^6 cells) in untreated mice. EGT led to a ca. threefold-increased response (186 ± 57 SFC/10^6 cells; Fig. 7). Delivery of a second DNA dose was almost ineffective in untreated animals (P = 0.1), whereas a significant boosting effect was observed in electroporated mice, where on average a fourfold enhancement of the CTL response (P = 0.0002) was obtained, with a final frequency of 1 HIV-1 gag-specific CD8+ T cell in 3,000 splenocytes (Fig. 7).

**DISCUSSION**

The rationale for choosing the genetic immunization approach for the development of an E2-based anti-HCV vaccine strategy is twofold: (i) to exploit the cell machinery for correct folding and posttranslational modification of the antigen which is essential for inducing antibodies against native viral structures and (ii) to induce a specific T-cell response. Toward this goal, we have engineered highly cross-reactive antigenic and immunogenic HVR1 mimotopes (46) into DNA plasmids able to induce anti-E2 antibodies upon intramuscular injection (Zucchelli et al., submitted). However, previous studies performed in mice and rabbits using synthetic peptides as immunogens led to higher immunization levels, possibly reflecting the lower efficiency of DNA immunization with respect to peptide immunization (46). In the present work we have approached this problem by developing an EGT protocol that can substantially increase the level of immunization by intramuscular injection of plasmid DNA.

We have previously shown that electrical treatment in the form of high-frequency, low-voltage electric pulses can significantly increase the in vivo production of biologically active recombinant erythropoietin and secreted alkaline phosphatase upon plasmid DNA injection in the mouse skeletal muscle (49, 50). The rationale behind the EGT approach to potentiating DNA vaccination against HCV E2 was to increase gene expression, assuming that this is one of the major limiting factors of naked DNA immunization. HCV E2 expression at least 10 times higher than background levels was obtained by EGT of 5 μg of plasmid DNA in the quadriceps muscle of BALB/c mice. This 10-fold increase of E2 in vivo gene expression is likely to be an underestimation, since no expression could be observed after simple naked DNA injection of 10 times more plasmid. These data are in good agreement with those previously obtained by performing similar experiments with a plasmid encoding different proteins (1, 35, 49, 50). It should be noted that while many of the characteristics of the electrical stimulation treatment were maintained (shape, amplitude, frequency and number of pulses and trains), the actual current settings used and voltages applied in the present study were different from those adopted in our previous report. Nonetheless, highly comparable data were obtained in terms of the fold increase in gene expression. This testifies to the flexibility of the EGT approach, which we believe can be adapted to different experimental settings without loss of efficacy. This conclusion is further supported by other published data, as well as our own experience on the successful use of EGT for improving DNA transduction of skeletal muscle fibers in different species (14, 35; E. Fattori, unpublished data). The EGT-mediated improvement of HCV E2 in vivo expression may be due to better transfection of muscle fibers, since no E2 synthesis was detected by inverting the order of electrical stimulation and DNA injection. Monitoring E2 expression over time measured significant protein levels already after 16 h from the injection, which continued to increase in a linear fashion and peaked around day 8. After this time point the amount of antigen detected in the muscle progressively diminished to reach background levels after 3 weeks, presumably because of the immune-mediated destruction of transfected muscle cells (11).

The major goal of this study was to improve the efficiency of HCV gene vaccination. In fact, a significant difference in the seroconversion frequency was observed between animals subjected to EGT and untreated animals. A second DNA dose was administered 3 weeks after the first injection, when the amount of E2 present in the muscle of EGT-treated mice becomes almost undetectable, in order to assess the contribution of newly synthesized antigen to the anamnestic response. Even after the booster injection, only a modest or suboptimal seroconversion frequency was observed in the untreated animals immunized with either 5 or 50 μg of DNA. In contrast, all EGT-treated animals showed a robust response against E2. The dose-response profile of the seroconversion frequency revealed that with the pH75E2 plasmid in BALB/c mice, the 5 μg dose represents a threshold below which an antibody response is not developed by all animals. However, when lower amounts of DNA were injected (2 and 1 μg), a marginally significant difference (P < 0.061 as determined by adjusted χ² analysis) in the number of responding animals between EGT-treated and untreated mice was still observed, and even at the 0.5-μg dose one animal in the EGT immunization group developed specific antibodies. Altogether, these data indicate that in this experi-
mental setting EGT can improve HCV E2 genetic vaccination by more than 1 order of magnitude. This higher immunization efficiency mediated by EGT is also reflected by a significant increase in antibody titers. As a result of this increase in anti-E2 antibody titers, a substantial improvement in the extent of anti-HVR1 cross-reactivity was obtained in EGT-treated versus untreated mice, in that the number of peptides reproducing the HVR1 of natural viral isolates recognized by sera from electrically stimulated animals was more than doubled. Furthermore, the level of cross-reactivity displayed by sera from EGT-treated BALB/c mice immunized with only two doses of 50 μg of the pF78E2 plasmid was comparable to that previously obtained by conventional naked DNA injection of four 100-μg doses of the same plasmid in the same mouse strain (35 and 28% of positive peptides, respectively [Zucchelli et al., submitted]).

