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ScienceDirect
Journal of Controlled Release 124 (2007) 81–87
journal of controlled release
www.elsevier.com/locate/jconrel
GENE DELIVERY

Optimisation of intradermal DNA electrotransfer for immunisation

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Received 18 June 2007; accepted 14 August 2007
Available online 19 August 2007

Abstract

The development of DNA vaccines requires appropriate delivery technologies. Electrotransfer is one of the most efficient methods of non-viral gene transfer. In the present study, intradermal DNA electrotransfer was first optimised. Strong effects of the injection method and the dose of DNA on luciferase expression were demonstrated. Pre-treatments were evaluated to enhance DNA diffusion in the skin but neither hyaluronidase injection nor iontophoresis improved efficiency of intradermal DNA electrotransfer. Then, DNA immunisation with a weakly immunogenic model antigen, luciferase, was investigated. After intradermal injection of the plasmid encoding luciferase, electrotransfer (HV 700 V/cm 100 μs, LV 200 V/cm 400 ms) was required to induce immune response. The response was Th1-shifted compared to immunisation with the luciferase recombinant protein. Finally, DNA electrotransfer in the skin, the muscle or the ear pinna was compared. Muscle DNA electrotransfer resulted in the highest luciferase expression and the best IgG response. Nevertheless electrotransfer into the skin, the muscle and the ear pinna all resulted in IFN-γ secretion by luciferase-stimulated splenocytes suggesting that an efficient Th1 response was induced in all case.

Keywords: Skin; DNA; Plasmid; Electrotransfer; Immunisation

1. Introduction

The skin is an attractive target for antigen delivery and immunisation [1]. It is accessible, easy to assay and to remove if problems occur. After gene transfer, the encoded protein may exert local or systemic effect. As the half-life of skin cells is short, other organs and particularly the muscle are more appropriate for long term expression of proteins [2]. Nevertheless in the context of vaccination, long term expression is not required. The skin acts as an immunological barrier, containing a high density of immunocompetent cells. Although Langerhans cells represent only 1 to 4% of the total cells in the epidermis, it is believed that they cover over 25% of the skin area [3]. These antigen-presenting cells greatly contribute to develop immune responses after DNA delivery.

For gene therapy, the use of non-viral DNA offers several advantages: (i) lack of immunogenicity of the vector, (ii) absence of size limit for the therapeutic cassette, (iii) simpler GMP (Good Manufacturing Practice) production and (iv) improved safety and toxicity profiles. However, topical application or intradermal injection of naked DNA has so far resulted in low transgene expression [4,5]. This is why different chemical, mechanical and physical methods have been developed to enhance non-viral DNA delivery to skin cells (for review [6,7]).

Electrotransfer is one of the most efficient and promising methods of non-viral gene transfer. It involves plasmid injection into the tissue and application of electric pulses. It is hypothesised that the electric field plays a double role in DNA transfection. First, it transitorily disturbs membranes, and thus increases cells permeability. Second, it promotes electrophoresis of negatively charged DNA [8,9]. However, the relation between these different effects of the electric field and transfection efficiency is controversial and still to be elucidated [10]. Volts, duration of pulses and the more appropriate type of electrodes must be evaluated for each tissue. A previous study has demonstrated that a combination of a short high-voltage pulse (HV) and a long duration low-voltage pulse (LV) was

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doi:10.1016/j.jconrel.2007.08.010

efficient for DNA electrotransfer in the skin [11]. For muscle transfection, we used a classical and validated procedure consisting in delivery of a series of identical electric pulses [12]. Widera et al. demonstrated that electroporation increased DNA vaccine delivery and immunogenicity in the muscle [13].

