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

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We describe an improved genetic immunization strategy for eliciting a full spectrum of anti-hepatitis C virus (HCV) envelope 2 (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 in 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 transfect 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

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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. Supercoiled, endotoxin-free pF78E2 plasmid, encoding the chimeric version of the E2 glycoprotein of HCV (S. Zucchelli, R. Roccasecca, A. Meola, B. Bruni Ercole, R. Tafi, J. Dubuisson, G. Galfré, R. Cortese, and A. Nicosia, submitted for publication), was supplied by BayouBiolabs (Harahan, La.). The human immunodeficiency virus type 1 (HIV-1) gag plasmid encoded the full-length HIV-1 gag gene under the control of the cytomegalovirus promoter-enhancer sequence. Four- to six-week-old female BALB/c mice (Charles River, Como, Italy) were used for immunizations. Mice received from 0.5 to 50 μg of plasmid DNA dissolved in 50 μl of 1× phosphate-buffered saline (PBS) into the left quadriceps muscle via insulin syringe (catalog number 329430; 0.03-ml Insulin Syringe Micro-Fine IV Needle, U-100 28G1/2; Becton Dickinson, Franklin Lakes, N.J.). Optionally, a booster immunization was performed on the opposite leg 3 weeks after the first injection. Sera were collected 2 weeks after each injection.

CD rats received a single dose of 200 μg of pF78E2 plasmid DNA or of a plasmid encoding the human ciliary neurotrophic factor (CNTF [12]) under the control of the cytomegalovirus promoter-enhancer sequence, and sera were collected 6 weeks after injection.

For rabbit immunizations, New Zealand female rabbits were used. Animals received 0.3 or 3 mg of the pF78E2 plasmid DNA dissolved in 0.5 ml of 1× PBS into the quadriceps muscle. Plasmid DNA solution was delivered by insulin syringes (250 μl/syringe). A booster immunization was performed at week 4. Sera were collected 3 weeks after each injection.

All animal procedures were conducted in conformity with national and international laws and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, 12 December 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana no. 40, 18 February 1992; *Guide for the Care and Use of Laboratory Animals*, NIH publication 85-23, 1985). In particular, mice and rats were fully anesthetized with ketamine (Imalgene 500; Merial Italia SpA, Milano, Italy) at 100 mg/kg of body weight and xylazine (Xilor, BIO 98 srl; S. Lazzaro, Bologna, Italy) at 5.2 mg/kg. Complete anesthesia in rabbits was obtained with three injections at ca. 15-min intervals. The first injection consisted of acepromazine (Prequillan P.A., FATRO SpA; Ozzano Emilia, Bologna, Italy) at 0.3 mg/kg of body weight; animals then received ketamine at 25 mg/kg, xylazine at 8 mg/kg, and finally ketamine at 40 mg/kg.

Electrical treatment. The mouse hind leg was shaved and the quadriceps muscle was injected with a predetermined amount of plasmid DNA in 50 μl of 1× PBS. The electric field was applied as previously described (49) with a few changes. A custom amplifier was constructed using an APEX PA-85 power operational amplifier in the output stage (APEX Technologies, Tucson, Ariz.). Signals were generated by an integrated custom signal generator and were monitored by using a two-channel 8-bit oscilloscope card (K7103 Velleman, Gavere, Belgium), the whole setup being controlled by a custom software package written in Java programming language running on a PC-compatible laptop (Extensa 501T; Acer America, San Jose, Calif.). Electric pulses were delivered through a pair of stainless steel electrodes (0.2-mm wires ca. 3 cm long) placed on the skin side to side around the muscle with a separation distance of 5 mm in parallel orientation with respect to the muscle fibers. The electric contact was assured using an electrocardiographic paste (E.C.G. Gel; CeraCarta SpA, Forlí, Italy). Voltage and current were sampled periodically during the experiment with the digital oscilloscope. Voltage was monitored across the lower resistor of a voltage divider (100,000-Ω resistor over a 10,000-Ω resistor) in parallel with the electrodes, while current was monitored by measuring the potential drop across a precision 1-Ω resistor in series with the electrodes.

