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Research Paper

Effect of Tape Stripping and Adjuvants on Immune Response After Intradermal DNA Electroporation

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Purpose. DNA vaccines require both efficient delivery methods and appropriate adjuvants. Based on their mechanisms of action, we hypothesised that some adjuvants could enhance vaccine immunogenicity or direct the response towards Th1 profile after intradermal DNA electroporation.
Methods. After intradermal electroporation of plasmid DNA encoding luciferase, mice received hyaluronidase, imiquimod, monophosphoryl lipid A or were tape stripped in order to modulate the immune response against the encoded protein. We measured total immunoglobulin G, IgG1, IgG2a titres and the cytokines produced by splenocyte cultures to assess both humoral and cellular response. The effect of tape stripping on the response against intradermally delivered ovalbumin protein was also assessed.
Results. Neither hyaluronidase nor imiquimod improved the immune response against the encoded luciferase. Monophosphoryl lipid A did not modify the cytokines production but increased the anti-luciferase IgG2a titres. Tape stripping significantly increased anti-luciferase IgG2a and IFN-γ responses. It also enhanced the humoral response after intradermal injection of the ovalbumin protein.
Conclusions. Tape stripping is able to increase the Th1 immune response against both DNA and protein vaccines. Therefore, tape stripping appears to have interesting adjuvant effect on intradermal vaccination.

KEY WORDS: adjuvant; DNA vaccine; electroporation; skin.

INTRODUCTION

The skin is the largest and the most accessible organ of the human body and plays a key role in protection against aggressions. It acts as an efficient physical barrier but also as an immunological barrier. The specific immunologic environment of the skin, known as the Skin Associated Lymphoid Tissue (SALT), consists mainly of (i) Langerhans cells and dermal antigen-presenting cells which circulate between the skin and the lymph nodes, (ii) keratinocytes and endothelial cells which produce a wide range of immune and growth regulatory cytokines and (iii) lymphocytes which extravasate from the circulation into the skin (1). The SALT provides both innate and adaptive immunity and efficiently protects the individual against aggressions. These immunological properties make the skin an attractive organ for the delivery of vaccines.

Plasmids are very stable and can be produced by generic methods, making the development and production process easy and cheap. Moreover, the delivery of several antigens on the same plasmid is possible. DNA vaccines are known to induce a strong protective immune response in small animal models but the response is lower in large animals. Consequently, the development of efficient DNA delivery technologies and appropriate adjuvants is essential for the future of genetic vaccination.

Electroporation has been widely used to introduce DNA into various types of cells *in vitro* and is one of the most efficient non-viral methods to enhance gene transfer in various tissues *in vivo*. Electroporation involves plasmid injection in the target tissue followed by application of electric pulses (2). It is easy to perform, inexpensive and very efficient. The pulses have to be adapted for each tissue. A combination between one short high voltage pulse and one longer low voltage pulse is sufficient for intradermal DNA electroporation (3). Electroporation increases transgene expression up to 100-fold more than the injection of naked DNA into the skin (3–6). Previous studies have shown that intradermal DNA electroporation resulted in a significant immune response against the encoded protein (3,6,7). However, this response was lower than that observed when other routes of delivery were used such as muscle or ear pinna (6). This is why the use of adjuvant could be useful for improving immune response after intradermal DNA electroporation.

Adjuvants increase and/or modulate the intrinsic immunogenicity of an antigen (8). Several adjuvants are known to strongly enhance immune responses generated by traditional vaccines, but less is known about the effects of adjuvants on vaccination with DNA (9).

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Toll-like receptors (TLR) are pathogen-recognition receptors that recognize pathogen-associated molecular patterns. TLRs are expressed in both dendritic cells and keratinocytes in the skin. TLR agonists are potent activators of the innate immune response. They activate dendritic cell maturation and inflammatory cytokine secretion by innate immune cells and, as a consequence, they promote the adaptive immune response when coadministered with foreign antigen. Ligands that stimulate TLRs represent therefore potential vaccine adjuvants (10). In most cases, signalling through the TLRs promotes the development of Th1 type response, which is required for an efficient immunisation against tumors, for instance.