The aim of this research was to optimise intra-dermal DNA immunisation by electrotransfer. The effect of parameters such as the injection method or the dose of DNA was investigated. The effect of different pre-treatments to promote plasmid distribution before the electrotransfer was also studied. The first pre-treatment consisted in the application of an iontophoretic current to enhance DNA diffusion. Iontophoresis consists in using a low electric field to promote the movement of ions into tissues. This technique has been used for many years to deliver drugs or oligonucleotides into the eye or into the skin [14–16]. The second pre-treatment consisted in hyaluronidase injection to break down extra-cellular matrix components and facilitate plasmid distribution. Hyaluronic acid is an ubiquitous glycosaminoglycan of the extra-cellular matrix present around muscular fibres and in the skin, which contains approximately one-half of the hyaluronic acid of the body [17]. As a pre-treatment, bovine hyaluronidase has been shown efficient to enhance electrotransfer into the muscle [18], but its efficacy into the skin had not yet been investigated.

The immune response was evaluated using luciferase as a model antigen. Luciferase gene is widely used as reporter gene. Usually this protein, which is expressed intra-cellularly, induces no immune response. However, immune response occurs when high luciferase expression is reached in the muscle [19]. Luciferase was chosen because we considered that a protein with limited immunogenicity was a better model of the tumor antigens, which are often poorly immunogenic. Because the route and gene administration parameters influence the immune response [20–23], electrotransfers into skin, muscle and ear pinna were performed.

2. Materials and methods

2.1. Plasmid DNA

Electrotransfer was performed using the pGL3 Luciferase Reporter Vector (Promega Benelux, Leiden, Netherlands) containing the cytomegalovirus (CMV) promoter or the CMV-actin-globin (CAG) promoter for the optimisation or the immunisation studies respectively. Plasmids were prepared using Endo-Free Qiagen Gigaprep kit, according to the manufacturer's protocol. The quality of resulting plasmid was assessed by the ratio of light absorption (260 nm/280 nm) and by 1% agarose gel electrophoresis. Light absorption at 260 nm was used to determine DNA concentration. All plasmid dilutions were done in Phosphate Buffer Saline (PBS). Plasmids were stored at −20 °C before use.

2.2. Animals

Except for the vaccination study, we used female NMRI mice, 6 weeks old (Université Catholique de Louvain, Brussels, Belgium). Mice were anaesthetized with 40 µl of a mixture of ketamine 50 mg/ml (Ketalar, Pfizer, Brussels, Belgium) and xylazine 5,6 mg/ml (Sigma, Bornem, Belgium). For the vaccination study, we used female BALB/c mice, 6 weeks old at the beginning of the experiment (Janvier, Le Genest St Isle, France). They were anaesthetized with 20 µl of the ketamine/xylazine mixture. The skin of the abdomen or the muscle was shaved 1 day prior to the experiments with a depilatory cream (Veet for sensitive skin, Belgium), in order to thoroughly remove all the hair.

All experimental protocols in mice were approved by the Ethical Committee for Animal Care and Use of the faculty of Medicine of the Université Catholique de Louvain.

2.3. Plasmid injection and electrotransfer

For intra-dermal electrotransfer, the plasmid was injected into the dermis using a Hamilton syringe with a 30-gauge needle. Unless stated, we injected 15 µl intradermally in two different sites, with a distance of about 5 mm (double injection protocol). Then, a cutaneous fold was performed and the sites of injection were placed between plate electrodes, 2 mm spaced. To study the effect of the injection method, the double injection protocol was compared to the injection of one 30 µl [11] or 100 µl volume [24]. The diameter of the bubble for each injection volume was measured by a caliper. After injection of 100 µl, we applied 4 mm spaced electrodes around the bubble formed by the injected volume (Fig. 1). A short HV pulse (700 V/cm 100 µs), immediately followed by a LV pulse (200 V/cm 400 ms) were applied approximately 1 min after plasmid injection [11]. There was no time interval between HV pulse and LV pulse. Except for the study of the dose–effect and for the immunisation studies, the dose of DNA was 12 µg. For immunisation studies, the dose injected into the skin was 50 µg.

