Transcutaneous subunit vaccine delivery. A combined approach of vesicle formulations and microneedle arrays

Ding, Z.

Chapter 3

Microneedle arrays for the transcutaneous immunization of diphtheria and influenza in BALB/c mice

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Introduction

The majority of vaccines are administered parenterally, however, multiple injections by syringes and needles require trained personnel and cause stress to children and parents in the pediatric vaccination program. In case of influenza, since the virus undergoes frequent antigenic variation, the current solution of controlling this disease by vaccination requires annual injections. Multiple injections (the pediatric vaccination program and influenza vaccination) raise the demand of a patient-friendly vaccine delivery. Transcutaneous immunization (TCI) offers an attractive route as it has the advantage of improved compliance and may lead to comparable or more potent immune responses than the conventional injection [1]. An enhanced immune response is expected because the skin is populated with a high number of immune-competent dendritic cells (DCs) in the dermis and Langerhans cells (LCs) in the epidermis [2, 3]. After activation, they migrate to the draining lymph nodes, present antigens to T cells and initiate systemic humoral and cellular immune responses [4-7].

As the natural function of the skin is to protect the body against water loss and unwanted environmental influences, it is also the major obstacle for dermal delivery of vaccines. The barrier function of the skin is located in the uppermost layer of the skin, the *stratum corneum*. It consists of corneocytes embedded in a highly organized crystalline lamellar structure of the intercellular lipid matrix. This unique arrangement of the 15-20 μm thick layer results in a practically impermeable barrier for many compounds. To overcome this barrier and achieve effective TCI, formulations with potent adjuvants, particular carrier systems, penetration enhancers as well as physical methods *e.g.* thermal ablation [8], microdermabrasion [9] electroporation [10] and cavitational ultrasound [11] have been studied in animal models and human, resulting in improved antibody titers compared to non-adjuvanted vaccine on intact skin and in some cases protective immunity was achieved. Recently, phase II trial of TCI using *E. coli* heat-labile exterotoxin applied on the skin after mild abrasion was successful in mitigating traveler's diarrhea [12]. However, low immunological efficiency, safety issue of adjuvants, poor patient compliance and complexity of application still pose severe problems hindering wide-spread introduction of TCI for mass vaccination campaigns.

A fast developing technique for TCI is to increase the skin permeability using a microneedle array. It was first proposed by Gerstel and Place in the 1970s [13]. Prausnitz resumed the study using microneedle arrays in transdermal drug delivery 10 years ago when the technology of fabrication in micron dimensions became readily available [14]. Currently, it is under active development in several groups at universities and companies. Based on the transient formation of mechanically produced conduits in the skin, antigens are able to pass through the *stratum corneum* and taken up by the LCs and/or the DCs [15]. These microneedles should be long enough to penetrate the stratum corneum, but short enough to avoid the contact with the nerves in the deeper skin tissue. In this way painless vaccination *via* the skin can be achieved. Successful TCI studies with equal or higher vaccination efficacy than i.m. injection have been reported by Mikszta *et al*. applying anthrax vaccine by intradermal injection with microneedle in rabbits [16] and Widera *et al.* using ovalbumin by an antigen-coated microneedle array in hairless guinea pigs [17]. However, practically the elasticity of the skin makes effective piercing by microneedle array very difficult. For reliable piercing in human skin, arrays with microneedle lengths typically of 500 μm and longer are required [18]. So far, effective and reproducible *stratum corneum* piercing by the microneedle array remains a very important issue, which influences the dose control of dermally delivered drugs including vaccines.

We have reported that microneedle arrays manufactured from commercially available 30G hypodermal needles applied by a handheld applicator are able to enhance the transport of hydrophilic molecules with a molecular weight up to 72 kDa through dermatomed human skin *in vitro* [18]. The microneedle arrays used in that study were rather long (from 550 to 900 μm). As a consequence they might cause pain sensation and the recovery of skin barrier may take longer time. The necessity to pierce the skin reproducibly with shorter microneedle arrays is therefore obvious. Recently, in our group an electric impact insertion system has been developed which drives microneedle array into the skin at a predetermined velocity [19]. The elasticity of the skin can thus be counteracted, allowing the penetration of the shorter microneedle arrays *in vitro* into human skin. In the current study, the piercing ability of shorter microneedle arrays was validated and optimized in mouse skin *in vivo* using handheld and electric insertion systems before performing a TCI study, since mouse skin is thinner but more flexible than human skin.

