J. DRUG DEL. SCI. TECH., 16 (1) 85-89 2006

Genetic vaccination by gene electro-transfer in non-human primates

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Muscle gene electro-transfer (GET) of plasmid DNA is a promising approach for gene therapy and genetic vaccination. Several protocols have been described which give good levels of gene transduction in small animals. However, to progress towards human applications, efficacy must be demonstrated in non-human primates. Here, we extensively explore several electrical and injection parameters in Rhesus monkeys and define a series of conditions for efficient vaccination.

Key words: Gene electro-transfer – DNA vaccines – Non-human primates.

Genes can be transferred to muscle cells of mice by simple plasmid injection and expression is long lasting [1]. However, various factors hinder muscle-based gene-transfer strategies, and transduction can be particularly difficult in large animals such as non-human primates [2]. Therefore it is highly desirable to develop new technologies that improve gene transduction and expression in skeletal muscle of large organisms. Recently, the susceptibility of skeletal muscle cells to electroporation at low voltage has been exploited to improve gene transfer and expression in mammals for gene therapy purposes. Several reports have shown the high efficiency of an electrical approach in delivering reporter genes such as β-galactosidase [3], luciferase [4] or secreted alkaline phosphatase (SeAP) [5], as well as genes of interest for therapeutic applications such as erythropoietin [6-8], FGF1 [9] growth hormone-releasing hormone [10, 11]. Additionally, levels of circulating proteins can be modulated upon gene electro-transfer (GET) by exploiting inducible elements such as the tetracycline regulated system [6, 12]. Genetic vaccination, one of the most efficient methods to elicit a cellular mediated immune response (CMI) [13], could also benefit from the application of electric pulses after DNA injection.

Hepatitis C (HCV) is the major cause of non-A non-B hepatitis and it is estimated that over 170 million people worldwide are infected [14]. Infection is characterized by a high tendency to chronicity which often progresses to cirrhosis and liver carcinoma [15]. Developing a good vaccine strategy for prevention or treatment of HCV infection would therefore be of great impact on human health. Although antibodies against all viral proteins can be detected in infected patients, these do not prevent the progression of the disease and recent evidence suggests that the progression of the infection to recovery versus chronicity correlates with the strength of the T-cell response [16-21].

We have previously shown [22] that GET of pVJE2F78, an HCV E2 encoding plasmid, results in a faster seroconversion rate and increases in anti E2 antibody titers with respect to simple naked DNA injection. Also, the strength of T-cell response is improved after electroporation [22]. Although the GET technology holds promise for gene therapy and genetic vaccination, several issues remain to be addressed before application in humans. The actual clinical setting, such as needle type, angle of injection, or electrical conditions can only be defined using the most suitable animal model such as non-human primates. In a previous experiment we explored the efficiency of gene delivery upon the variation of different electrical parameters such as pulse length, frequency, and peak voltage while monitoring both short and long term protein production. We designed a short electrical treatment that gives high expression levels of plasmid encoded protein in different species such as mice, rabbits, and monkeys and efficient immunization in mice. In this experiment, we have used an HCV non-structural region (NS) encoding plasmid and performed a large study comparing different electrical conditions for efficient vaccination of non-human primates to guide parameters for GET to be used in humans.

I. MATERIALS AND METHODS

1. Animals and treatments

The SeAP monkey study was performed on young adult Cynomolgus monkeys (*Macaca fascicularis*) and immunization studies were conducted in Rhesus macaques (*Macaca mulatta*). The Cynomolgus subjects were colony-born individuals from a closed colony established in 1981 and were maintained and utilized at the CNR breeding center in Rome. The present research was ethically approved by the Italian Ministry of Health. Rhesus macaques were housed at the Merck WP facility (PA, USA). All animal care and treatment were in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council).

All the subjects appeared clinically healthy at the time of the experiment. Prior to treatment and blood sampling, each animal was restrained and anesthetized with 10 mg/kg of ketamine hydrochloride, injected intramuscularly. Blood samples were obtained from the femoral vein. During the study the subjects were housed in stainless-steel primate cage in indoor facilities and were fed a standard monkey diet ad libitum, supplemented with fresh fruit. No variation in the body weights were observed during the experiment.