By using the electrical conditions previously set up for EGT treatment on muscle fibers exposed by surgical intervention (establishing a fixed voltage at 45 V/cm and a floating current [49]), we did not detect E2 expression upon injection of 5 μg of DNA in the quadriceps of BALB/c mice (data not shown). However, due to the increased overall resistance (about 4,500 Ω at 1.0 kHz), a much lower current was measured in the tissue with respect to the previous protocol (20 versus 50 mA). By contrast, fixing the actual current going through the tissue 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 a depth of 8 mm, in parallel orientation with respect to the muscle fibers. With this single device we first injected intramuscularly the plasmid DNA solution and then delivered electric pulses 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 μg 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; KinematicaAG Littau-Luzern) of muscles in 1 ml of lysis 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). Different muscle extracts were normalized over the total amount of proteins, and E2 expression was determined by enzyme-linked immunosorbent assay (ELISA). The 96-well plates (Nunc-Immuno Plate MaxiSorp Surface, catalog number 439454; Nunc A/S, Roskilde, Denmark) were coated with 1 μg of Galanthus Nivalis Lectin (GNA; catalog number L-8275; Sigma Chemical Co., St. Louis, Mo.) per well in coating buffer (50 mM $NaHCO_3$, pH 9.6). After overnight incubation, the plates were washed (1× PBS, 0.05% Tween 20) and incubated for 1 h at 37°C with 250 μl of blocking buffer (2% bovine serum albumin [BSA], 1× PBS, 0.05% Tween 20) per well. Normalized amounts of muscle extracts were added and incubated for 3 h at room temperature. E2 protein was revealed after an overnight incubation with anti-E2 monoclonal antibody (MAb) 185 (S. Zucchelli, unpublished data) and revealed with anti-mouse immunoglobulin G (IgG; Fc-specific) alkaline phosphatase-conjugated antibody (Sigma catalog number A-7434). Plates were developed at 37°C with a 1 mg/ml solution of *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets, catalog number 104-105) in ELISA substrate buffer (10% diethanolamine buffer, 0.5 mM $MgCl_2$; pH 9.8). Results were expressed as the difference between 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 μg 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 μl of blocking buffer (2% BSA, 1× PBS, 0.05% Tween 20) per well. Saturating amounts of E2 protein produced by transient transfection of 293 cells (Zucchelli et al., submitted) were incubated in blocking buffer for 3 h at room temperature. A fixed amount (100 μl/well) or threefold dilutions (from 1:100 to 1:72,900) of sera were used in seroconversion or titration assays, respectively. Sera were preincubated for 2 h at room temperature with 1 μl of extract from mock-transfected cell per well, aliquoted into the wells, and incubated overnight at 4°C. Bound antibodies were detected using anti-mouse IgG Fc-specific alkaline phosphatase-conjugated antibody (catalog number A-7434; Sigma) diluted 1:2,000 in blocking buffer and visualized as described above.

Rat and rabbit antibodies were measured following the same ELISA protocol and were revealed with anti-rat IgG (whole molecule) alkaline phosphatase-conjugated antibody (Sigma catalog number A-8438) diluted 1:2,000 or with anti-rabbit IgG (whole molecule) alkaline phosphatase-conjugated antibody (Sigma catalog number A-8025) diluted 1:5,000. Reactivity to CNTF was determined on purified bacterially expressed recombinant CNTF (12) coated at 1 μg/well. Each serum dilution was tested in duplicate in two wells of a 96-well plate. An arithmetic mean of the absorbance OD_{405} values obtained in the two wells was used for calculations. Serum dilution versus OD values were plotted and fit with a Michaelis-Menten curve (Abelbeck Software KaleidaGraph, version 3.0.1). The titer was defined as the serum dilution from the fit that would have resulted in an absorbance of 0.2 OD. Nonresponders were conservatively assigned a titer of 50 (50% of the initial dilution tested).

ELISA assays to determine the serum cross-reactivity on synthetic peptides representing the HVR1 of natural viral isolates were performed as previously described (46). Equivalent amounts of sera from seroconverted mice were pooled, and the pool was tested at a 1:100 dilution.

ELISPOT assays. Cells secreting IFN-γ in an antigen-specific manner were detected using a standard enzyme-linked immunospot (ELISPOT) assay (36). Spleen cells were prepared from immunized mice (5 μg of plasmid DNA with [+] or without [−] EGT treatment) and resuspended in R10 medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U of penicillin per ml, 50 μg of streptomycin per ml, 10 mM HEPES, 50 μM 2-mercaptoethanol). Multiscreen 96-well filtration plates (catalog number MAIPS4510; Millipore Corp., Bedford, Mass.) were coated with purified rat

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. Splenocytes 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 1a 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 mimotope 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 assays. p815 cells (ATCC TIB64) expressing the H-2^d class I molecule were maintained in culture in R10 medium at 37°C and 5% CO_2 and then used as target cells for cytotoxicity assay.