Diphtheria is an example of a disease that is readily preventable by vaccination but remains a threat to public health due to short protection period and improper vaccination schedule. This had led to studies investigating TCI as a vaccination platform for diphtheria [20-22]. These studies revealed that dermal vaccination induced systemic response when applied with adjuvants multiple times on physically disrupted skin. In our current studies, diphtheria toxoid (DT) and influenza subunit vaccine (H3N2), average MW of 68 kDa and 228 kDa (trimmer), respectively, were both used in TCI studies aiming to examine the potential advantage of microneedle array pretreatment. Cholera toxin (CT), a well-known potent adjuvant, was co-administered to further improve the immune responses of these two vaccines. Our results show that microneedle arrays, with a needle length of 300 μm applied by the electric applicator, were able to pierce the mouse skin. It was very effective in increasing the immune response of DT. For the influenza TCI, co-administration of CT significantly improved the immune response against influenza, however, independent of microneedle array pretreatment of the skin.

Materials and methods

Materials

The human influenza vaccine is composed of the mono-valent bulk subunit vaccine A/Panama/2007/99 RESVIR-17 (H3N2, trimer, larger aggregates of trimmers exist due to the cluster of its hydrophobic trans-membrane domains) and was kindly provided by Solvay Pharmaceuticals (Weesp, The Netherlands). Antigen concentration was based on haemagglutinin content. Diphtheria toxin (DTa 79/1) and diphtheria toxoid (batch 98/40, protein content 12.6 mg/ml by BCA assay, 1 μg equal to approximately 0.3 Lf) were provided by The Netherlands Vaccine Institute (NVI, Bilthoven, The Netherlands). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ1 chain specific) and IgG2a (γ2a chain specific) were purchased from Southern Biotech (Birmingham, US). Chromogen 3, 3', 5, 5'-tetramethylbenzidine (TMB) and the substrate buffer were purchased from Biosource B.V (Nivelles, Belgium). O-phenyldiaminedihydro-chloride (OPD), lyophilized bovine serum albumin (BSA), Tween 20, Trypan blue and cholera toxin were purchased from Sigma (Zwijndrecht, The Netherlands). Tween 80 was purchased from Merck (Darmstadt, Germany). Nimatek® (100 mg/ml Ketamine, Euovet Animal Health B.V., Bladel, The Netherlands),

Visagel® from Eurovet, Rompun® (20 mg/ml Xylazine, Bayer B.V., Mijdrecht, The Netherlands) from Bayer and the injection fluid (0.9% NaCl) were obtained from a local pharmacy. All other chemicals used were of analytical grade and all solutions were prepared with distilled water.

Female BALB/c mice (H2d), 8-week old at the start of the experiment were purchased from Charles River (Maastricht, The Netherlands), and maintained under standardized conditions in the animal facility of the Leiden/Amsterdam Centre for Drug Research, Leiden University. The study was carried out under the guidelines compiled by the Animal Ethic Committee of The Netherlands.

Methods

Microneedle arrays and applicators

Three types of microneedle arrays were used in this study. First, the assembled microneedle arrays were manufactured from commercially available 30G hypodermic needles (BD, Alphen a/d Rijn, The Netherlands) as described previously [18]. The needles were assembled as a 4×4 array on a polymer plate with the surface area of around 0.5 cm^2. Serials of assembled microneedle arrays were fabricated with needle lengths of 300, 550, 700 and 900 μm (Fig. 1A). The second type was made of stainless steel wire with a diameter of about 200 μm and a length of 300 μm, with a tangentially cut tip [19]. This type will be referred to as solid microneedle array (Fig. 1B). The third one is silicon hollow microneedle arrays with a length of 245 μm, available as 4x4 and 9x9 arrays (Fig. 1C) and produced as described previously [19].