2. Plasmid construction and preparation

Plasmids pV1JNSopt/mut and pCMVSeAP were previously described [5, 6, 23]. Large amounts of endotoxin-free, supercoiled, purified plasmids were prepared by Bayoubiolabs (LA, USA) and resuspended in endotoxin-free saline solution at the desired concentration. Serum SeAP levels were monitored by Phospha-Light (chemiluminescent reporter assay for secreted alkaline phosphatase), Tropix with some modifications [6].

3. Electrical apparatus and electrical conditions

Three electrotherapy devices were constructed and used. Each contains a programmable arbitrary waveform generator; periodic

wave characteristics (frequency, amplitude, and duty cycle) and the waveform shape can be individually programmed. Dedicated high power amplifiers produce current-controlled or voltage-controlled output up to hundreds of milliamps (mA) and hundreds of volts (V) if desired. Applied waveforms and tissue impedance are determined from digital acquisition boards or digital oscilloscopes.

4. Injection devices

The needles used to inject monkeys are as follows. (A) Double needle array: coupled hypodermic needles are soldered on a printed circuit board that is a mechanical support and connects the needles to the electrical field generator by means of appropriate cables. Two independent insulin syringes are used for injecting drugs through the needles connected to the generator. (B) Triple needle array: a polymer cassette supports two needle electrodes and holds the syringes within. After injection, the cannula automatically retracts into the syringe, so only two electrodes are inserted during electrical stimulation. The DNA vaccine is therefore injected in two sites in the case of the double needle and in a single site with the triple needle (*Figure 1*).

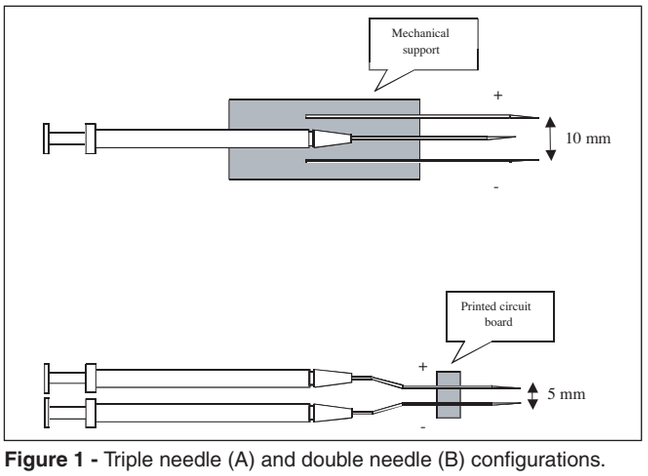

Figure 1 - Triple needle (A) and double needle (B) configurations.

5. Ad6 NS vector construction and characterization

The construction of the HCV NS expression cassette and the insertion into Ad6 vector generating the MRKAd6NS mut have been described [23].

6. PBMC preparation

Blood samples were collected from the femoral vein using aseptic techniques; Vacutainer blood collection systems (Becton Dickinson) were used. Peripheral blood mononuclear cells (PBMCs) from EDTA-treated peripheral blood were isolated by lymphocyte separation medium (LSM) density gradient centrifugation (Organon Teknika). Cells at the interface were collected and washed twice in RPMI-1640 medium. To lyse erythrocytes the cell pellets were resuspended in 5 ml of erythrocyte shock medium (ACK lysing buffer, Gibco-BRL, custom-made), and left at room temperature for 5-10 min. PBMCs were washed once, resuspended in R10 medium (RPMI medium 1640, supplemented with 10 mM HEPES buffer, 2 mM L-glutamine, 50 U/ml of penicillin and 50 μg/ml of streptomycin, 50 μM 2-mercaptoethanol [all Gibco-BRL] and 10% fetal bovine serum [HyClone]) and then used in the assays.