Spleen cells from immunized animals were cultured to amplify effector CTLs. A total of 4×10^6 spleen cells were resuspended in R10 supplemented with 5 μg of $CD8^+$-specific peptide (pep1323) and 10 U 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 (Multi-well-Tissue Culture Plate, Falcon, catalog number 3047, Becton Dickinson). After 7 days of in vitro restimulation, cultures were assayed for cytotoxic activity in a standard ^{51}Cr release assay. Briefly, target cells were labeled with $Na^{51}CrO_4$ (catalog number CSJ.1; Amersham Pharmacia Biotech, Buckinghamshire, England) and pulsed with either pep1323 or medium alone for 2 h at 37°C and 5% CO_2. 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% CO_2. Next, 30-μl samples of supernatants were transferred to a LumaPlate-96 (catalog number 6005164; Packard Instrument Company, Meridien, Conn.), allowed to dry overnight, and then counted (Top-Count, Microplate Scintillation Counter; Packard Instrument Company) to determine the amount of ^{51}Cr 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 of the geometric mean of the +EGT animals divided by the geometric mean of the −EGT cohort). *P* values are conservatively reported as two-tailed values, and any *P* value of <0.05 was considered significant. Response rates were compared between treatment groups using an adjusted χ^2 test.

RESULTS

Increased in vivo expression of HCV E2 protein by EGT of plasmid DNA in the mouse muscle. Plasmid pF78E2 was used to verify the effect of EGT on the in vivo expression of the encoded antigen after intramuscular injection in mice. This vector encodes for a chimeric version of the E2 glycoprotein ectodomain fused to a highly cross-reactive HVR1 mimotope (F78 [46]). BALB/c mice were injected in the quadriceps muscle with 5 μg of pF78E2, and half of the animals were subjected to EGT just after the injection. An additional group of animals receiving a saline solution was used as a negative control. At different times after DNA injection, groups of three mice were sacrificed, and protein extracts were prepared from injected muscles for the testing of E2 expression by ELISA. As shown in Fig. 1, expression of the E2 protein in the −EGT and mock-injected animals was undetectable at all tested time points. In contrast, mice subjected to EGT already displayed measurable levels of E2 protein by 16 h after DNA injection. E2 expression increased progressively over time, reaching a peak at 8 days postinjection and declining by day 14, to be completely undetectable by day 23 (Fig. 1). We performed a follow-up experiment by injecting 50 μg of the same plasmid. Based on the data obtained with 5 μg, E2 expression was assayed at 3 and 8 days postinjection. Also in this case, we detected E2 protein production only in the EGT-treated mice (Fig. 1). Due to the undetectable E2 expression in the muscles of untreated mice, we could not determine the degree of improvement in in vivo protein production by EGT treatment. However, data previously obtained in our laboratory by injecting 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).

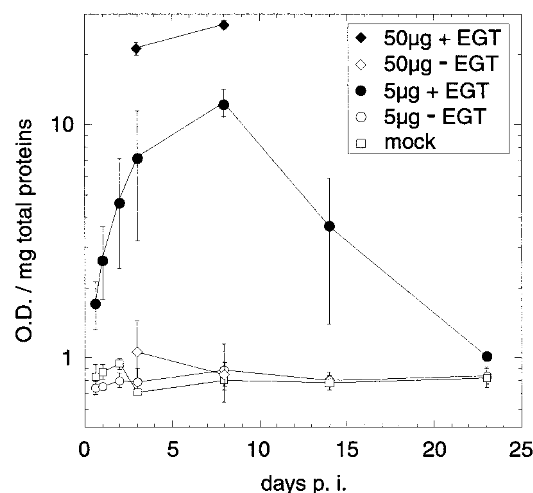


FIG. 1. EGT of vaccine DNA vector increases the efficiency of antigen expression in mouse muscle. HCV E2 in vivo expression was measured by ELISA on crude muscle extracts from mice injected with 5 μg of pF78E2 plasmid as described in Materials and Methods. A follow-up experiment was performed by injecting 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 (A_{405}) from two replicates 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 (◆, 50 μg + EGT), 50 μg of pF78E2 plasmid without EGT (◇, 50 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 (□, mock).