As a first step toward assessing the feasibility of this approach for human vaccination, we tested the EGT protocol in larger animals than mice and showed that it can improve genetic immunization in rats and rabbits. In the latter species, EGT dramatically improved immune responses, as evidenced by the injection of two doses of 0.3 mg of pF78E2 (corresponding to ca. 100 μg/kg), followed by EGT treatment, that induced antibody titers 10-fold higher than those obtained by injecting 10 times more DNA in the absence of electrical stimulation. These data are particularly important in light of what can be envisaged as the most reasonable DNA dosage for human vaccination, which should be in the 20- to 200-μg/kg range, for both safety and economical reasons.

In the case of the HCV E2 plasmid immunization, the EGT-mediated enhancement of humoral response was observed over a variety of DNA doses in both mice and rabbits. However, it appears that after multiple injections antibody titers reach a plateau and cannot be further improved. This phenomenon of immune response "saturation" was most evident in mice at the higher DNA dose, where similar antibody titers were induced after one injection with or without EGT. On the other hand, it would appear that this phenomenon is specific for the plasmid DNA delivery mode of gene vaccination, since viral delivery of the same E2 expression cassette results in 10-fold-higher antibody titers (A. Folgori, unpublished data).

To ascertain whether EGT is also effective in increasing anti-E2 cellular response, we measured the number of antigen-specific T-cell precursors by quantitative IFN-γ ELISPOT assay. The most important result of these experiments was the finding that induction of both CD4+ and CD8+ T cells specific for HCV E2 epitopes was significantly enhanced by EGT, leading to the conclusion that this method can be used to improve genetic vaccination for eliciting a full spectrum of immune responses. HCV E2 DNA was previously shown to be a poor immunogen when delivered intramuscularly in several mouse strains (30). Also in the present study, only a weak CD8+ response was induced in BALB/c mice that were not subjected to EGT treatment. In contrast, both the frequency of responding animals and the number of CD8+ precursors specific for the 1323 peptide were increased by the EGT procedure. Relevant to a vaccine application is the finding that a corresponding improvement in effector function was observed when these CD8+ T cells were assayed for their ability to induce lysis of peptide-pulsed target cells.

By using a plasmid encoding for HIV-1 gag, we provided additional evidence of the efficacy of EGT in enhancing cell-mediated immunity and confirmed that the described procedure can be used for different antigens. Demonstration of a strong increase in HIV-1 gag CD8+ T cells in the spleen of EGT-treated mice further supports the conclusion that this approach may hold the key for the future development of genetic vaccines against a wide spectrum of human pathogens requiring both arms of the immune system to be efficiently activated.

While preparing this manuscript, we became aware of similar results obtained by in vivo electroporation of plasmids encoding the HBV surface antigen and HIV-1 env and gag proteins (64). Even though the electrical conditions used in the present study were different, our data confirm and expand on these observations. However, in contrast to the findings of these authors, we already obtained an EGT-mediated increase of HIV-1 gag-specific CD8+ T cells after a single DNA injection without boosting the response by vaccinia virus encoding gag. We chose to do so to avoid any bias on the assessment of the efficiency of DNA immunization that could result from either the antigen-specific or the inflammation stimuli delivered by the viral vector.

What is the basis for EGT-mediated enhancement of the immune response? Our initial hypothesis was that the naked DNA transduction of muscle fibers is a limiting factor that EGT improves on. This interpretation is supported by the good correlation between the amount of gene product measured in the transduced muscle tissue and the strength of the humoral response. However, additional factors may contribute to the increased DNA vaccination efficacy by EGT. Relevant to this point is the observation that one hundred times more DNA molecules are found in the inguinal lymph nodes of EGT-treated than in untreated mice 1 week after plasmid DNA injection (S. Zucchelli et al., unpublished data). This finding reflects the higher efficiency of transfection by EGT, which may lead not only to more muscle cells being transduced but also to better targeting of other cell types, including bystander professional antigen-presenting cells. Finally, we cannot exclude that the low and transient tissue damage induced by muscle cell electrical treatment (49) might contribute to the extent of the response as a result of local inflammation, providing for a more proficient immunological milieu.

The method described in this work is likely based on the transient modification of certain physical properties of the target cells that presumably enable the injected DNA molecules to better penetrate the nuclei. Therefore, it is predictable that EGT may produce an additive enhancement if used in conjunction with other means of improving genetic vaccination, such as adjuvants or costimulatory molecules (20, 25, 28, 56, 61), by employing more-efficient vectors such as self-replicating genomes (5, 21), or by adopting different injection devices (19, 57).

ACKNOWLEDGMENTS

We thank Immacolata Zampaglione and Patrizia Costa for help in animal immunizations, Anna De Martis for sharing unpublished results, and Daniela Frasca for technical advice. We are grateful to Timothy Schofield for providing insightful guidance on the statistical analysis. We also thank Iacob Mathiesen for helpful discussions and Janet Clench for critical reading of the manuscript.

REFERENCES