7. Peptides for T cell assays

The peptide sequence, spanning the NS3-NS5B region reproduced the amino acid sequence of the HCV BK 1b strain. Peptides were purchased by Bio-Synthesis Inc. (Lewisville, TX, USA). All peptides were synthesized with free N-terminal amine and free C-terminal carboxylate and were purified by preparative HPLC. The peptides, 15 amino acids (aa) in length and overlapping by 11 aa (NS3-NS5b), were reconstituted in 100% DMSO at 40 mg/ml (NS3-NS5b) and mixed in pools so that each peptide is equally represented in the mixture. To facilitate the analysis, the 491 NS3-NS5b peptides were combined in seven pools covering NS3 protease (NS3p), NS3 helicase (NS3h), NS4, NS5A and NS5B (split in two pools NS5B-I and NS5B-II).

8. IFNγ ELISpot

The IFNγ ELISpot assays with Rhesus PBMCs were performed as described [23]. Spots were quantified by an automated ELISpot reader system (ELR03 AID Elispot Scientific). The ELISpot response was considered positive when all of the following conditions were met: IFNγ production was present in Con-A stimulated wells; the number of specific spots/million PBMCs to at least one HCV peptide pool was greater than 55 and three times the number detected in the mock control wells (DMSO); and in serial dilutions of PBMCs, the responses were titratable.

9. Statistical analysis

Immune parameters were compared by evaluating group means via Tukey HSD or Student T-test, as appropriate. Data analyses were carried out using JMP 5.0.1 software (SAS Institute, Cary, NC, USA).

II. RESULTS AND DISCUSSION

1. Efficiency of GET in different muscles of non-human primates

To monitor the efficiency of gene electro-transfer in non-human primates we used previously described electrical conditions [24] consisting of 8 trains of 1,000 bipolar pulses delivered every other second at a frequency of 1 kHz. The pulse length was 0.2 msec/phase and amplitude ± 100 V/cm (short pulses/high frequency or SP/HF conditions). In a preliminary experiment we injected four groups of three Cynomolgus monkeys in different muscles – tibialis, semitendineous, rectus femoralis and deltoid – with 400 μg of a SeAP encoding plasmid divided between the two legs and monitored SeAP levels in circulation 3 and 10 days after GET. As a control, two animals were injected in the deltoid with the same amount of plasmid and were not subjected to electrical stimulation. SeAP was detectable in serum 4 days after injection and further increased after 10 days (*Table I*). Although the small number of animals/group used did not allow a significant analysis, we concluded that gene electro-transfer can be applied with success to different muscles.

Table I - SeAP levels (ng/ml) 3 and 10 days post-injection in different muscles with and without electrical stimulation (ES).

Monkey #	Weight (kg)	Mg/kg	Muscle	ES	SEAP 3 days	SEAP 10 days
216	3.40	117.7	tib	yes	3.5	157
250	3.60	111.0	tib	yes	7.0	144
					5.3*	150.5*
237	3.15	127.0	semitend	yes	8.5	211
240	2.90	138.0	semitend	yes	46.7	1286
					27.6*	748.5*
224	3.90	102.0	rect. fem.	yes	ND	194
245	3.50	114.0	rect. fem.	yes	3.8	543
					3.8*	368.5*
229	3.10	129.0	deltoid	yes	35.2	361
242	2.50	160.0	deltoid	yes	12.0	102
					23.6*	231.5*
218	3.50	114.3	deltoid	no	ND	ND
228	3.20	125.0	deltoid	no	ND	ND

*Average.

In a previous experiment [24], we had found that, by optimizing the pulse length, we were able to deliver an electric treatment in which the time of exposure was reduced from 8 to 3 s and yet the efficiency was optimal in term of gene expression in various species. We have called this type of electric treatment, consisting in two trains of 100 square bipolar pulses with pulse length 2 msec/phase, frequency 100 Hz, short conditions (SC) [24].

To verify the efficiency of the SC versus the SP/HF conditions in non-human primates we injected three groups of three Cynomolgus monkeys with the same DNA dose/body weight (100 μg/kg) followed by either type of treatment or left without electrical stimulation. Four days after injection SeAP was undetectable in monkeys that did not receive any type of treatment and similar between the SP/HF and SC groups (*Table II*).

Table II - SeAP levels (ng/ml) 7 days post-injection and stimulation with the SP/HF or SC conditions.

