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Skin targeted DNA vaccine delivery using electroporation in rabbits I: Efficacy

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Abstract

Genetic immunization through skin is highly desirable as skin has plenty of antigen presenting cells (APCs) and is easily accessible. The purpose of this study was to investigate the effects of electroporation pulse amplitude, pulse length and number of pulses on cutaneous plasmid DNA vaccine delivery and immune responses, following intradermal injection in vivo in rabbits. Expression of the delivered plasmid was studied using a reporter plasmid, coding for β-galactosidase. The efficiency of DNA vaccine delivery was investigated using a DNA vaccine against Hepatitis B, coding for Hepatitis B surface antigen (HBsAg). Serum samples and peripheral blood mononuclear cells (PBMC) were analyzed for humoral and cellular immunity, respectively, following immunization. The expression of transgene in the skin was transient and reached its peak in 2 days post-delivery with 200 and 300 V pulses. The expression levels with 200 and 300 V pulses were 48- and 129-fold higher, respectively, compared with the passive on day 2. In situ histochemical staining of skin with X-gal demonstrated the localized expression of β-galactosidase with electroporation pulses of 200 and 300 V. Electroporation mediated cutaneous DNA vaccine delivery significantly enhanced both humoral and cellular immune responses ($p < 0.05$) to Hepatitis B compared to passive delivery. The present study demonstrates the enhanced DNA vaccine delivery to skin and immune responses by topical electroporation. Hence, electroporation mediated cutaneous DNA vaccine delivery could be developed as a potential alternative for DNA vaccine delivery.

Keywords: DNA vaccine; Cutaneous DNA vaccine delivery; Electroporation; Skin; Gene delivery

1. Introduction

DNA vaccines offer a novel way to immunize individuals against deadly diseases using genetic material

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coding the antigens. The development of DNA vaccines is one of the most promising applications of recent advances in gene based therapy (Srivastava and Margaret, 2003). DNA vaccines are easy to produce, do not replicate and encode only the antigen of interest, as opposed to live attenuated or viral carrier systems. DNA vaccines express antigens in vivo, thus conserving the native conformation of the antigenic epitopes, which is critical for the induction of specific humoral antibody and cellular immune responses. The endogenously synthesized antigens can access both major histocompatibility complex (MHC) classes I and II pathways for antigen presentation (Leitner et al., 2000). DNA vaccines are shown to induce both cell mediated and humoral immunity, which are long lasting (Donnelly et al., 1997). They may be constructed to have more than one antigen-coding gene, thus potentially decreasing the number of vaccinations required.

Vaccination through skin is highly desirable as skin is most accessible and has a large pool of antigen presenting cells (APCs) that can process and present the antigen to appropriate lymphocytes efficiently. Skin contains plenty of functional bone marrow derived epidermal Langerhan's cells and dermal dendritic cells, which are specialized for induction of immune responses (Tuting et al., 1998). To be therapeutically useful, the DNA vaccine must be delivered inside the nucleus before it can express antigen molecules. This requires efficient membrane permeabilization to allow the DNA vaccines to enter the cells (Nishikawa and Huang, 2001). Following the report of gene expression after direct plasmid DNA injection (Wolff et al., 1990), several studies examined the possibility of vaccination using plasmid DNA coding antigens (DNA vaccines) in vivo (Ulmer et al., 1993; Raz et al., 1994; Lagging et al., 1995). Although direct injections of DNA vaccines do induce immune response in smaller animals, the delivery of the DNA to target cells is not optimal, especially in higher animals (Whalen, 1996; Waalen, 1997; Mumper and Ledebur, 2001). And also the amount of DNA used to induce immunity with direct injections is higher (Wang et al., 1998). The potency of immunization using DNA vaccines by topical route or intradermal injection has proven to be quite low (Donnelly et al., 1994; Fan et al., 1999; Shi et al., 1999). Several nonviral methods have been reported to enhance the immunogenicity of DNA vaccines, primarily by increasing the transfection efficiency. Most of these methods are not suitable for routine use either because of the safety concerns or due to the complex procedures and expenses involved (Herweijer and Wolff, 2003). Consequently, there remains a need to improve the skin targeted DNA vaccine delivery for efficient use of DNA vaccines for human immunization.