Piercing of mouse skin with microneedle arrays

The abdominal skin of the mouse was shaved 24 h prior to microneedle array treatment. Just before the treatment, mice were anaesthetized by intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine. The shaved skin area was wiped with gauze soaked in 70% ethanol. A skin fold of the shaved abdominal region was supported by styrofoam and pierced by microneedle arrays. The handheld applicator was pressed 1 min manually with the assembled microneedle arrays ranging from 300-900 μm microneedle length. The electric applicator was used with a velocity of 3 m/s and a retention time of 1 s for all types of microneedle arrays not longer than 300 μm [19].

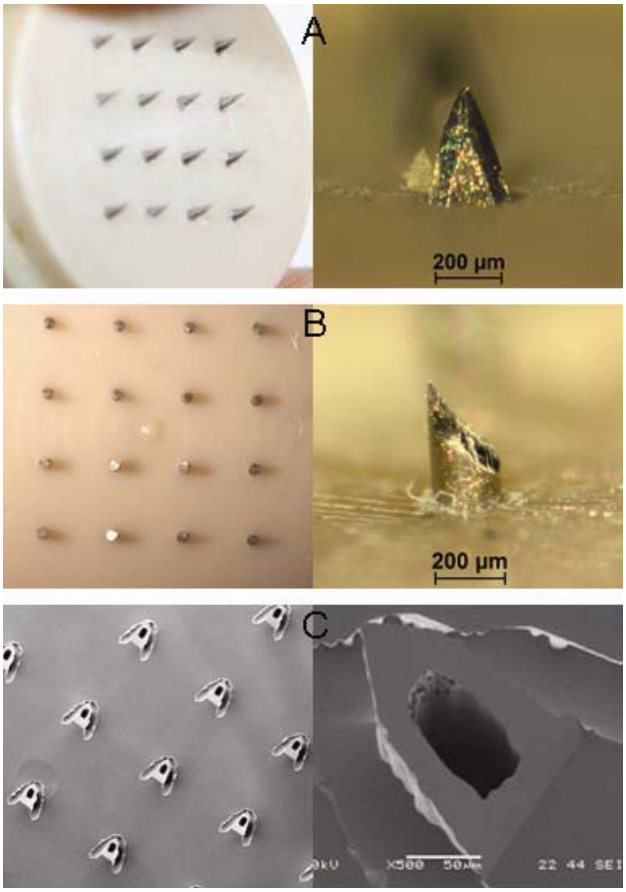

Figure 1. Three types of microneedle arrays; (A) assembled (B) solid and (C) silicon hollow microneedle arrays.

In vivo evaluation of piercing

Figure 2. Experimental setup for visualization of microneedle array piercing *in vivo*.

Immediately after piercing of the mice, the trans-epidermal water loss (TEWL) was measured by a Tewameter (TM 210, Courage + Khazaka, Köln, Germany). After placement of the probe on the pierced surface of the skin, the TEWL values were measured for 1 min and a mean value was calculated. To evaluate the uniformity of skin piercing, the site of piercing was covered with a hollow Hilltop® chamber (Miamiville, US) and filled with 300 µl 0.4% Trypan blue PBS solution for 1 h (Fig. 2). Subsequently the dye solution and chamber were removed and the mouse was sacrificed by cervical dislocation. The pierced skin was excised and examined under a magnifier. Successful

piercing of the microneedle arrays through the skin was scored by the appearance of blue dots in arrays at the dermal side of the skin.

Dermal diphtheria and influenza vaccination

The mice were shaved on their abdomen and rested for 24 h. On the day of vaccination, the mice were anaesthetized; the shaved skin area was wiped with 70% ethanol. A microneedle array was pierced into the skin using the electric applicator. For diphtheria vaccination, only the 300 µm-long solid microneedle array was investigated. Groups of 8 mice were immunized with 100 µg DT or 100 µg DT plus 100 µg CT with and without microneedle array pretreatment. DT and CT were applied on the skin in 70 µl 0.01 M citrate buffer at pH 5.0. DT formulations were carefully spread to wet the entire skin area of application (microneedle array-pretreated or untreated, ~ 2 cm^2 area restricted by a metal ring) and incubated occlusively for 1h. For the control group, 5 µg DT absorbed by $AlPO_4$ (Adju-Phos®; Brenntag Biosector, Denmark) in 100 µl saline solution was injected subcutaneously. The $AlPO_4$ adsorbed DT (DT-alum) was prepared as previously described and the adsorption of DT was between 70% and 80% [23].