Conditions	Monkey ID	SeAP levels (ng/ml)
SP/HF	255 303 280	1.26 0.22 3.26
SC	281 302 275	0.59 0.39 1.23
no GET	210 322 275	< 0.02 < 0.02 < 0.02

2. Immunization with HCV-NS

To verify the efficiency of the SC in vaccination we assessed the immunological potency with a plasmid vector expressing the non-Structural region of HCV (NS) [23] in Rhesus. Two groups of 4 Rhesus received 5 mg plasmid DNA injected in the quadriceps followed by GET with the SP/HF or the SC conditions. The vaccine was administered in three doses at T = 0, 4, and 8 weeks. T-cell mediated immune response was measured by IFNγ ELISpot on total PBMCs isolated at weeks 4, 12, and 25 from the injected monkeys following stimulation with six different pools of overlapping peptides covering the entire NS region (see Material and Methods section). Cell mediated immune response was detected in all of the treated monkeys 4 weeks after the first injection (*Figure 2*, T = 4) and peaked 4 weeks after the third injection (*Figure 2*, T = 12) reaching in some animals levels over 7,000 IFNγ spot forming cells (SFC)/10^6 PBMCs. Responses declined 25 weeks after the start of the study (*Figure 2*). The overall response was comparable in the two groups of monkeys (*Figure 2*) thus suggesting that the SC conditions can be efficiently used for DNA based vaccination.

3. Test of different injection modalities, electrical conditions and devices in Rhesus

Having demonstrated that we can considerably shorten the electric treatment and yet maintain the immunogenic potency of the DNA vaccine, we examined several other variables, such as the injection device (double needle array or triple needle array, *Figure 1*), the angle of injection, 20° or 90° with respect to the skin surface; and waveform of the applied electric current, i.e. continuous sinusoidal or pulsed square. Seven groups of three monkeys each were treated as indicated in *Table III*. The first four groups of animals were electro-

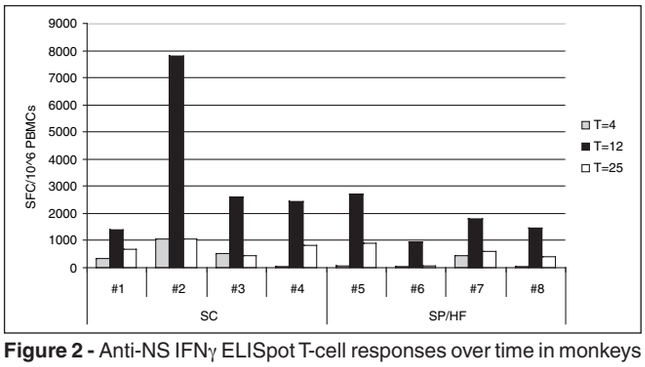

Figure 2 - Anti-NS IFNγ ELISpot T-cell responses over time in monkeys immunized with the pV1JNSopt/mut plasmid followed by electrical stimulation with SC or SP/HF conditions as indicated. Total anti-NS response is calculated by summing the mock-corrected response to each of 6 peptide pools covering the entire vaccine encoded NS. SFC = IFNγ spot forming cells.

Table III - Test of different injection and electrical conditions in non-human primates.

Group #	Monkey #	Protocol	Current (mA) dose 1, 2, 3	Time	Needle	Angle of injection
1	96-0233 96-0239 96-R061	SC	100 (lim) 100 (lim) 100 (lim)	3 s	double-needle array	20°
2	98-0186 92-0088 94-R014	SC	120 79 79	3 s	double-needle array	20°
3	98-0191 94-R001 94-R006	SC	100 100 100	3 s	double-needle array	20°
4	99-R008 96-0104 92-0145	SC	100 100 100	3 s	double-needle array	90°
5	92-0147 94-0125 94-0149	sinusoidal continuous, 420 Hz	100 100 100	10 s	triple-needle array	90°
6	94-0217 94-R004 96-R044	sinusoidal continuous, 420 Hz	100 100 100	10 s	double-needle array	90°
7	96-R045 96-R047 96-R063	sinusoidal continuous, 250 Hz	71 71 71	3 s	triple-needle array	90°