Recently, DNA vaccine delivery by electroporation has shown to be efficient (Selby et al., 2000; Widera et al., 2000; Zucchelli et al., 2000; Babiuk et al., 2002). Electroporation is a physical method, which involves application of short, high voltage electric pulses to permeabilize the target cell/tissue reversibly for macromolecules such as genes/proteins. Electroporation of cell membrane has been studied extensively and used for DNA transfection of the cells by reversibly permeabilizing the cell membranes (Neumann and Rosenheck, 1972; Kinosita and Tsong, 1977). The efficiency of transformation using electroporation is reported to be greater than the transformation efficiency by chemical methods (Andreason and Evans, 1988). Electroporation has been evaluated in animals and humans for the delivery of chemotherapeutic agents with high efficiency (Mir et al., 1998; Sersa et al., 2000). Furthermore, it has been employed in studies involving delivery of plasmid DNA in vivo to different types of tissues with improved transfection efficiency (Zhang et al., 1996; Muramatsu et al., 1997; Glasspool-Malone et al., 2000; Matsumoto et al., 2001; Blair-Parks et al., 2002; Liu and Huang, 2002). Electroporation mediated DNA vaccine delivery to muscle was shown to be a potential alternative to enhance the immunization efficiency (Widera et al., 2000; Zucchelli et al., 2000). But this procedure involves insertion of electrode needles into the muscle after intramuscular injection, which may not be feasible for use in humans. Cutaneous gene delivery using topical electroporation needs no specialized procedures, as the pulses would be applied topically with tweezer type of electrodes, following the injection of plasmid DNA. This was shown to be efficient for skin targeted gene transfer (Zhang et al., 2002).

Lack of safe and effective methods for delivering DNA vaccines may be the main reason for lower efficacy of these agents observed in higher animals and humans. This could be overcome by developing effective delivery methods that can improve the transfection and expression of DNA vaccines in vivo. In the present study, we investigated the expression and immune re-

sponses with skin targeted DNA vaccine delivery using topical electroporation for the delivery of DNA vaccine against Hepatitis B virus. The effects of electroporation pulse amplitude, pulse length and number were studied. Development of such a non-viral DNA vaccine delivery system is critical for the successful use of these agents for effective immunization against deadly infectious diseases.

2. Materials and methods

2.1. Materials

The plasmid DNA coding for β-galactosidase, gWiz™ β-gal, and the plasmid coding for Hepatitis B surface antigen (HBsAg), pCMV-S (HBV DNA vaccine), were generously provided by Aldevron LLC (Fargo, ND, USA). The plasmids have an optimized human cytomegalovirus (CMV) promoter followed by intron A from the CMV immediate-early (IE) gene and a high efficiency polyadenylation transcription terminator. The purity of the plasmids was checked by measuring the ratio of A_{260}/A_{280}, which was higher than 1.85, and the integrity was checked by agarose gel electrophoresis. The β-galactosidase enzyme assay system with reporter lysis buffer was obtained from Promega Corp. (Madison, WI, USA). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was obtained from Calbiochem (La Jolla, CA, USA). The HBsAg protein (adw subtype) was obtained from Aldevron LLC (Fargo, ND). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). Deionized water obtained with a Barnstead Nanopure Infinity® ultrapure water system (Barnstead, Boston, MA, USA), having resistivity of ≥18 MΩ cm, was used to prepare all solutions and buffers.

2.2. Animals

New Zealand White (NZW) rabbits (*Oryctolagus cuniculus*), 10 weeks old and about 2.0–3.0 kg body weight, were used in the study. The animals were housed in North Dakota State University (NDSU), Department of Veterinary Technology Animal Care Facilities and cared for in accordance with the "Guide for the Care and Use of Laboratory Animals" (NRC, 1996). All the animal experiments were performed according to the protocols approved by Institutional Animal Care and Use Committee of NDSU.

2.3. Effect of electroporation on cutaneous delivery of reporter plasmid/DNA vaccine

The hairs on the back of the rabbits were closely clipped using an electric clipper (Oster Golden A5® single speed clipper using size 40 blade) carefully without any damage to the skin 24 h prior to the beginning of the study. The rabbits were anesthetized using pentobarbital sodium (Nembutal®, 40 mg/kg body weight) given intraperitoneally. An area of the skin was swabbed with 70% (v/v) isopropyl alcohol. A 50 μg (20 μl of 2.5 mg/ml in phosphate buffered saline, PBS) of either reporter plasmid or DNA vaccine was injected intradermally on the back of the rabbit, using a microsyringe with 28-gauge needle (Hamilton Company, Reno, NV, USA), on the back of the rabbit. The injected site was pinched and clamped with stainless steel tweezer electrode (10 mm × 5 mm, CUY 663B, NEPA Gene Co, Chiba, Japan). The electrode was clamped on the skin fold such that the cathode was on the skin side injected with the DNA. The distance between the electrode plates was kept constant at 3 mm.