For electrotransfer into the ear, we injected two volumes of 15 µl into the external side of the ear pinna, for a total dose of 50 µg, using a Hamilton syringe with a 30-gauge needle. The

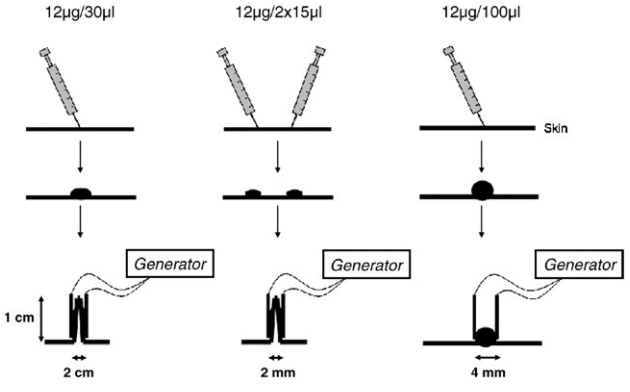

Fig. 1. Effect of intradermal DNA injection method on gene expression after electrotransfer. Illustration of the differences between the three protocols: injection of 30 µl followed by electrotransfer with 2 mm spaced electrodes (left), double injection protocol with two 15 µl injected volumes followed by electrotransfer with 2 mm spaced electrodes (centre) and injection of one 100 µl volume followed by electrotransfer with 4 mm spaced electrodes (right). The total injected dose was 12 µg of DNA in both cases.

ear was placed between 2 mm spaced electrodes. Then, we applied HV–LV pulses (700 V/cm 100 μs, 200 V/cm 400 ms).

For electrotransfer into the muscle, we injected a volume of 30 μl into the tibial cranial muscle, and we placed the leg between 4 mm spaced electrodes. We delivered 8 square-wave electric pulses (200 V/cm 20 ms 2 Hz) [12,19]. For pre-treatment studies with iontophoresis or hyaluronidase, the dose of DNA injected into the muscle was 1 μg. For immunisation study, the dose injected into the muscle was 50 μg.

For all experiments, conductive gel was used to ensure electrical contact with the skin (EKO-GEL, ultrasound transmission gel, Egna, Italy). The pulses were delivered by a Cliniporator system (Cliniporator, IGEA, Carpi, Italy) using 2 mm or 4 mm plate electrodes (IGEA, Carpi, Italy).

2.4. Iontophoresis pre-treatment

Just after injection of plasmid, the skin or the muscle was placed between two plate electrodes and a 0.5 mA/cm^2 current [25] was applied during 30 min. Conductive gel was applied between the electrodes and the skin. Electrotransfer was performed as described above approximately one minute after the end of this pre-treatment.

2.5. Hyaluronidase pre-treatment

Two hours before plasmid injection and electrotransfer, we injected either 2×25 μl into the dermis or 50 μl into the tibial cranial muscle of a 300 μg/ml saline solution of bovine hyaluronidase (Sigma H4272, 750–1500 units/mg). Control groups were treated with saline solution (NaCl 0.9%) [18]. Injection of DNA and electrotransfer were performed as described above.

2.6. Luciferase assay

Two days after the electrotransfer, the mice were sacrificed and the electrotransfered areas of the skin or the tibial cranial muscles were removed. The samples were cut into pieces and homogenized in 1 ml cell culture lysis reagent solution (CCLR, Promega Benelux) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) using a Duall® tissue grinder (Cofraz, Essene, Belgium). After centrifugation at 12,000 g for 10 min at 4 °C, we assessed the luciferase activity of 10 μl of the supernatant (diluted in CCLR if needed) after the addition of 50 μl of Luciferase Assay Substrate (Promega), using a TD-20/20 luminometer (Promega) (adapted from [11]). The results were expressed in relative light units (RLU). Dilutions of purified firefly luciferase protein (Sigma L4899) were used as standard. Based on standard curve performed for each experiment, 10,000 RLU represent the luciferase activity of approximately 330 pg of luciferase protein.

2.7. Immunisation studies

BALB/c mice were injected with 50 μg of plasmid encoding luciferase and electric pulses were applied as described. Mice were also immunised by intradermal injection of 1 μg of luciferase recombinant protein (Promega). The choice of the protein dose is detailed in the dose–effect results section.