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

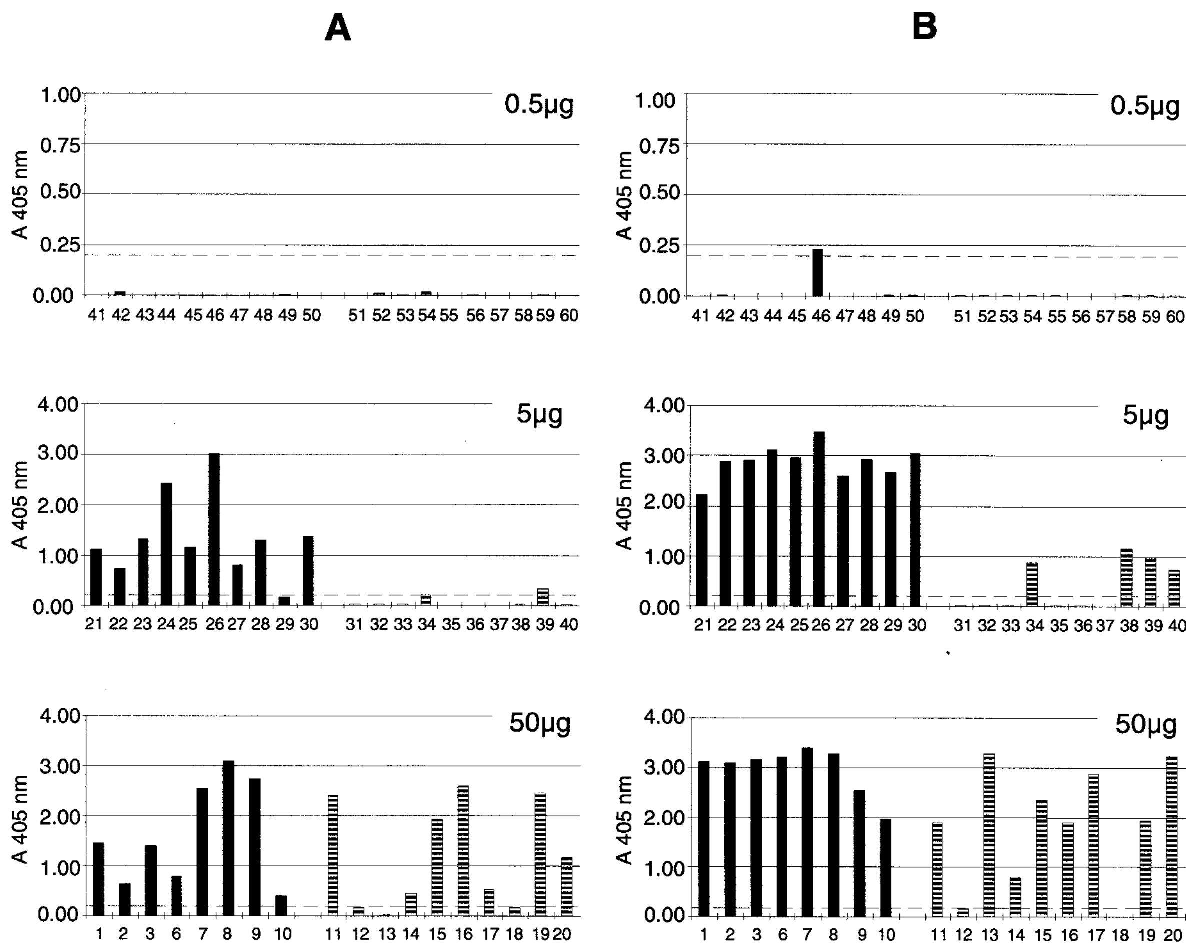


FIG. 2. EGT increases the frequency of seroconversion of pF78E2-injected mice. Seroconversion was measured by ELISA on recombinant E2 as described in Materials and Methods. Sera from individual mice (indicated on the horizontal axis) were tested in duplicate at a 1:100 dilution, and average values (A_{405}) are reported. Results from EGT-treated mice are represented by black bars; hatched bars indicate the reactivity of sera from untreated mice. Values below the dotted lines (two times the background) were considered negative. Results from animals injected with 0.5, 5, and 50 μg are shown in the upper, medium, and lower panels, respectively. (A) Post-dose 1 sera. (B) Post-dose 2 sera.