2.4. Skin electroporation

A square wave electroporator (CUY21 EDIT Version, NEPA Gene Co, Chiba, Japan) was used to generate the electric pulses for skin electroporation. To study the effect of electroporation pulse amplitude, five pulses of 10 ms pulse length with a 1 s interval between each pulse were applied. The pulse voltages investigated were 100, 200 and 300 V with the other pulse parameters kept constant. The group that received injection of plasmid without any electroporation pulses was treated as passive. The group that received neither plasmid nor pulses was treated as control and the group that received just the electroporation pulses (300 V) without any plasmid DNA was treated as electroporation control (E. control). We also studied the effect of clamping the electrode (without any pulses, pressure) to skin after plasmid injection on the cutaneous gene delivery. Whereas for investigating the effect of electroporation pulse length on cutaneous gene delivery, we applied five pulses of 100 V with 1 s in-

terval between each pulse. The pulse lengths investigated were 10, 20 and 30 ms. To investigate the pulse number, we used 10 pulses of 50 V having a pulse length of 30 ms and compared with the other pulse parameters.

2.5. Vaccination of animals

For studying the immune responses with the DNA vaccine, the rabbits were primed with 50 μg of HBV DNA vaccine at the beginning of the study. The animals were then boosted with 50 μg of HBV DNA vaccine on 21st day using the same treatment protocol, which was used to prime the animals.

2.6. Quantification of β-galactosidase expression

The area of the skin treated with the reporter plasmid DNA was excised at different time points, post-delivery, and was analyzed for β-galactosidase expression. The skin samples were collected with a surgical scalpel immediately after euthanizing the animals. The samples were immediately frozen by keeping in liquid nitrogen and then stored at −70 °C until further use. These samples were weighed, minced into small pieces and homogenized with reporter lysis buffer (Promega Corp., Madison, WI, USA) using a homogenizer. During homogenization, the samples were kept on ice. The tissue homogenate was then centrifuged at 15,000 rpm and 4 °C for 15 min and the clear supernatant was separated for further analysis. The total protein content of the samples was measured with the Micro BCA™ protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA). The β-galactosidase activity in the samples was measured using a β-galactosidase enzyme assay system (Promega Corp., Madison, WI, USA). The activity of β-galactosidase was determined by adding an equal volume of assay buffer containing the substrate, *o*-nitrophenyl β-D-galactopyranoside, to the sample and incubating the samples at 37 °C. During incubation, β-galactosidase hydrolyzes the colorless substrate to *o*-nitrophenol, which is yellow. The reaction was terminated by the addition of 1 M sodium carbonate and the absorbance of the samples was measured using Shimadzu UV-1601 spectrophotometer (Shimadzu Corp., Columbia, MD, USA) at 420 nm. Finally, the enzyme activity in the samples was expressed as milliunits/mg of protein.

2.7. In situ histochemical staining of skin for localized expression of β-galactosidase

To demonstrate the localized expression of β-galactosidase, the skin was stained in situ histochemically using X-gal following the method of Alexander and Akhrust (1995). The area of the skin treated with the plasmid and control skin was removed using a surgical scalpel at different time points after euthanizing the rabbit and the skin was cut into 1–2 mm strips. These strips were fixed in freshly prepared ice-cold 2% (v/v) formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$ in phosphate buffered saline (pH 7.4) overnight at 4 °C. The tissues were then washed three times with PBS containing 2 mM $MgCl_2$, 0.1% sodium deoxycholate and 0.02% Nonidet P-40. The tissues were then stained in the dark at 37 °C overnight with 1 mg/ml X-gal (4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside) in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% NP-40 and 0.1% sodium deoxycholate at pH 8. The stained tissues were post-fixed in 10% formalin for 2 h, sectioned into 10 μm sections, counterstained with hemotoxylin and eosin. The stained tissue sections were observed under light microscope (Meiji Microscope, Osaka, Japan) with 40× magnification and photographed by Polaroid® Microcam camera on 339 Polaroid Auto Film.

2.8. Measurement of anti-HBsAg IgG

Blood samples were collected from the rabbits at different time points after immunization and the serum was separated to measure the level of anti-HBsAg IgG. The anti-HBsAg IgG levels in the individual rabbit sera (diluted 1:1000) were measured, in triplicate, by enzyme linked immunosorbent assay (ELISA) using 96-well Nunc-Immuno MaxiSorp microplate (Nalge Nunc International, Rochester, NY). For ELISA, the wells of the plate were coated with 100 μl of 10 μg/ml of HBsAg in coating buffer overnight at 4 °C. After washing three times with PBS-Tween, the plate was blocked with 3% BSA in coating buffer for 2 h at room temperature. The plate was then incubated with rabbit sera at room temperature for 2 h. Sera were removed and the plate was washed with PBS-Tween and was incubated with 1:10,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Pierce biotechnol-

ogy, Rockford, IL, USA.). After washing the conjugate solution, the plate was incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen peroxide (Pierce biotechnology, Rockford, IL, USA.) for half an hour. The reaction was stopped with 2 M H_2SO_4 and the optical density was read at 450 nm. The optical densities of different groups of animals were compared.