Two boosts were similarly applied two and four weeks after the priming. Two weeks after the last boost, blood samples were collected by retro orbital puncture and sera were separated by centrifugation at 700 g for 20 min at 4 °C. Anti-luciferase antibodies were measured by ELISA [19]. For the determination of total immunoglobulin G (IgGtot) concentration, we performed the assay in duplicate and we converted the mean absorbance value for each mouse to IgGtot concentration using a monoclonal anti-luciferase antibody standard (Sigma L2164). Limit of quantification (LOQ), defined as the blank mean value plus 10 standard deviations of the blank mean, was 0.007 μg/ml. Isotypes of anti-luciferase antibodies (IgG1, IgG2a) were determined using appropriate secondary antibodies (LO-MG1-13, LO-MG2A-9 and LO-MGCOC-2 labelled with peroxidase, IMEX, UCL, Brussels, Belgium).

For cytokine assays, mice were sacrificed, and their spleens were removed aseptically. Splenocytes were adjusted to a concentration of 5×10^6 cells/ml and cultured 500 μl per well in 48-well tissue culture plates (Becton Dickinson, Belgium) in RPMI 1640 medium supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% sodium pyruvate, 5×10^{-5} M 2-mercapto-ethanol and 10% MEM (Gibco, Merelbeke, Belgium). Cells were stimulated by the addition of 10 μg of luciferase recombinant protein (Promega) per well. Unstimulated cells were used as control. Cells were incubated at 37 °C in a humidified 5% CO_2 incubator and supernatants were collected either after 48 h for interferon-gamma (IFN-γ) assay or after 72 h for interleukin-4 (IL-4) assay (adapted from [26]). We measured cytokine concentrations in the supernatants using mouse IFN-γ and IL-4 DuoSet ELISA development kits (R&D Systems Europe Ltd, Abingdon, UK) according to the manufacturer's protocols.

2.8. Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). *T*-test or one-way ANOVA and Tukey's post test were performed on log normalised data to demonstrate statistical

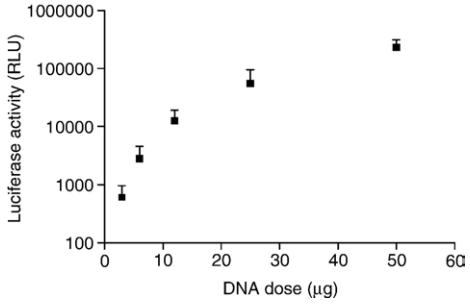

Fig. 2. Effect of DNA intradermal dose on gene expression after electrotransfer. Squares represent the mean values (±SEM) of luciferase activity determined biochemically from tissue sample after injection of 3, 6, 12, 25 or 50 μg of DNA, $n=5$. Statistical analysis: one-way ANOVA.

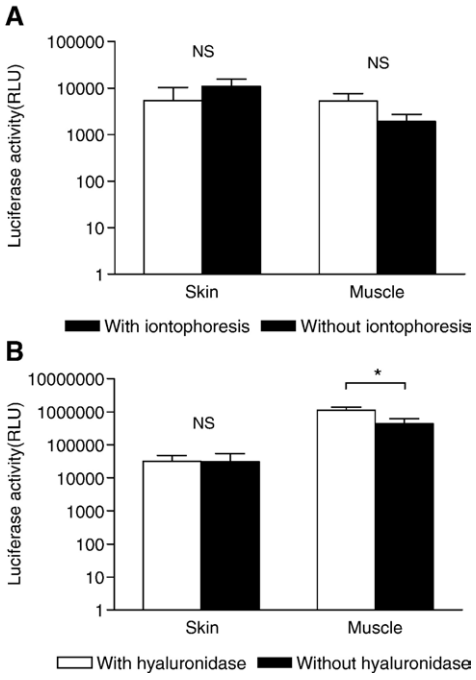

Fig. 3. Influence of pre-treatment with iontophoresis (A) and hyaluronidase (B) on gene expression after electrotransfer. Bars represent the mean values (±SEM) of luciferase activity determined biochemically from tissue sample, $n=10$ (Fig. 3.A) and $n=7$ (B). Statistical analysis: t-test. NS, not significant. $^{*}P<0.05$.

differences ($P<0.05$), using the software GraphPad Prism 4 for Windows.