For influenza vaccination, after piercing the array was withdrawn and a hollow Hilltop® chamber was attached to the skin with medical tape. The chamber was filled with 30 µg influenza haemagglutinin in 300 µl PBS (surface area around 1.2 cm^2) for 1 h incubation. Groups of 6 mice were immunized as follows: (a) PBS with solid microneedle array (300 µm-long, 4×4) treatment; (b) vaccine alone on untreated skin; (c) vaccine with silicon hollow microneedle array (245 µm-long, 4×4) treatment; (d) vaccine with silicon hollow microneedle array (245 µm-long, 9×9) treatment; (e) vaccine with solid microneedle array (300 µm-long, 4×4) treatment; (f) vaccine with 100 µg CT and solid microneedle array (300 µm-long, 4×4) treatment and (g) vaccine with 100 µg CT on untreated skin. The control group received an intramuscular injection of 15 µg influenza haemagglutinin in 100 µl physiological saline solution per mouse.

In both studies, after 1 h incubation the mice were extensively washed with lukewarm tap water, patted dry and washed again. The TCI and control groups were boosted twice using the same protocol at day 21 and day 42 (approximately the same skin region for the TCI groups), and sacrificed at day 56. Blood samples were obtained from the tail vein one day before each immunization and total blood was collected from the femoral artery under

anesthesia before euthanasia. Blood samples were collected in MiniCollect® tubes (Greiner bio-one, Alphen a/d Rijn, The Netherlands) till clot formation and centrifuged 10 min at 10,000*g* to obtain cell-free sera, then transferred to new tubes as aliquots and kept at -80 ºC until further use.

Vero cell test

The diphtheria toxin-neutralizing antibodies in mouse sera were assessed by a Vero cell test as previously described [24]. Complement in the serum was inactivated by heating at 56 ºC for 45 min. Then 2-fold serial dilutions of individual sera were prepared with complete medium 199 (CM199, Gibco, Breda, The Netherlands) and applied to microtitre plates (50 µl/well). Then, 50 µl diphtheria toxin, DTa 79/1 (0.0005 Lf/ml) in CM199, was added to each well. The plates were incubated for 2 h at 37 ºC for neutralization. Subsequently, 50 µl CM199 with 2.5×10^5 Vero cells/ml was added to each well. A reference antitoxin as well as an untreated cell control was included in each plate. The plates were covered with a plate sealer and incubated at 37 ºC in 5% CO_2 for 6 days. Each well was checked for the presence of living cells by microscope. The neutralizing antibody titer was expressed as the dilution factor of the most diluted serum that still had living Vero cells.

Serum IgG, IgG1 and IgG2a assay

The IgG subtype profile of influenza-specific antibodies was checked on day 20, 41 and 56 with ELISA as previously described [25]. Briefly, ELISA plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight at 4 °C with 200 ng/well of influenza subunit antigen (H3N2) in coating buffer (0.05 M sodium carbonate/ bicarbonate, pH 9.6). Plates were subsequently washed twice with PBS containing 0.05% Tween 20, pH 7.6 (PBST) and then blocked by incubation with 1% (w/v) BSA in PBST for 1 h at 37 °C. Thereafter the plates were washed three times with PBST. Two-fold serial dilutions of sera from individual mouse were applied to the plates and incubated for 2 h at 37 °C. Plates were then washed three times with PBST and incubated with HRP-conjugated goat antibodies against either mouse IgG1 or IgG2a for 1.5 h at 37 °C. Antibodies were detected by OPD and expressed as the reciprocal of the calculated sample dilution corresponding with absorbance of 0.2 above the background at 492 nm [26]. DT specific antibodies (IgG, IgG1 & IgG2a) and CT-specific IgG titers were determined with the same procedure by coating the plate with 140 ng DT or 66.7 ng CT per well, detecting antibody by

TMB and measuring optical absorbance at 450 nm. If a reading was above or below the relevant standard curve, further measurements were taken with lower or higher dilutions.