2.9. Cellular immune responses

Cellular immune responses with electroporation mediated HBV DNA vaccine was investigated by isolating peripheral blood mononuclear cells (PBMC) from freshly collected heparinized blood by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich). The animals vaccinated with a 50 μg of DNA vaccine using 300 V electroporation pulses were used in this study. The blood samples were collected 9 weeks after the booster dose was administered. Cellular immune responses were studied by measuring lymphoproliferation, induction of IL-4 and IFN-γ levels by PBMC.

2.9.1. Lymphoproliferative assay

The isolated PBMC were cultured at 1×10^5 cells/well in 96-well microtitre plates using RPMI 1640 (10% FBS). These cells were stimulated with HBsAg (1 μg/well) immediately and allowed to grow for 72 h. At the end of incubation, 25 μl of MTT (2 mg/ml) was added to the wells and incubated for another 3 h before dissolving the formazan crystals using acidified isopropanol and finally the absorbance at 570 nm was recorded.

2.9.2. Cytokine measurements

To further characterize the cellular immune responses with the electroporation mediated DNA vaccine delivery, the production of IL-4 and IFN-γ was determined. The PBMCs were treated in the same way as for lymphoproliferation assay and at the end of 72 h incubation, the supernatants were collected for cytokine quantification. ELISA kits were used to measure the levels of IL-4 (Biosource International Inc., Camarillo, CA) and IFN-γ (BD Biosciences Pharmingen, San Diego, CA).

2.10. Experimental design and statistics

The expression of β-galactosidase with different electroporation parameters at a given time point and the cellular immune response were compared using ANOVA, followed by Student's *t*-test. A probability value of less than 0.05 was considered significant.

For DNA vaccine studies, the experiments were designed using the repeated measures design to test the change in treatment means and treatment differences over time. There were two factor levels: treatments and time (treatments: A—control; B—electroporation control [300 V, 10 ms pulses were applied without any DNA]; C—passive; D—100 V, 30 ms; E—200 V, 10 ms; F—300 V, 10 ms; time: 4, 6 and 8 weeks). The treatments were randomly assigned to the experimental subjects. The results were analyzed by the profile analysis method using the multi analysis of variance (MANOVA) approach. The level of significance used was 5%. The results were analyzed with the help of SAS software.

3. Results

3.1. Effect of electroporation pulse amplitude on cutaneous gene delivery

Table 1 shows the effect of electroporation pulse amplitude on the expression of β-galactosidase in the skin, 24 and 48 h after the cutaneous delivery of plasmid DNA. The reporter gene expression following the passive delivery (injection of plasmid without any pulses) and 100 V pulses was not significantly different ($p > 0.05$) from that of the control (no plasmid and no pulses). However, electroporation pulses of 200 and 300 V significantly enhanced ($p < 0.05$) the expression of β-galactosidase in comparison with the control, passive as well as 100 V pulses. The expression with 200 and 300 V electroporation pulses was observed to be 35.79- and 86.29-fold higher, respectively, compared to passive at 24 h post-delivery (Table 1). At 48 h, the expression was 48.38- and 129-fold higher with 200 and 300 V pulses, respectively, in comparison to passive injection (Table 1). This demonstrates the enhanced plasmid delivery to the skin with electroporation pulses, particularly when the pulse amplitude is higher than a threshold voltage.

Table 1
Effect of electroporation pulse amplitude/pulse length on cutaneous gene delivery

Treatment	β-Galactosidase expressed (mU/mg of protein)		Enhancement in gene expression	
	Day 1	Day 2	Day 1	Day 2
Passive	0.02 ± 0.00	0.03 ± 0.01	–	–
100 V, 10 ms	0.10 ± 0.07	0.10 ± 0.06	4.20	3.22
100 V, 20 ms	0.02 ± 0.00	0.02 ± 0.00	0.83	1.12
100 V, 30 ms	0.05 ± 0.01[a]	0.05 ± 0.03	2.25	1.87
200 V, 10 ms	0.85 ± 0.11[b]	1.50 ± 0.17[b]	35.79	48.38
300 V, 10 ms	2.07 ± 0.66[c]	4.00 ± 0.61[c]	86.29	129.03

The values are expressed as mean ± S.D. of three determinations. Enhancement in gene expression $= \frac{\text{quantity of } \beta\text{-galactosidase expressed by electroporation}}{\text{quantity of } \beta\text{-galactosidase expressed by passive}}$.