3. Results

3.1. Optimisation of the intradermal electrotransfer injection method

To define the best method of DNA injection for DNA electrotransfer into the skin, different injection protocols were compared, injection of two volumes of 15 µl of the plasmid solution, injection of a volume of 30 µl or injection of a larger volume of 100 µl (Fig. 1). Injection of 15, 30 µl and 100 µl resulted in formation of a bubble with a diameter of 2.78±0.32 mm, 3.52±0.12 mm and 5.45±0.37 mm respectively ($n=3$).

We observed significant differences between these treatments. Luciferase expression after the injection of two 15 µl volumes resulted in 3-fold increase compared to only one injection of 30 µl. The injection of a 100 µl volume induced 20-fold higher luciferase expression level compared to the double injection protocol. Of note, with this treatment mice skin presented burns at the points of contact between the extremities of the electrodes and the skin. The double injection protocol for a total dose of 12 µg DNA resulted in a measured expression of about 40 ng of the protein and seemed to be well tolerated (neither burn nor red spot beyond the electrotransfered area). Hence, this injection protocol was considered as the best one for our experiments.

3.2. Effect of DNA dose on intradermal electrotransfer

In order to study the influence of the DNA dose on the gene expression after electrotransfer into the skin and to determine the optimal dose to use, a dose–effect study was performed. Expression of luciferase was determined two days later, and a one-way ANOVA statistical analysis underlined the influence of the dose (Fig. 2). The expression increased with the dose up to 25 µg of DNA and then levelled off. Based on these results, we injected 12 µg DNA per mice to study the effect of pre-treatments. The injection of 50 µg of DNA resulted in mean luciferase activity measure of 2.3×10^5 RLU. This approximately corresponded to the activity measure of 1 µg of purified firefly luciferase protein. Thus, these doses of 50 µg DNA and 1 µg protein were chosen for the immunisation studies.

3.3. Iontophoresis and hyaluronidase pre-treatment studies

In an attempt to further improve the efficacy of gene transfer, one electrical and one enzymatic pre-treatment were used to promote the diffusion of the plasmid into the tissue before the electrotransfer.

Iontophoresis was used to promote electrophoresis driven DNA diffusion in the skin. Following the injection of DNA into skin or muscle, we applied a 0.5 mA/cm^2 current during 30 min before electrotransfer. Significant enhancement of gene expression was detected neither in the skin nor in the muscle (Fig. 3A).

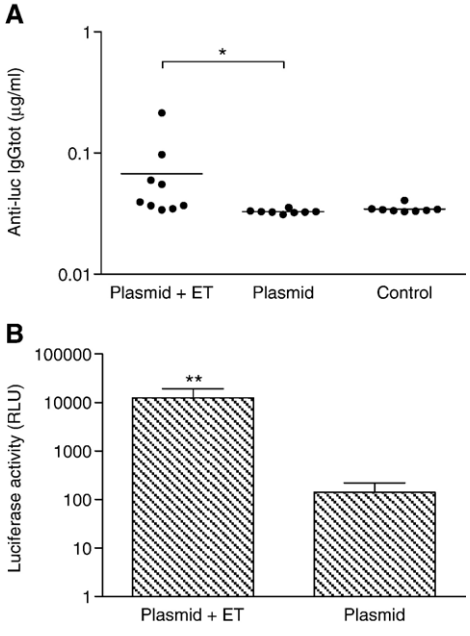

Fig. 4. Immune response after intradermal injection of plasmid with or without electrotransfer (ET). Panel A: Determination of anti-luciferase IgGtot concentrations. Circles represent individual concentration 6 weeks after the first immunisation and lines represent the mean values. Mice of control group were immunised with PBS. Statistical analysis: t-test. $^{*}P<0.05$. Panel B: Expression after injection of 12 µg pGL3CMVLUC in the skin with or without electrotransfer. Bars represent the mean values (±SEM) of luciferase activity determined biochemically from tissue sample, $n=5$. Statistical analysis: t-test. $^{**}P<0.01$.