Hemagglutination inhibition (HI) assay

Serum samples were collected on day 20, 41 and 56 after influenza TCI. HI assay were performed at ViroClinics (Rotterdam, The Netherlands), according to WHO standard procedure using haemagglutinin antigens representing the stains of virus included in the vaccine [27]. Two-fold serial dilutions of the serum samples in PBS were incubated with the titrated antigen solution and 1% Turkey erythrocytes. The results are given as titers, meaning the highest dilutions of the serum which achieves complete inhibition of hemagglutination. All sera were titrated simultaneously in duplicate. Detection limit of this assay was 10 and non-responding sera were assigned an arbitrary titer of half the detection limit.

Statistical analysis

Statistical comparisons were made using two-tailed unpaired Student's *t*-test or one way ANOVA with Tukey post test where suitable using the software Prism Graphpad. A *p* value less than 0.05 was considered to be significant.

Results

In vivo evaluation of piercing in mouse skin

In the first set of experiments, the piercing capacity of microneedle arrays *in vivo* on mouse skin was evaluated using assembled microneedle arrays (300 to 900 μm long) and the handheld applicator. Figure 3a shows a clear trend between the TEWL and the needle length of the microneedle arrays, and demonstrated higher TEWL than those of the untreated control.

The 4x4 microneedle arrays with a needle length > 550 μm formed patterns of blue dots on both the *stratum corneum* side and the dermal side of the skin while no blue spots could be observed after application of 300 μm-long ones (Fig. 3b). This clearly indicates that microneedle with needle lengths of 550 μm and longer applied manually pierced the mouse skin and created conduits, while the 300 μm-long one did not.