We hypothesised that the use of adjuvants, acting on different targets, might increase the immune response obtained after intradermal DNA electroporation. In order to determine the best way to elicit strong Th1 immune response, four potential adjuvants were assessed: (i) hyaluronidase, (ii) imiquimod, (iii) monophosphoryl lipid A (MPLA) and (iv) tape stripping.

Hyaluronidase is responsible for the degradation of hyaluronan that is a ubiquitous glycosaminoglycan of the extracellular matrix present around muscular fibres and in the skin. As a pre-treatment, bovine hyaluronidase enhanced the expression of the encoded protein after electroporation into the muscle (11) but failed to enhance expression after intradermal DNA electroporation (6). Small hyaluronan fragments induce the production of inflammatory cytokines as well as the migration and maturation of dendritic cells in the skin by signaling through the TLR 4 receptor and activation of NF-κB (12–15).

Imiquimod is a synthetic agonist of TLR 7/8 that upon topical application, induces increased production of IFN-α, IL-12, TNF-α and promotes Th1 immune response (16). It can safely and strongly enhance both antibody and CD8+ T cell responses and may provide an effective method to enhance the immunogenicity of vaccines (17,18). A 5% cream (Aldara, 3M) was the first TLR agonist approved for treatment of anogenital warts, actinic keratosis and small superficial basal cell carcinomas (19).

MPLA, which is isolated from LPS, retains much of the immunostimulatory properties of the parent lipopolysaccharide without its inherent toxicity. It has been widely used as an adjuvant in various vaccine formulations (20,21). MPLA is thought to function through activation of TLR 2 and TLR 4 for the induction of TNF-α, IL-10, and IL-12 (22). LPS is well known for generating Th1 response (23).

Tape stripping consists of partially removing the stratum corneum, the external layer of the skin, by the use of strips that are applied on the skin and then removed. By disrupting the skin-barrier, a "natural" adjuvant effect can be achieved through activation of the Langerhans cells (24,25). Recently, it has been demonstrated that tape stripping can induce TLR 9 expression (25,26). Epicutaneous immunization on tape stripped skin induces potent antigen-specific systemic IgG2a responses against a topically applied antigen. Until now, the use of tape stripping to modulate the response against DNA vaccine has never been assessed.

These adjuvants were applied or injected after electroporation for several reasons: (i) tape stripping and imiquimod (cream) could not be delivered together with plasmid because they would affect electrical properties of the skin; (ii) concerning the other adjuvants, their major target (TLR 4) is extracellular. The kinetics of TLR expression and APC activation by adjuvants varies with adjuvant and end point but usually peaks between 6 and 48 h (22,26–28) whereas luciferase expression peaks after 24 to 48 h (3). Hence, electroporation was applied immediately before their injection or tape stripping.

The objective of this study was to check the hypothesis that the four selected adjuvants, acting on different targets, could enhance Th1 immune response after intradermal electroporation of plasmid encoding a weakly immunogenic protein, the luciferase.

MATERIAL AND METHODS

Plasmid DNA

Electroporation was performed using the pGL3 Luciferase Reporter Vector (Promega Benelux, Leiden, Netherlands) containing the CMV-actin-globin (CAG) promoter. The plasmid was 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 (260/280 nm) and by 1% agarose gel electrophoresis. Light absorption at 260 nm was used to determine the DNA concentration. All plasmid dilutions were done in Phosphate Buffer Saline (PBS). Plasmid was stored at −20°C before use.

Animals

For the vaccination studies, female BALB/c mice, 6 weeks old at the beginning of the experiment were used (Janvier, Le Genest St Isle, France). For the measures of TransEpidermal Water Loss (TEWL), we used female NMRI mice, 6 weeks old (Université Catholique de Louvain, Brussels, Belgium). Mice were anesthetized with a mixture of ketamine 50 mg/mL (Ketalar, Pfizer, Brussels, Belgium) and xylazine 5.6 mg/mL (Sigma, Bornem, Belgium). The skin of the abdomen was depilated 1 day prior to the experiments with a depilatory cream (Veet for sensitive skin, Belgium), in order to thoroughly remove all of 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.