[a] Siginificantly higher ($p < 0.05$) than that of passive.

[b] Significantly higher ($p < 0.05$) than the passive and 100 V electroporation.

[c] Significantly higher ($p < 0.05$) than passive, 100 and 200 V electroporation.

3.2. Effect of electroporation pulse length on cutaneous gene delivery

Table 1 shows the effect of electroporation pulse length on cutaneous gene delivery on days 1 and 2 post-delivery. Five electroporation pulses of 100 V having different pulse lengths were applied for studying the effect of pulse length. The expression of β-galactosidase with the pulses of 100 V, 30 ms was significantly higher ($p < 0.05$) than that of passive at 24 h following the delivery. However, the enhancement in gene expression was just 2.25-fold than passive (Table 1). Electroporation pulses of 10 and 20 ms did not show any enhancement in the expression of β-galactosidase.

3.3. Effect of number of electroporation pulses on cutaneous gene delivery

Fig. 1 shows the effect of pulse number on cutaneous gene delivery. The effect of number of electroporation pulses on cutaneous gene delivery was investigated using 10 pulses of 50 V, 30 ms; 5 pulses of 100 V, 30 ms and 5 pulses of 300 V, 10 ms. Theoretically, all of the above electroporation parameters should deliver the same amount of electrical charge to the target tissue. We found no enhancement ($p > 0.05$) in the β-galactosidase expression with 50 V pulses in comparison to that of passive. However, the expression of β-galactosidase with 300 V, 10 ms was significantly higher than that of passive and 50 V, 30 ms at 1 and 2 days post-delivery. No effect was observed when electric pulses of 300 V were applied to the skin without any plasmid DNA.

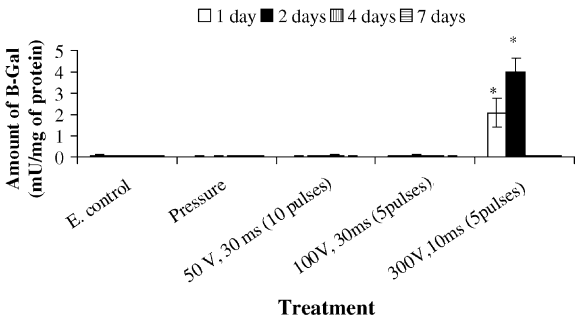

Fig. 1. Effect of electroporation parameters on reporter gene delivery up to 7 days. All the values shown are mean ± S.D. ($n = 3$ animals). *Significantly greater ($p < 0.05$) than that of passive, 50 V, 30 ms, 10 pulses and 100 V, 30 ms.

The clamping of electrodes (pressure), without applying any electroporation pulses, did not show any difference ($p > 0.05$) in the β-galactosidase expression in comparison to that of passive.

3.4. Time-course of transgene expression

The effect of electroporation pulse amplitude on time-course of gene expression shown in Fig. 2 indicates that the expression of transgene in the skin was significantly higher with electroporation pulses of 200 and 300 V on days 1 and 2, post-plasmid delivery. The expression of transgene in the skin reached its peak level in 2 days post-delivery with 200 and 300 V pulses and the expression of β-galactosidase was at control levels after 4 days of plasmid deliv-

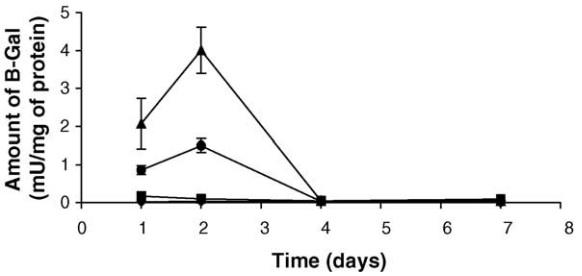

Fig. 2. Effect of pulse amplitude on time-course of expression of β-galactosidase in skin. Key: (♦) passive (injection without pulses), (■) 100, (●) 200 and (▲) 300 V. Five pulses of the specified voltage and 10 ms pulse length were applied. The values shown are mean ± S.D. ($n = 3$ animals).

ery. With any of the treatment conditions, the expression did not differ significantly ($p > 0.05$) from that of the control on days 4 and 7 after the plasmid delivery (Figs. 1 and 2).