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 −EGT 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 OD_{405} 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 +EGT mice with a cohort GMT of 6500, whereas the −EGT cohort measured a GMT of 650. This boosting of the +EGT 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

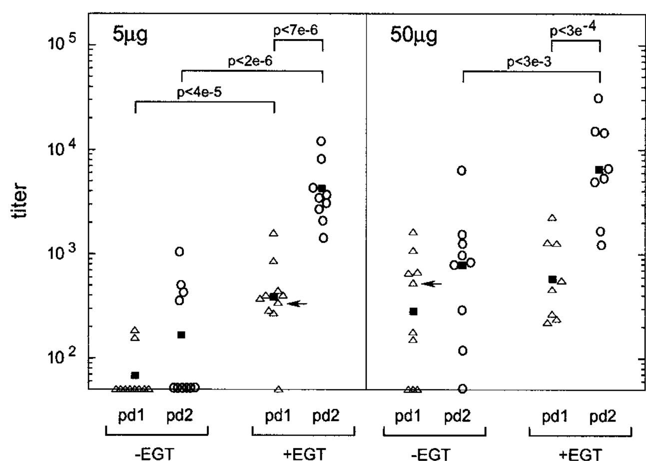


FIG. 3. EGT increases antibody titers of pF78E2 injected mice. Individual titers against recombinant E2 were measured by ELISA on each serum from EGT-treated (+EGT) and untreated (−EGT) animals after one (pd1, △) or two (pd2, ○) intramuscular injections of 5 or 50 μg of pF78E2 plasmid. The doses were 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. Samples that did not seroconvert were assigned an endpoint titer of 50. 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).

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). A nonparametric analysis (Wilcoxon test) of the low-responder groups yielded similar conclusions.

Rats and rabbits were immunized to verify the efficacy of EGT in larger species. EGT-treated rats developed anti-E2 titers more than sevenfold higher than untreated animals (data not shown), supporting the trend observed in mice, although the differences were not found to be statistically significant due to the small number of animals used in this experiment (groups of two or three animals). By using a plasmid encoding human CNTF for DNA immunization of rats, we verified that the improvement in antibody response mediated by EGT (sixfold enhancement) is an antigen-independent phenomenon (data not shown). Groups of seven (non-EGT-treated) and eight (EGT-treated) rabbits were immunized with 3 mg of pF78E2 plasmid at time zero and at 4 weeks. Sera were assayed at 3 weeks after each dose. Significant enhancement was achieved by EGT after both one and two doses (Fig. 4). In particular, animals treated with EGT reached a cohort GMT of 340 after one injection, whereas non-EGT control animals did not measurably respond (cohort GMT = 60). We observed after the second immunization a dramatic enhancement in the EGT-treated cohort (GMT = 25,600), while the non-EGT-treated cohort was now measurable (GMT = 790). The administration of the second dose turned a roughly sixfold enhancement after one dose (3.0- to 11.8-fold, 95% confidence interval) into a >30-fold enhancement after two doses (4.7- to 220-fold, 95% confidence interval). These conclusions were confirmed by performing a nonparametric analysis (Wilcoxon test) of the low-responder groups.

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). 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

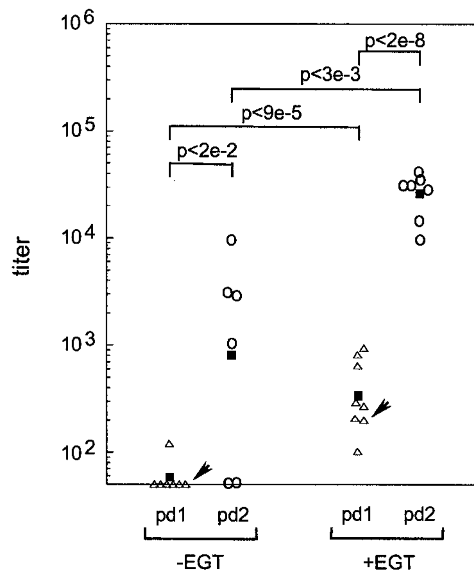


FIG. 4. Enhanced antibody response in EGT-treated rabbits immunized with pF78E2. Individual animal anti-E2 antibody titers in sera from rabbits immunized with the 3-mg dose of the pF78E2 plasmid were determined by ELISA on serum samples collected 3 weeks after the first (pd1, △) or the second (pd2, ○) 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).