Immunisation Studies

For immunisation by intradermal DNA electroporation, 50 μg of the plasmid encoding luciferase was injected into the dermis of BALB/c mice using a Hamilton syringe with a 30-gauge needle. We injected plasmid intradermally 15 μL at two different sites, with a distance of about 5 mm. Then, a cutaneous fold was performed and the sites of injection were placed between plate electrodes. A short high voltage (HV) pulse (700 V/cm 100 μs), immediately followed by a low voltage (LV) pulse (200 V/cm 400 ms) was applied approximately 1 min after plasmid injection. There was no time interval between HV pulse and LV pulse. 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 spaced plate electrodes (IGEA, Carpi, Italy) (3,6). For immunisation with the ovalbumin protein, 50 μg of ovalbumin (Sigma A5503) in 30 μL PBS was injected intradermally. No electric pulses were applied. Each mouse received priming and two boosts, 2 and 4 weeks after the priming.

Adjuvants

Hyaluronidase was injected just after plasmid injection and electroporation. We delivered 2×25 μL of a 300 μg/mL saline solution of bovine hyaluronidase (Sigma H4272, 750–1,500 U/mg) into the dermis.

Imiquimod was obtained as a 5% cream (Aldara, 3M) in packets containing 12.5 mg of imiquimod in an oil-in-water base. Imiquimod was applied topically over the sites of injection immediately after each immunisation. Each mouse received approximately 1.25 mg imiquimod. The cream was rubbed into the skin for 15 s (18).

MPLA (Sigma, L6895) was used as aqueous dispersion in 0.5% (*v*/*v*) triethanolamine solution. We performed two intradermal injections of 12.5 μL of a 1 mg/mL MPLA solution just after each immunisation.

Tape Stripping and TEWL

For the immunisation studies, the abdominal skin of anesthetized mice was stripped ten times with tape (Tesa® Film Crystal Clear, Germany) just after the plasmid injection and electroporation.

To demonstrate the efficacy of tape stripping to partially and reproducibly remove the stratum corneum, TEWL was measured with a Tewameter TM300 (Courage-Khazaka, Köln, Germany). The probe was placed on the skin and the TEWL values were recorded at room temperature with controlled humidity. As expected, a significant enhancement was observed when the abdominal skin was stripped ten times (P value=0.0078, Wilcoxon signed rank test). The TEWL values before and after tape stripping were 9.8±1.1 and 79.1±3.7 g/h/m^2 respectively (mean±SEM).

Evaluation of the Immune Response

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 or anti-ovalbumin antibodies were measured by ELISA (3,6,29). Titres were defined as the highest dilution to give an optical density of 0.2 at 492 nm. 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, Université Catholique de Louvain, Brussels, Belgium) (6). Responding mice are defined as the number of mice showing total immunoglobulin G titres higher than the background value.

For cytokine assays, mice were sacrificed 2–3 weeks after the last boost and their spleens were removed aseptically. 500 μL of splenocytes at 5×10^6 cells/mL were cultured 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) or 10 μg of ovalbumin (Sigma) 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) and interleukin-2 (IL-2) assays or after 72 h for interleukin-4 (IL-4) and interleukin-10 (IL-10) assays (6). We measured cytokine concentrations in the supernatants using mouse DuoSet ELISA development kits (R&D Systems Europe Ltd, Abingdon, UK) according to the manufacturer's protocols.

Statistical Analysis

All results are expressed as mean±standard error of the mean (SEM). Statistical analyses were performed using the software GraphPad Prism 5 for Windows.

RESULTS

Effect of Chemical Adjuvants on Immune Response After Intradermal DNA Electroporation

We immunised mice against the weakly immunogenic luciferase protein by intradermal DNA electroporation, in order to study the influence of adjuvants on the immune response. The potential adjuvants were applied just after the priming and each boost. Anti-luciferase total immunoglobulin G tended to be lower when hyaluronidase was injected or when imiquimod was applied on the skin after DNA electroporation (Fig. 1A). These conditions resulted in a decreased number of responding mice. The number of responding mice was also lower when intradermal DNA electroporation was followed by injection of MPLA.