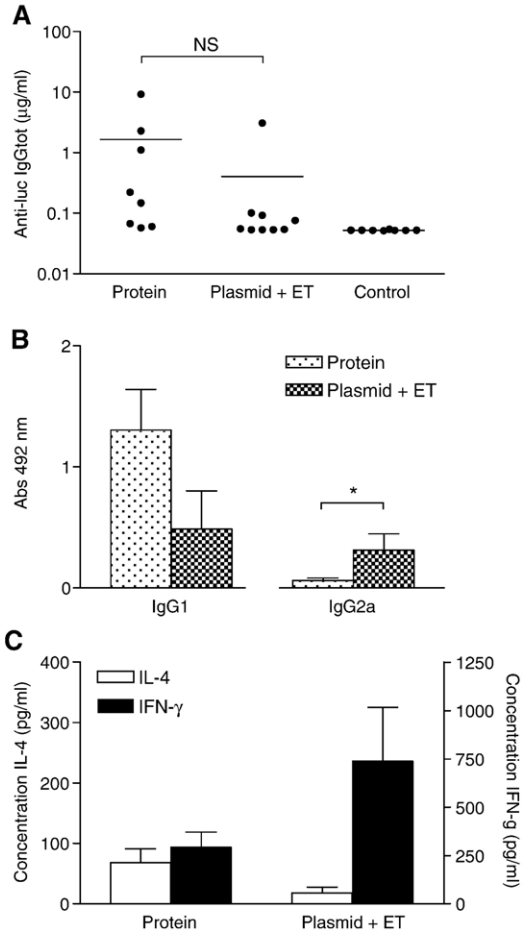

Fig. 5. Immune response after immunisation with intradermal protein or with intradermal DNA followed by electrotransfer (ET). Protein group was immunised by intradermal injection of 1 µg of luciferase recombinant protein while plasmid+ET group was immunised by electrotransfer of 50 µg of plasmid into the skin. Panel A: Determination of anti-luciferase IgGtot concentrations. Circles represent individual concentration 6 weeks after the first immunisation and lines represent the mean values. Mice of control group were immunised with PBS. Statistical analysis: *t*-test. NS, not significant. Panel B: Determination of antibody isotypes in sera, 6 weeks after the first immunisation. Bars represent the mean values of absorbance for responding mice (±SEM). Sera samples were diluted 1/10. Statistical analysis: *t*-test. $*P<0.05$. Panel C: Concentrations of IL-4 and IFN-γ determined after mice sacrifice and luciferase-stimulated splenocyte culture. Bars represent the mean values for responding mice (±SEM). Statistical analysis: *t*-test.

Skin and muscle were also pre-treated by injection of bovine hyaluronidase 2 h before electrotransfer to decrease the viscosity of extra-cellular matrix and facilitate DNA diffusion. As expected, significant enhancement of gene expression was observed after pre-treatment of the muscle [18], but the expression after pre-treatment of the skin remained unchanged (Fig. 3B). A two-fold increase of the hyaluronidase concentration in the skin did not have any influence on this result (data not shown).

3.4. Anti-luciferase immunisation studies

After immunisation by intradermal injection of 50 µg pGL3CAGLUC, anti-luciferase IgGtot concentration measured was similar to PBS control. When electrotransfer was applied after plasmid intradermal injection, we showed a significant increase of anti-luciferase IgGtot antibodies (Fig. 4A). Expression of luciferase was two log-fold higher when intradermal electrotransfer was applied (Fig. 4B).

The immune response after immunisation with the recombinant protein was compared to the response after DNA vaccination. The concentrations of anti-luciferase total immunoglobulin G (IgGtot), and the number of responding mice tended to be lower after DNA immunisation, but the difference was not statistically significant (Fig. 5A). Concerning IgG isotypes, we showed an IgG1 decrease and an IgG2a increase with intradermal electrotransfer of plasmid compared to