3.5. In situ histochemical staining

We studied the localized expression of the β-galactosidase in the skin following the electroporation-mediated delivery of reporter plasmid along with the passive and control. Fig. 3 shows the light micrographs of the skin sections stained with X-gal at 2 days post-delivery. We did not observe any expression in the case of the control (Fig. 3A), with passive delivery and the skin treated with 50 and 100 V electric pulses. However, the skin treated with electric pulses of 200 and 300 V showed local expression of β-galactosidase. Fig. 3(B and C) shows the light micrographs of the skin, which was histochemically stained after plasmid delivery with 200 and 300 V, respectively. The blue colored spots observed due to the histochemical reaction mediated by the expressed β-galactosidase were present in the lower dermis region of the skin. This indicates that the pulses of 200 and 300 V enhanced cutaneous trans-

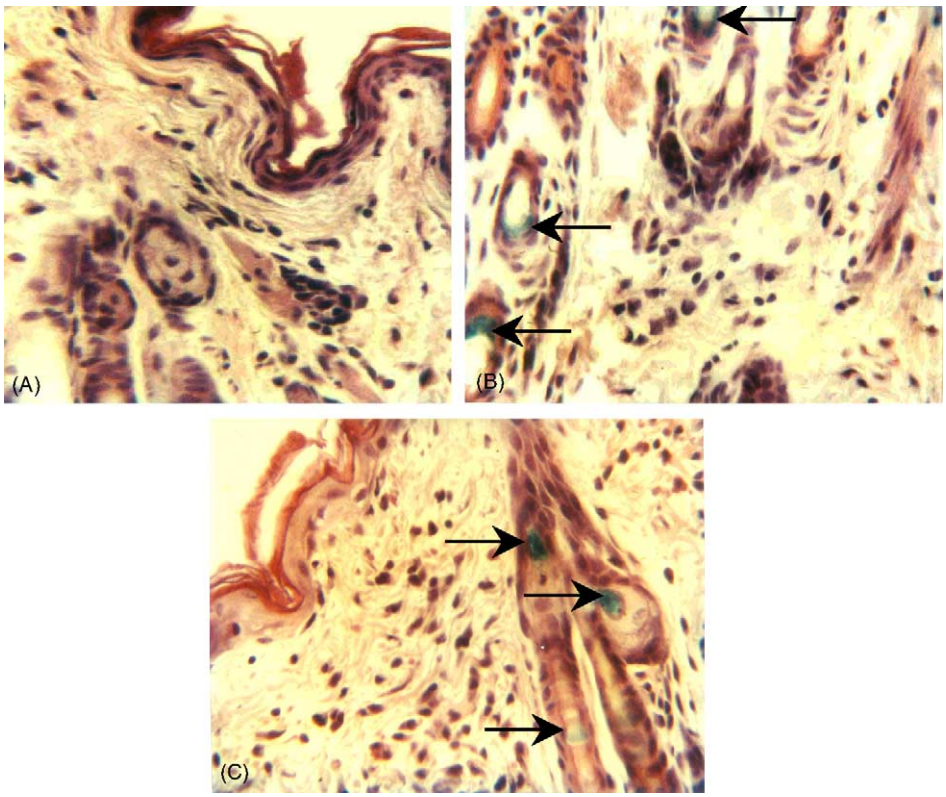

Fig. 3. In situ histochemical staining with X-gal for local expression of β-galactosidase in skin. Key: (A) control skin, (B) 200 V pulses and (C) 300 V pulses. Blue stain indicates the expression of β-galactosidase (as shown by arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fection with the reporter plasmid and corroborates the results obtained from quantification of β-galactosidase expressed in the skin.

3.6. Effect of electroporation on cutaneous delivery of HBV DNA vaccine coding for HBsAg

The optical densities of sera samples (diluted 1:1000) read at 450 nm at the end of ELISA were taken as the immune response and compared. The profile analysis is shown in Fig. 4. The immune response to the electroporation pulses (Fig. 4B) alone is not different from that of control (Fig. 4A). The profile analysis using MANOVA approach indicates that the treatments are significantly different ($p < 0.05$) and follow the hierarchy F, E, D, C, B, A with C being the transition treatment, which implies that the treatments D–F are significantly different ($p < 0.05$) from that of A–C.

These results indicate that electroporation mediated cutaneous DNA vaccine delivery enhanced the humoral immune response in rabbits compared to the passive delivery. In the case of β-galactosidase reporter plasmid delivery, the expression was found to be significantly higher ($p < 0.05$) with 100 V, 30 ms; 200 V, 10 ms and 300 V, 10 ms pulses than the passive. This might be true with DNA vaccine also. Therefore, the enhanced immune response may be due to increased transfection