IgG isotypes were determined, in order to characterise the immune response. Even though the sera of hyaluronidase, imiquimod and MPLA treated mice contained lower level of IgG1 than the mice which received only the plasmid injection and the pulses, IgG2a titres were significantly higher for the mice treated with MPLA (Fig. 1B). The IgG1/IgG2a ratios were 10.6±8.4 and 0.5±0.4 for mice immunised without and with MPLA respectively.

The cellular immune response was assessed by the cytokine concentrations in the supernatant of luciferase-stimulated splenocyte cultures. There was no significant difference in IFN-γ and IL-2 production between the mice immunised only by injection of the plasmid and electroporation and the hyaluronidase, imiquimod or MPLA treated mice (Fig. 1C). Concentrations of IL-4 and IL-10 were very low (under 20 pg/mL) for all the mice (data not shown).

Effect of Tape Stripping on Immune Response After Intradermal DNA Electroporation

As tape stripping stimulates TLR9 expression in the skin and enhances the antigen presentation function of Langerhans cells (25,26), the potential of tape stripping as adjuvant of intradermal DNA electroporation was studied. After immunisation, the anti-luciferase total immunoglobulin G

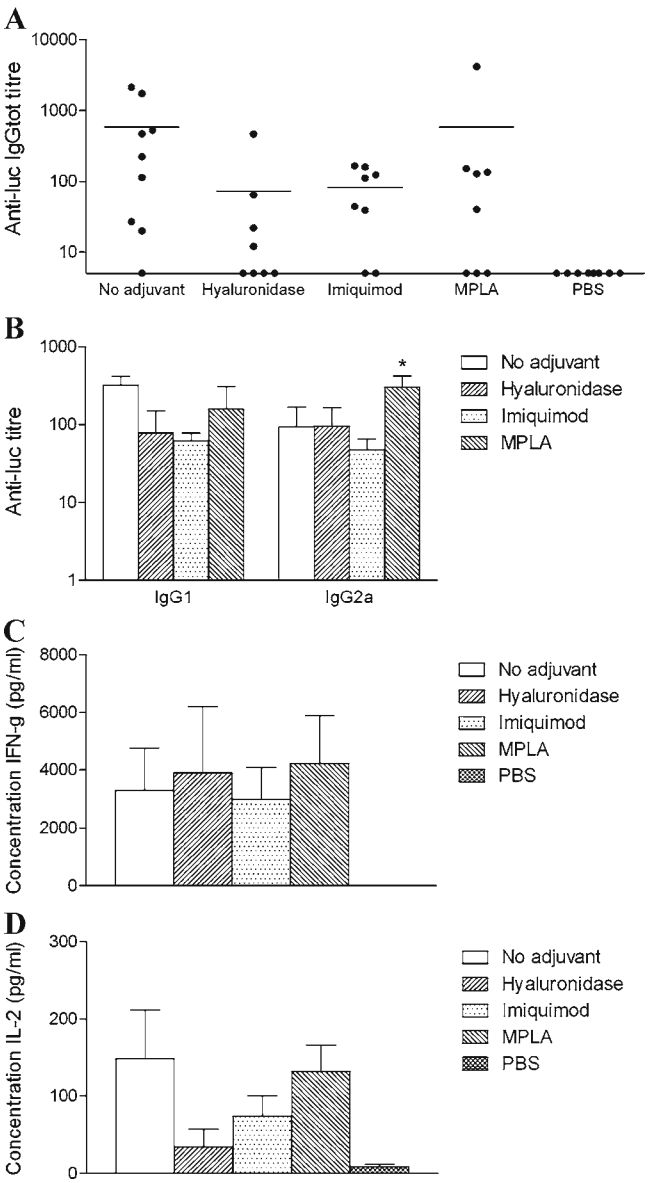

Fig. 1. Immune response after intradermal electroporation of luciferase plasmid without post-treatment (no adjuvant) or followed by hyaluronidase, imiquimod or MPLA delivery (n=8 to 9). **A** Determination of anti-luciferase total immunoglobulin G titres. *Circles* represent individual titres 6 weeks after the first immunisation and *lines* represent the mean values. **B** Determination of antibody isotypes in sera, 6 weeks after the first immunisation. *Bars* represent the mean values for responding mice (±SEM). **C** Concentration of IFN-γ determined in luciferase-stimulated splenocyte culture. *Bars* represent the mean values for responding mice (±SEM). **D** Concentration of IL-2 in luciferase-stimulated splenocyte culture. *Bars* represent the mean values for responding mice (±SEM). Statistical analysis: two-tailed t test compared to no adjuvant. *P<0.05.