stimulated with the SC conditions with the following differences: a) group 1 was electro-stimulated in constant voltage mode and current limited at 100 mA, group 2 and 3 at constant current mode; b) current levels were equal (100 mA) for all three injections in groups 1, 3, 4 but higher for the first injection (120 mA) and lower in the following two injections (79 mA) in group 2; c) in group 4 the angle of injection with respect to the skin was 90° instead of 20°. The 90° needle insertion angle was chosen because it is more convenient in medical practice. The last three groups (5, 6, 7) were treated with a continuous sine wave; groups 5 and 6 received a peak current of 100 mA, frequency 420 Hz, and a total treatment duration of 10 s. Group 5 was injected with a triple needle array and group 6 with a double needle array. In group 7 we tested a 250 Hz sinusoidal treatment lasting only 3 s as with SC conditions. The immunization schedule consisted of three administrations at weeks 0, 4, and 8 of 5 mg pV1JNSopt/mut plasmid divided between the two quadriceps.

T-cell mediated immune response was measured by IFNγ ELISpot on total PBMCs isolated monthly from the injected monkeys. Immune response was detectable at four weeks after the first injection (*Figure 3*, T = 4), increased after the third injection (*Figure 3*, T = 12) and slightly declined at the time of boosting, 24 weeks after the initiation of the study (*Figure 3*). Using Tukey HSD to control for the many group mean comparisons, responses were not different ($p > 0.05$) between any of the treated groups at any time point up to and including 24 weeks.

Minor changes in the electrostimulation conditions (double vs. triple needle, 90° vs. 20° injection angle, sinusoidal vs. square wave pulse, etc.) did not make a statistically detectable difference in the immunogenicity in this model, at least within the range of parameters explored here.

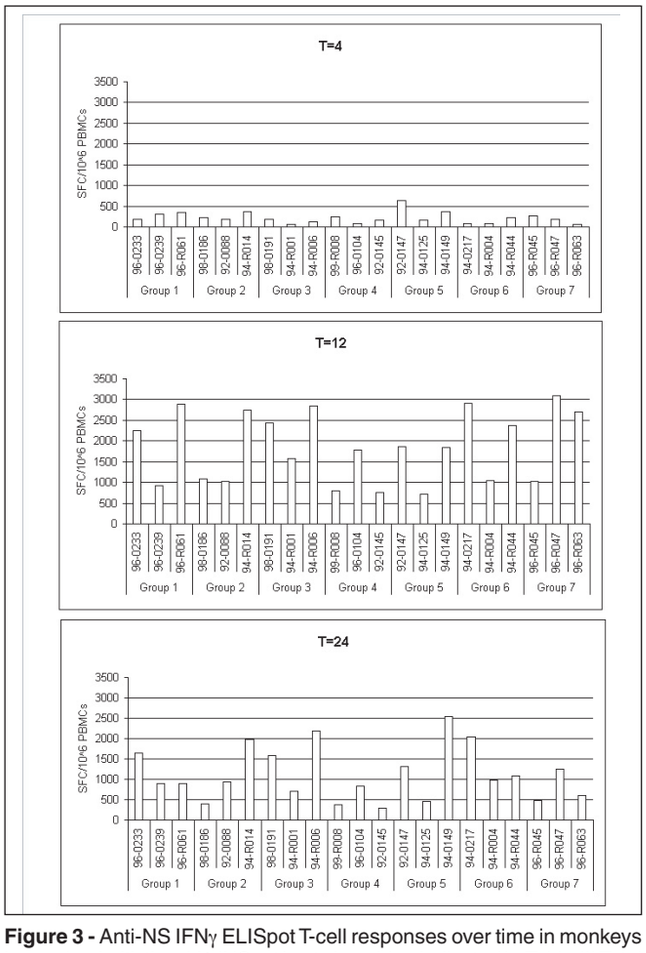

Figure 3 - Anti-NS IFNγ ELISpot T-cell responses over time in monkeys immunized with the pV1JNSopt/mut plasmid as indicated in *Table III*. Total anti-NS response calculated by summing the mock-corrected response to each of 6 peptide pools covering the entire vaccine encoded NS. SFC = IFNγ spot forming cells. DMSO is the mock response.