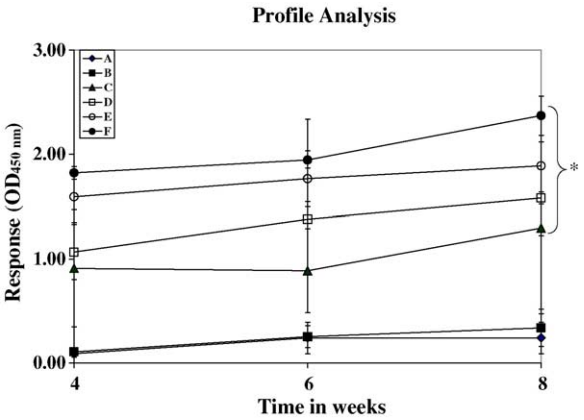

Fig. 4. Profiles of immune response (anti-HBsAg IgG levels) with different treatments. The mean values (±S.D.) for the response was plotted against the time. Key: (A) control; (B) electroporation control; (C) passive; (D) 100 V, 30 ms; (E) 200 V, 10 ms; (F) 300 V, 10 ms ($n = 3$ in each group). *Significantly different from the other treatments.

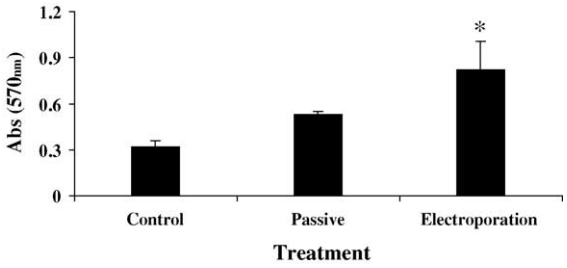

Fig. 5. Lymphoproliferation assay. Proliferation of PBMC was determined using MTT assay. PBMC were stimulated with 1 μg of HBsAg. All the values are shown as mean ± S.D. ($n = 3$). *Significantly greater ($p < 0.05$) than that of control and passive.

and expression of the antigen in the skin, which induces production of high levels of antibody. Higher antibody levels were observed with 300 V pulses, as soon as 1 week after the booster dose (i.e., at 4 weeks) in comparison to the passive injection and the higher antibody levels were maintained up to atleast 8 weeks (the end of the study period) without any further DNA vaccine administrations.

3.7. Cellular immune responses

The group that showed highest antibody levels (Group F) was studied for the cellular immune responses and compared with the control (Group A) and passive group (Group C). Fig. 5 shows the proliferation of PBMCs isolated from the passive and electroporation treated animals upon stimulation with HBsAg. The animals vaccinated using electroporation displayed significantly higher ($p < 0.05$) proliferation than the passive and control groups. Cytokine profiling was also performed to distinguish Th1 versus Th2 response. Fig. 6(A and B) shows the IL-4 and IFN-γ levels, respectively. The IL-4 levels were not different from that of the control group. However, the IFN-γ levels of the electroporation group were found significantly higher ($p < 0.05$) than those of both control and passive groups.

4. Discussion

Electroporation mediated gene delivery is shown to be highly efficient in various studies with different mammalian cells in vitro (Toneguzzo and Keating, 1986; Andreason and Evans, 1988; Espinos et al.,

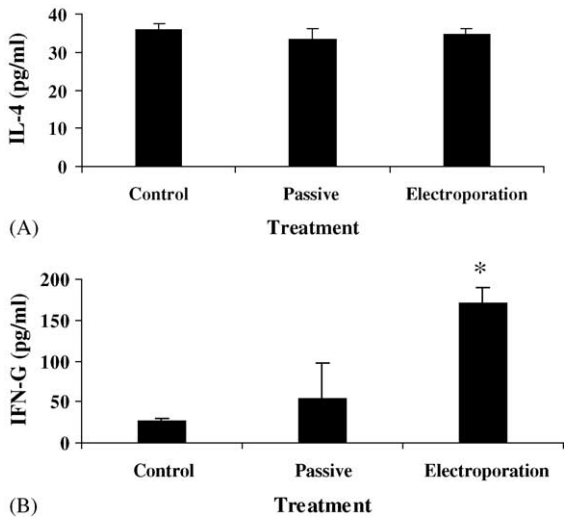

Fig. 6. Measurement of cytokine levels (A) IL-4 levels determined in cell culture supernatants of PBMC stimulated with 1 μg of HBsAg. (B) IFN-γ levels determined in cell culture supernatants of PBMC stimulated with 1 μg of HBsAg. All the values are shown as mean ± S.D. ($n = 3$). *Significantly greater ($p < 0.05$) than that of control and passive.