titres tended to increase when tape stripping was performed (Fig. 2A). IgG2a titres for tape stripping treated mice were significantly higher (Fig. 2B). The IgG1/IgG2a ratios were 10.6±8.4 and 1.5±0.9 for mice immunised without and with tape stripping respectively. The IFN-γ concentrations in the supernatant of luciferase-stimulated splenocyte cultures were also increased when intradermal DNA electroporation was followed by tape stripping (Fig. 2C). Tape stripping did not significantly modify the IL-2 secretion.

Effect of Tape Stripping on Immune Response Against Ovalbumin

We measured the effect of tape stripping on the immunogenicity of the model antigen ovalbumin injected

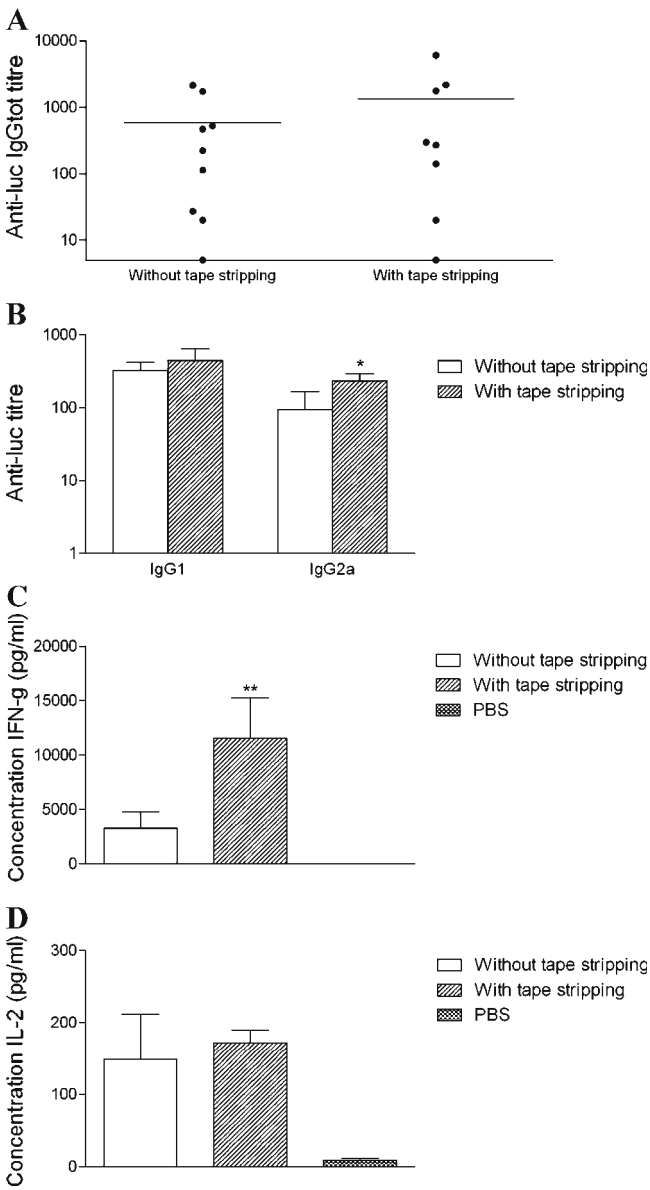

Fig. 2. Immune response after immunisation with intradermal electroporation of luciferase plasmid with (n=9) or without tape stripping (n=8). **A** Determination of anti-luciferase total immunoglobulin G titres. *Circles* represent individual titres 6 weeks after the first immunisation and *lines* represent the mean values. **B** Determination of antibody isotypes in sera, 6 weeks after the first immunisation. *Bars* represent the mean values for responding mice (±SEM). **C** Concentration of IFN-γ determined in luciferase-stimulated splenocyte culture. *Bars* represent the mean values for responding mice (±SEM). **D** Concentration of IL-2 in luciferase-stimulated splenocyte culture. *Bars* represent the mean values for responding mice (±SEM). Statistical analysis: two-tailed t test compared to tape stripping *P<0.05; **P<0.01.