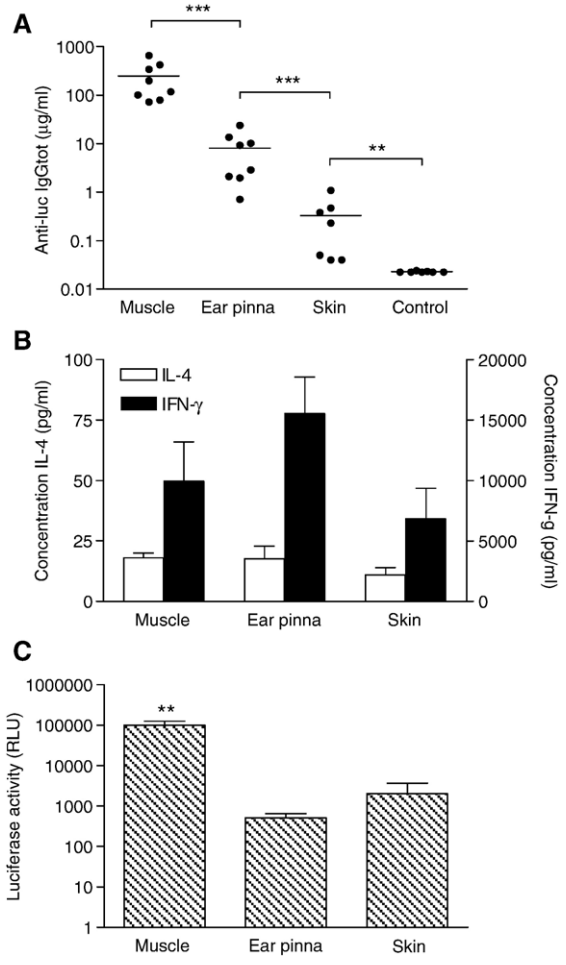

Fig. 6. Immune response after DNA electrotransfer immunisation into skin, ear pinna or muscle. Panel A: Determination of anti-luciferase IgGtot concentrations. Circles represent individual concentrations 6 weeks after the first immunisation and lines represent the mean values. Mice of control group were immunised with PBS. Statistical analysis: one-way ANOVA and Tukey's post test. $**P<0.01$, $***P<0.001$. Panel B: Concentrations of IL-4 and IFN-γ determined after mice sacrifice and luciferase-stimulated splenocytes culture. Bars represent the mean values for responding mice (±SEM). Statistical analysis: one-way ANOVA and Tukey's post test. Panel C: Expression after electrotransfer of 12 µg pGL3CAGLUC in the muscle, the skin or the ear pinna. Bars represent the mean values (±SEM) of luciferase activity determined biochemically from tissue sample, $n=5$. Statistical analysis: one-way ANOVA and Tukey's post test. $**P<0.01$ versus ear pinna and skin.

intradermal injection of recombinant protein (Fig. 5B) suggesting a Th1-shifted response with DNA. The cytokine profile obtained on luciferase-stimulated culture of splenocytes showed lower concentration of IL-4 and higher concentration of IFN-γ in the case of intradermal electrotransfer of plasmid, confirming the Th1 orientation of the response (Fig. 5C).

In order to compare electrotransfer into the skin to other accessible routes for DNA delivery, we performed a study where the immune response after electrotransfer in the skin was compared to the response after electrotransfer in the ear pinna and the muscle. After priming and two boosts of 50 μg DNA, immunisation performed into the muscle showed the highest anti-luciferase IgGtot concentration (Fig. 6A). Electrotransfer into the muscle showed also a higher expression for equal DNA dose (Fig. 6C). The IgG1 and IgG2a isotypes were both present after electrotransfer into the muscle, the ear pinna and the skin, but the IgG1/IgG2a ratio varied with the site of delivery. The highest IgG1/IgG2a ratio was measured for the intradermal route and the lowest for the intra-pinna route (data not shown). Immunisation into the muscle, the ear pinna and the skin resulted in IFN-γ secretion by luciferase-stimulated splenocytes suggesting an efficient Th1 response in all case. The production of IL-4 was low for the three groups of mice (Fig. 6B).

4. Discussion

Increasing knowledge in the field of molecular biology has led DNA vaccine to become an accessible and attractive approach, very promising in particular in the field of cancer therapy (for review [27]). However, the development of this type of vaccine requires appropriate DNA delivery technologies.

The aim of our study was to optimise intradermal DNA electrotransfer for immunisation based on the immunological properties of the skin [1] and the high efficacy of DNA electrotransfer to enhance transfection [8–11] and immune response [13].