4. Comparison of Ad or GET boosting

Next we decided to evaluate the efficacy of boosting with an Ad-based vector or with either of the two shortest electrical conditions (2 and 7) presumably most suitable for human treatment in terms of angle of injection, short treatment times and low current levels. The 21 animals were therefore randomized into three groups (A, B and C) with comparable pre-boost ELISpot counts and injected with either 10^8 vp of MRKAd6NSmut (group A) or with pV1JNSopt/mut plasmid followed by electrical stimulation with the SC at 79 mA, double needle, angle of injection 90° (group B) or with a continuous sine wave at 250 Hz, 71 mA, angle of injection 90° (group C). Immune response increased in all the animals receiving the boosting injection (*Figure 4*) reaching levels up to 13,000 IFNγ producing antigen-specific SFC/10^6 PBMCs corresponding to 1.3% of total PBMCs. Responses were indistinguishable between groups B and C (DNA+GET boost). Considering all DNA+GET-boosted animals together versus Ad-boosted (group A) animals, DNA+GET-boosted animals had higher mean log ELISpot responses ($p = 0.0303$). No significant differences were found when all three groups were compared at once (Tukey HSD, $p > 0.05$).

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In conclusion, we have defined a series of protocols of DNA vaccination by GET in non-human primates that give efficient cell-mediated immune response and could be suitable for human applications. The angle of injection/stimulation does not affect immunogenicity and this is particularly important in clinical practice since a certain degree of variability in this parameter is expected in routine vaccination. Also,

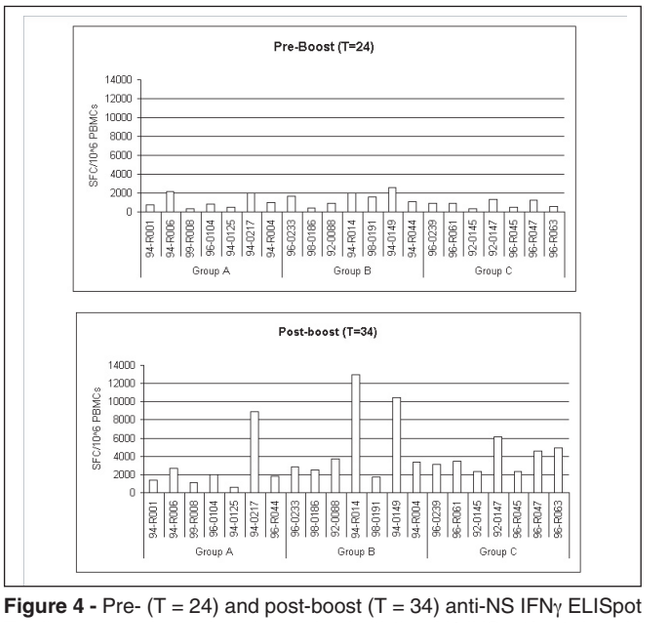

Figure 4 - Pre- (T = 24) and post-boost (T = 34) anti-NS IFNγ ELISpot T-cell responses in monkeys immunized with the pV1JNSopt/mut plasmid as indicated in *Table III* and boosted with MRKAd6NSmut (group A) or with pV1JNS/OPTmut followed by electrical stimulation with the SC at 79 mA (conditions 2 in *Table III*) (Group B) or sinusoidal continuous, 250 Hz, 71 mA (Group C). Total anti-NS response calculated by summing the mock-corrected response to each of 6 peptide pools covering the entire vaccine encoded NS. SFC= IFNγ spot forming cells. DMSO is the mock response.

needle configuration is irrelevant and both triple needle and double needle array can be used for efficient vaccination. Although the double needle could be somehow more comfortable in clinical practice, it could however be problematic and expensive to manufacture in a large scale setting. The length of treatment is also relevant for the applicability of GET to routine vaccination, both for potential pain during current application and for prolonged electrode insertion in the muscle. We show that a 3-s treatment is as efficient as a 10-s treatment thus minimizing potential discomfort. Vaccination based on viral vectors is considered one of the most efficient methods for T-cell based vaccine. We demonstrate instead that a homologous prime-boost strategy based on GET can be as efficient as a heterologous DNA prime and Ad boosting thereby providing an alternative strategy to viral vectors..

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MANUSCRIPT

Received 25 July 2005, accepted for publication 13 January 2006.