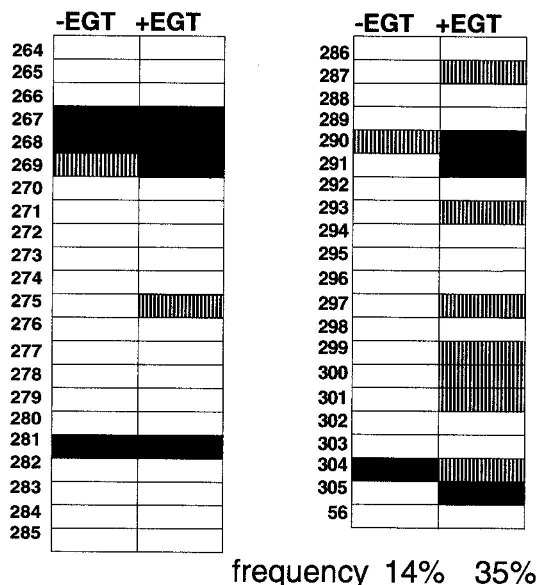
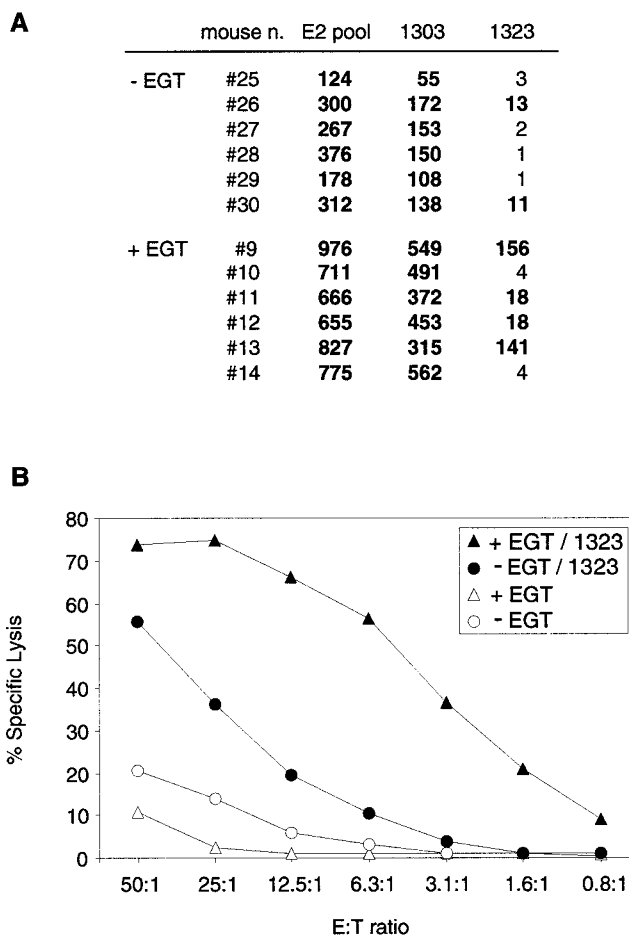


FIG. 5. EGT increases the level of serum cross-reactivity against different HVR1 sequences from natural viral isolates. The cross-reactivity of pools of sera from pF78E2-immunized BALB/c mice with (+EGT) or without (−EGT) electrical stimulation was measured by ELISA using 43 synthetic peptides reproducing different HVR1 sequences (indicated in the left column) as coated antigens. The data reported refer to post-dose 2 sera from animals immunized with two injections of 50 μg of DNA. For each serum pool, average values (A_{405}) from two replicates have been determined. Results are expressed as the difference between the average value of the tested peptide and that of an unrelated peptide. Positive values were differing more than 3σ from the background signal observed on the unrelated peptide. White boxes represent values below the background; hatched boxes indicate signals differing from those observed on the unrelated peptide between 0.1 and 0.4 OD_{405}; black boxes indicate values differing by more than 0.4 OD_{405}. The level of cross-reactivity of each serum is indicated at the bottom of each column and is expressed as the percentage of positive peptides over the total number of tested peptides (frequency).