2001). Recently, this method has been shown to be effective for gene transfer in vivo in many types of tissues including the skin (Zhang et al., 1996; Muramatsu et al., 1997; Glasspool-Malone et al., 2000; Matsumoto et al., 2001; Blair-Parks et al., 2002; Liu and Huang, 2002). Most of these in vivo studies used needle electrodes to administer the electroporation pulses. In the present in vivo study, using NZW rabbits, we demonstrated that the intradermal injection of plasmid DNA vaccine followed by electropermeabilization of the site using noninvasive electrodes enhanced the cutaneous transfection and subsequently immune responses. The expression with the 100 V electric pulses did not show any enhancement. This may be due to the well-known fact that the electric pulses applied should be higher than a threshold voltage in order to permeabilize the cells/tissue for macromolecules to enter the cells (Rols and Teissie, 1990; Neumann et al., 1999). In this case the threshold value was higher than 100 V, as the expression with 200 V was significantly higher than the control as well as passive and 100 V pulses. This is corroborated by the results of in situ histochemical staining of the skin samples, where the skin treated with 100 V did not show any expression. However, the skin showed local expression of β-galactosidase after plasmid delivery using 200 and 300 V. The expression was observed in the dermis area. This indicates that there was an increased cutaneous transfection with electroporation pulses of 200 and 300 V. Increased pulse length has shown enhanced cutaneous gene delivery as evidenced by the enhanced expression at 24 h, but the effect was far less than that of pulse amplitude. The increase in the expression was a mere 2.25-fold than the passive. Higher number of pulses at a lower voltage did not show any effect on cutaneous gene delivery. The time course of transgene expression demonstrated that the expression was transient with the highest levels observed at 2 days post-delivery in the case of 200 and 300 V pulses and the expression of β-galactosidase disappeared with the activity at control levels by 4 days post-delivery. Similar results of transient gene expression were reported in several studies with the non-viral gene delivery (Alexander and Akhrust, 1995; Dujardin et al., 2001). The application of electroporation pulses without plasmid DNA has no effect on the expression of β-galactosidase. This further confirms that the enhanced expression was due to the increased transfection of the plasmid DNA with electroporation mediated gene transfer.

Electroporation pulses have been reported to increase the gene expression up to 100-fold compared with the injection of naked plasmid DNA without electroporation (Aihara and Miyazaki, 1998). In the present study, the increase in the transgene expression with the electroporation mediated cutaneous gene transfer was more than 100-fold when 300 V pulses were applied. These results are in agreement with the previously reported results (Glasspool-Malone et al., 2000). Our results demonstrate the enhanced cutaneous non-viral gene delivery by topical electroporation using tweezer electrodes, without any visible damage to skin (a detailed study is being undertaken to address the safety of the technique). The present study also demonstrates that the electroporation pulses of higher pulse amplitude enhance transfection than that of pulses having higher pulse length or number of pulses (Fig. 1), when the total electrical charge delivered was the same.

Electroporation parameters that showed enhanced expression in the reporter plasmid delivery studies were tested for their efficacy with HBV DNA vaccine. The antibody levels with the electroporation mediated DNA vaccine delivery were higher than that of the passive

(injection without any pulses). Electroporation pulses enhanced the humoral immune response at the earliest measurement time (4 weeks) and maintained higher antibody levels than passive group until the end of the study (8 weeks), showing the efficacy of electroporation. Lymphoproliferation results show that the electroporation mediated DNA vaccine delivery induces larger cellular immune responses than the passive delivery. The elevated levels of IFN-γ with the electroporation indicates the development of Th1 type of immune response with the electroporation mediated DNA vaccine delivery, which might be helpful to eliminate the virus from the body. Earlier in this study, we have observed that these pulses enhanced the delivery of a reporter plasmid although transiently. Therefore, the enhanced immunogenicity with electroporation might be due to the increased transfection and expression of the DNA vaccine in the skin with these electroporation parameters. We would expect similar enhancement of immune responses in humans as well by increasing the transfection of DNA vaccine. Even though the expression was observed to be transient, the amount of antigen expressed may be sufficient to induce long lasting and strong immune responses (Donnelly et al., 1997; Selby et al., 2000). The enhanced immune responses with electroporation mediated DNA vaccine delivery may also be due to the direct transfection of APCs such as Langerhan's cells and dermal dendritic cells present in the skin, thereby, greatly enhancing the efficacy of DNA vaccines in inducing cellular immune responses. Further studies aimed at optimizing the electrical parameters for gene transfer and assessing the safety of this technique are warranted.

5. Conclusions

The major problem with the non-viral gene transfer is the low levels of transgene expression. The present study demonstrates that the injection of naked plasmid DNA followed by topical electroporation improves transfection efficiency in vivo by several folds, especially after a threshold voltage. The results show the efficacy of skin targeted DNA vaccine delivery using electroporation to induce strong humoral as well as cellular immunity against a disease. Although the expression of the transgene was found to be transient, it can be a potentially useful method for skin targeted non-viral gene delivery, especially for the delivery of DNA vaccines in genetic immunizations.

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