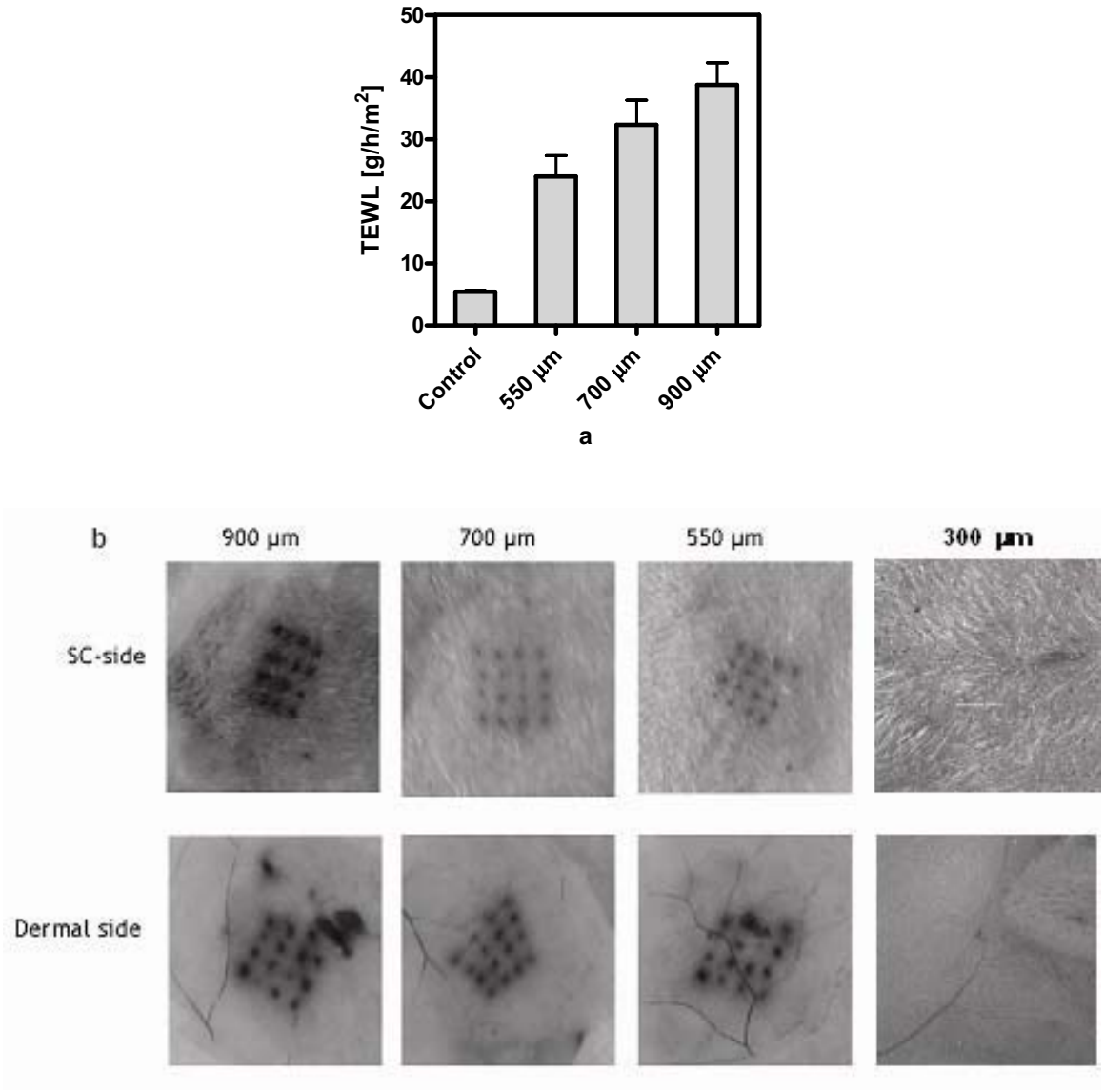

Figure 3. Assembled microneedle arrays with needle length of 300, 550, 700 and 900 µm were applied by the handheld applicator on mouse skin *in vivo*; (a) TEWL before and after microneedle arrays; (mean + SD, n=3). (b) Trypan blue staining. Pictures were taken from the stratum corneum and the dermal side of excised mice skin.

As manual piercing of 300 µm-long microneedle was not successful, it was decided to optimize the piercing of shorter microneedle array (*i.e.* ≤ 300 µm) by applying the array at an adjustable speed. For this an electric applicator was designed. A proper velocity and the sharpness of needles were expected to counteract the elasticity of the skin and pierce the *stratum corneum* more effectively as was already demonstrated in human skin [19]. Immediately after piercing, TEWL was measured (Fig. 4a). Elevated TEWL compared to the

control is consistent with the results obtained by the Trypan blue staining, namely short microneedles were able to pierce the skin when projected at the speed of 3 m/s. For all microneedle arrays including 245 µm-long silicon ones (4x4 and 9x9), blue spot arrays were clearly visible at the dermal side of the skin (Fig. 4b), indicating that piercing of all tested microneedle arrays was successful. Therefore the short microneedle arrays and the electric applicator were chosen for the *in vivo* vaccination studies.