The injection method influenced the luciferase expression after electrotransfer in the skin but also the appearance of side effects like burns. Injection method influence adding to the well-known influence of pulses parameters [9,11] make results from different publications and research groups difficult to compare. However, burns at the levels of the electrode contacts with the skin were also observed by Pedron-Mazoyer et al. after injection of 100 μl of plasmid solution and delivery by 6 mm electrodes of 8 pulses lasting 20 ms above 210 V [24]. A close contact between the DNA-containing bubble and the electrodes without any contact with the skin seems to be optimal. Either a skin fold with two injection sites close to two parallel plate electrodes or a new four plate electrode model surrounding the bubble [28] have been shown to be efficient and safe for electrotransfer after intradermal injection.

The enhanced gene expression after intradermal injection of a larger volume or after repartition of plasmid solution on two injection sites could be an effect of plasmid distribution into the tissue resulting in DNA transfer of more cells. A similar effect was described in intramuscular electrotransfer [29]. Two pre-treatments were investigated in order to promote plasmid distribution before doing electrotransfer but neither iontophoresis nor hyaluronidase improved luciferase activity after electrotransfer into the skin, in contrast to muscle hyaluronidase pre-treatment as already described [18]. We hypothesised that the effect of hyaluronidase is not sufficient to modify the viscosity of the skin or the DNA diffusion in this tissue.

Electrotransfer enhanced the immunogenicity of DNA vaccine even with a low immunogenic antigen. We demonstrated that our electrotransfer protocol led to anti-luciferase immune response contrary to intradermal injection of plasmid without electrical pulses. The higher luciferase expression measured in the skin when electrotransfer was applied explains at least partially this difference of immunogenicity.

The higher IgG2a and IFN-γ concentrations observed after immunisation by plasmid electrotransfer as compared to immunisation by recombinant protein suggested development of a Th1 immune response after DNA vaccination only. As reported previously, CpG motifs present on plasmids, act as a danger signal and provide a Th1-biased response [30,31]. The localisation of the immune protein (intracellular after DNA vaccination and extracellular after protein vaccination) could also explain the difference between these two vaccination protocols [32].

Beside the choice of DNA or protein vaccine, the choice of the delivery route is also essential. DNA vaccination into the skin, the ear pinna and the muscle led to an immune response. Intramuscular immunisation resulted in the highest production of immunoglobulin nevertheless immunisation into the muscle, the ear pinna and the skin led to IFN-γ response. These immunoglobulin concentration differences might be partially explained by the magnitude and the duration of expression [33], which varies from one tissue to another after electrotransfer of a same plasmid dose. Luciferase expression after electrotransfer into the muscle was significantly higher confirming this hypothesis. The ear pinna exhibits two layers of epidermis and dermis, separated by cartilage, thereby doubling the amount of antigen presenting cells [21]. The increased number of these cells could explain the obtained response.

To conclude, we have optimised DNA electrotransfer into the skin and demonstrated the importance of the method of injection. Iontophoretic pre-treatment and pre-treatment with hyaluronidase did not influence intradermal DNA electrotransfer efficiency. Immunisation by injection of two volumes of 15 μl of luciferase plasmid followed by electrotransfer was efficient to induce anti-luciferase immune response. We obtained a Th1 shifted immune response after intradermal DNA electrotransfer vaccination compared to vaccination with the recombinant protein. Finally, we compared immunisation into the skin to two other sites of administration. The present data point to the muscle as a tissue of choice for plasmid DNA electrotransfer immunisation when high immunoglobulin titres are required. But, intramuscular, intra-pinna or intradermal electrotransfer all seemed to be appropriate to obtain a Th1 profile of response.

Acknowledgments

This research was supported by the European Commission under the 6th framework under the grant Moleda and by the

FRSM (Fonds de la Recherche Scientifique Médicale, Belgium). Gaëlle Vandermeulen is FNRS Research Fellow (Fonds National de la Recherche Scientifique, Belgium). The authors thank Prof. Benoît Van den Eynde for helpful discussions.

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