intradermally, to confirm the immunostimulatory properties of tape stripping. The mice that underwent tape stripping after each immunisation showed higher anti-ovalbumin total immunoglobulin G titres (Fig. 3A). The IgG1 were equivalent in both groups (Fig. 3B). Moreover, the level of anti-ovalbumin IgG2a in sera after intradermal injection of ovalbumin without tape stripping was below the background limit, whereas three of out seven mice treated with tape stripping had anti-ovalbumin IgG2a in their sera (Fig. 3C).

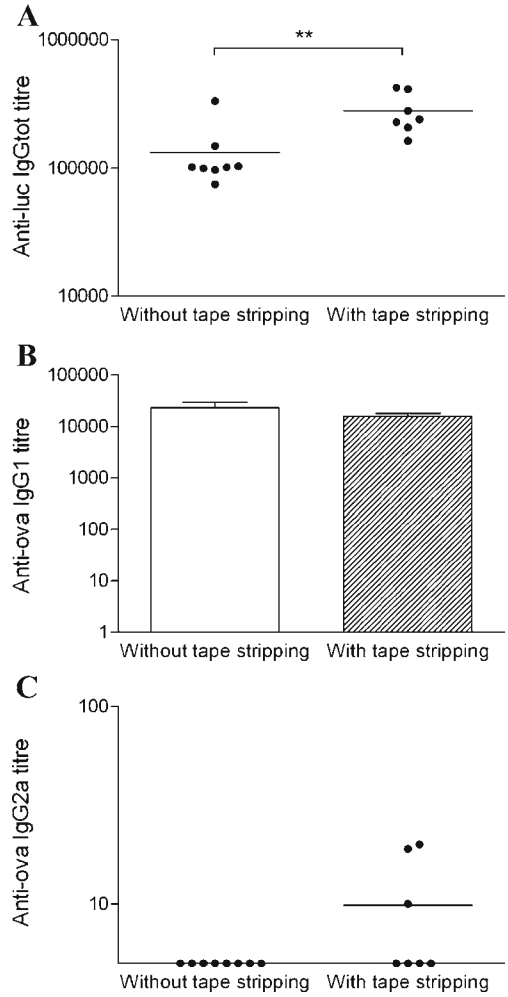

Fig. 3. Immune response after immunisation by intradermal injection of 50 μg of ovalbumin followed by tape stripping (n=8) or not (n=7). **A** Determination of anti-ovalbumin total immunoglobulin G titres. *Circles* represent individual titres 6 weeks after the first immunisation and *lines* represent the mean values. Statistical analysis: two-tailed t test, P value=0.0023. **B** Determination of anti-ovalbumin IgG1 titres in sera, 6 weeks after the first immunisation. *Bars* represent the mean values for responding mice (±SEM). Statistical analysis: two-tailed t test. **C** Determination of anti-ovalbumin IgG2a titres in sera, 6 weeks after the first immunisation. *Circles* represent individual titres and *lines* represent the mean values.

DISCUSSION

Several factors are known to influence the immune response after DNA vaccination. Firstly, the choice of the route of delivery appears to be paramount (6,30,31). The abundant presence of APCs together with the accessibility makes the skin an attractive target organ. However, the expression after intradermal injection of naked DNA is rather low. Different methods of DNA delivery have been developed to overcome this problem (for review (32)). Intradermal DNA electroporation is a very efficient method to increase the expression of encoded proteins in the skin and to obtain immune response against the encoded antigens (3,4,6). Secondly, the gene delivery method influences the type of response. In the skin, various studies showed that gene gun immunisation induces a Th2 profile (33,34) contrary to electroporation which usually elicits a Th1 response (6,7,35). Thirdly, the plasmid vectors themselves may display adjuvant activity because of their intrinsic immunostimulatory properties due to unmethylated CpG dinucleotides, which can trigger the TLR 9 pattern recognition receptor (36–38).