A

	mouse n.	E2 pool	1303	1323
- EGT	#25	**124**	**55**	3
	#26	**300**	**172**	**13**
	#27	**267**	**153**	2
	#28	**376**	**150**	1
	#29	**178**	**108**	1
	#30	**312**	**138**	**11**
+ EGT	#9	**976**	**549**	**156**
	#10	**711**	**491**	4
	#11	**666**	**372**	**18**
	#12	**655**	**453**	**18**
	#13	**827**	**315**	**141**
	#14	**775**	**562**	4

FIG. 6. EGT improves the T-cell response against HCV E2. (A) BALB/c mice receiving two doses of 50 μg of pF78E2 plasmid with (+EGT) or without EGT (−EGT) were analyzed for the induction of E2-specific cellular immunity. At 3 weeks after the boosting injection, the number of IFN-γ-secreting anti-E2 T cells was determined by ELISPOT on splenocytes from individual mice (indicated in the first column) using a pool of overlapping 20mer synthetic peptides encompassing the HCV E2 sequence (strain H) from aa 371 to 700 (E2 pool). IFN-γ-secreting $CD4^+$ T cells were evaluated using peptide 1303, and the number of E2-specific IFN-γ-secreting $CD8^+$ T cells was measured using peptide 1323. Two independent experiments were performed, with each one testing two different amounts of splenocytes (2.5×10^5 and 5×10^5) and two replicates for each tested amount of splenocytes. Average values were calculated, from the background level determined in the absence of peptides (typically less than 10 SFC/10^6 total splenocytes) was subtracted, and the result was expressed as the number of SFC/10^6 total splenocytes. Numbers corresponding to more than three times the background measured in control experiments without antigenic peptides were considered positive values and are indicated in boldface. (B) E2-specific CTL response in pF78E2-immunized representative mice from the +EGT (triangles) and −EGT (circles) groups. Splenocytes from immunized mice were restimulated in vitro and tested for cytotoxic activity against p815 cells pulsed with the HCV peptide 1323 (1323; closed symbols) or dimethyl sulfoxide (open symbols). Effector cell/target cell ratios are indicated in the abscissa. The percentage of specific killing is reported on the vertical axis. Each number represents the average of two independent experiments.

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 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 ($P < 1e^{-5}$). 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

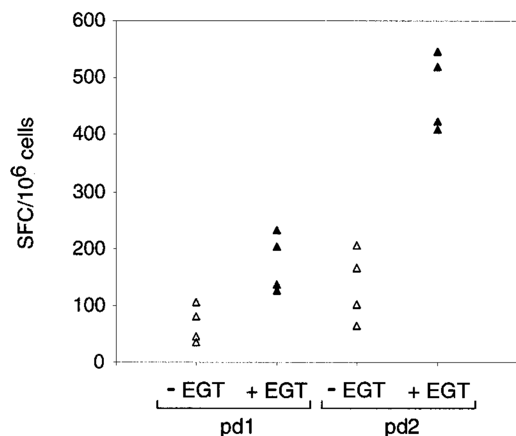


FIG. 7. EGT improves T-cell response against HIV-1 gag. BALB/c mice were injected with one (pd1) or two (pd2) doses of 5 μg of pHIV-1 gag plasmid. At 3 weeks after each injection, the number of IFN-γ-secreting anti-HIV-1 gag $CD8^+$ T cells was determined by ELISPOT on pooled splenocytes from two immunized mice using a synthetic peptide-reproducing gag amino acid sequence between residues 197 and 205. Two different amounts of splenocytes (2.5×10^5 and 5×10^5) were tested with three replicates for each tested amount of splenocytes. Average values were calculated, the background level determined in the absence of the gag peptide (typically less than 10 SFC/10^6 total splenocytes) was subtracted, and the result was expressed as the number of SFC/10^6 total splenocytes. Black triangles represent the number of SFC/10^6 cells in EGT-treated mice (+EGT); open triangles indicate the IFN-γ-secreting anti-HIV-1 gag $CD8^+$ T cells in untreated animals (−EGT). The difference between −EGT and +EGT groups are statistically significant ($P < 0.01$ and $P < 0.0002$ after one or two doses, respectively).

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 pF78E2 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 χ^2 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).

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