The objective of this study was to find novel adjuvants to enhance Th1 immune response after intradermal DNA electroporation. Four potential adjuvants were successively studied: (1) hyaluronidase, (2) imiquimod, (3) MPLA and (4) tape stripping acting through the activation of TLR 4, 7/8, 4 and 9 respectively as well as APC activation. They were expected to promote a Th1 response.

Based on their mechanism of action, we hypothesised that hyaluronidase, imiquimod and MPLA could increase the immune response after DNA delivery by intradermal electroporation. However, these potential chemical adjuvants failed to enhance the immune response. Only MPLA enhanced IgG2a response. This is consistent with the induction of a Th1 response by LPS (23). Several hypotheses can be formulated to explain their lack or low adjuvant effect: It is know that electroporation itself can activate proinflammatory chemokine and inflammatory cells (39). Moreover, the role of CpG motifs, recognized by TLR 9, as “built-in” adjuvant for DNA vaccine is generally accepted (37). The plasmid used contained 7 CpG-rich islands including 1 GACGTT particularly immunostimulatory for mice. (3) Physical trauma such as intradermal injection can also induce TLR9 expression (27). Hence, the fact that injection, DNA and electroporation per se may promote Th1-directed responses during vaccination could explain the lack of efficacy of these adjuvants.

The timing of DNA electroporation and adjuvant delivery or tape stripping might also be an issue. As justified in the introduction, we delivered the adjuvant or tape stripped the skin just after electroporation assuming that their effect on TLR expression and APC activation would be concomitant with luciferase expression. However, TLR 9 expression peaked around 9 h after physical trauma or CpG (26,27) while TNFα induction by DC activated *in vitro* by hyaluran oligosaccharides or MPLA was higher after 12 to 24 h (22,28). Hence, other timing, repeated administration and/or higher adjuvant doses could be required. For hyaluronidase, the size of the hyaluronan fragments might be too high for DC activation (14).

Zuber *et al.* demonstrated that imiquimod can act as a Th1 adjuvant after gene gun delivery but not after intradermal injection of a DNA vaccine against HIV (40). These results suggest that, in addition to the desired orientation of the immune response, the method of delivery should be carefully considered for the choice of an adjuvant.

We also hypothesized that disruption of the skin barrier by tape stripping could have an adjuvant effect on DNA vaccination. Tape stripping activates Langerhans cells and keratinocytes (24,25) and ensures effective immune surveillance in the epidermis and repair of the barrier (41). Tape stripping also induces expression of TLR 9 and was an effective way to induce a Th1-type immune response after topical application of CpG-ODN and antigen (26). Here, we demonstrate that tape stripping could also exert an interesting adjuvant effect for both DNA and protein vaccines. The higher anti-luciferase IgG2a titres and IFN-γ concentrations when tape stripping followed intradermal DNA electroporation suggested that tape stripping promotes the development of Th1 response against the encoded antigen. Previous studies on so-called epicutaneous vaccination have already demonstrated that tape stripping facilitated percutaneous penetration of antigens applied on the skin and modulated antigen-specific immune responses when coadministered with Cholera toxin or CpG ODN. (24,25). A recent publication showed that stratum corneum disruption was a feasible and well-tolerated procedure in human for vaccination purpose (42). Here we also demonstrate that, after intradermal injection of an antigen, the humoral response could be enhanced and shifted towards a Th1 response with an increase of IgG2a when tape stripping was applied. Based on previously studies published, we assume that TLR 9 expression due to tape stripping is involved. However the precise mechanism of the enhancement and of the modulation of immune response remains to be investigated.

CONCLUSION

This study assessed the possibility of increasing the immune response obtained after intradermal DNA electroporation by the use of four potential adjuvants and aimed at determining the best way to elicit a strong Th1 immune response. We demonstrated the particular interest of tape stripping which is able to modulate immune response against both DNA and protein vaccines inducing a Th1-oriented response.

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