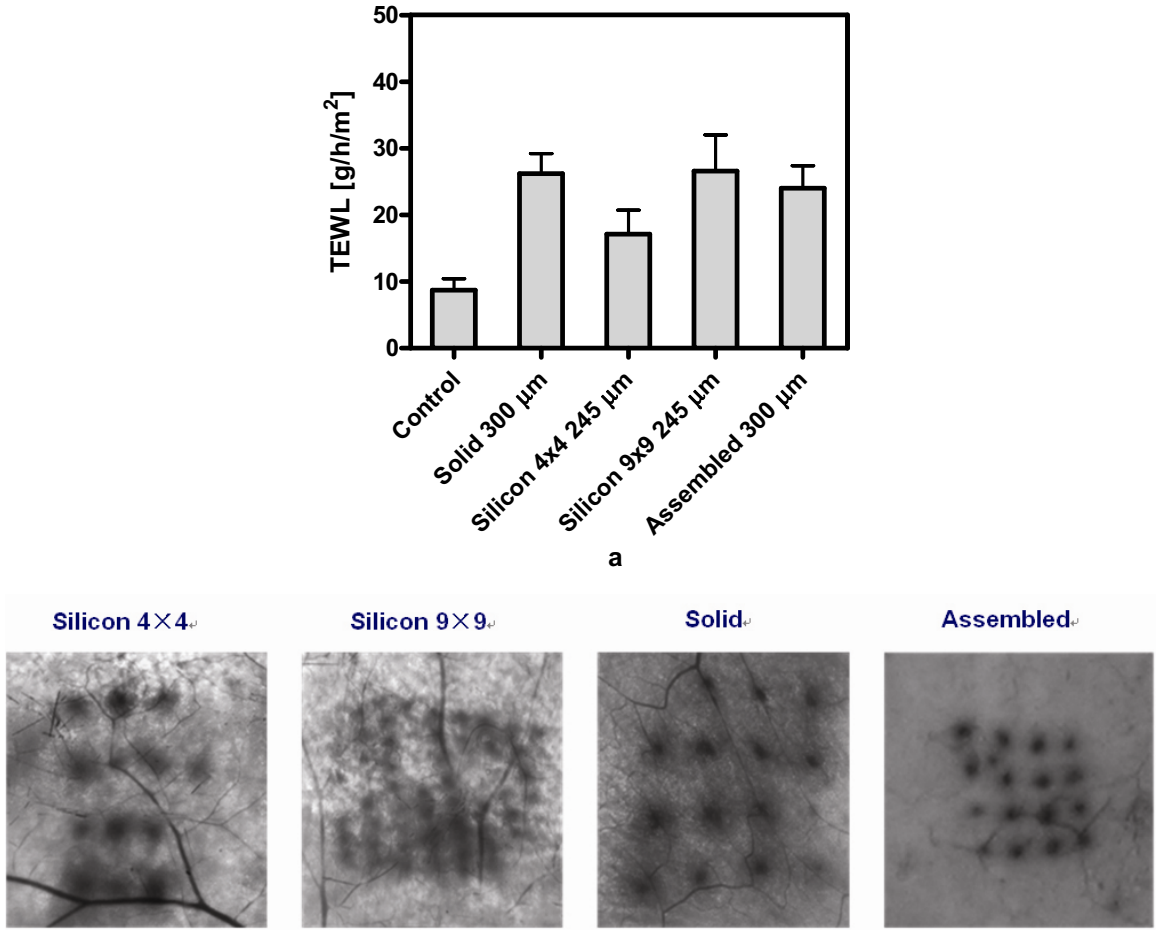

Figure 4. 245 µm-long silicon microneedle arrays (4×4 and 9×9), 300 µm-long solid and 300 µm-long assembled microneedle arrays were applied by the electric applicator on mouse skin *in vivo*; (a) TEWL before and after microneedle arrays treatment (mean ± SD, n=3); (b) Trypan blue staining after treatment. Pictures were taken from the dermal side of excised mice skin.

Dermal diphtheria vaccination

During the *in vivo* studies, there was no adverse effect from the shaving, anesthetizing, piercing, immunization, or washing procedure observed.

Neither erythema nor induration was seen at the immunization site after the antigen exposure.

TCI of DT was performed with or without microneedle array pretreatment, in the presence or absence of CT as adjuvant. Plain DT applied on intact skin induced low IgG titers. However, when DT was applied on microneedle pretreated skin, titers of all IgG subtypes were increased significantly (Fig. 5a, $p<0.01$). The presence of CT on microneedle pretreated skin (after prime, the 1st boost and the 2nd boost) induced respectively 4, 20 and 4 fold higher IgG titers than those without CT. However, CT made less significant difference in DT-specific IgG titers on untreated skin. IgG levels of all TCI groups increased after each boost, while in the group of CT with microneedle pretreatment, IgG titers reached the maximum level after the 1st boost and remained at a plateau after the 2nd boost. IgG titers of this group showed the same level as compared to the group with DT-alum injection. The ratio of IgG1 to IgG2a titers indicate whether the immune responses are biased towards T helper type 1 (Th1) or T helper type 2 (Th2). Th2 biased immune responses were found in the TCI groups with microneedle pretreatment where similar IgG1/IgG2a ratios to the injection group were detected (Fig. 5b). It appears that the CT lowered the IgG1/IgG2a ratio and therefore skewed the immune response to Th1 direction. Without microneedle array pretreatment, no protective effect was induced from TCI on intact skin in the presence and absence of CT as indicated from the Vero cell test (Fig. 5c). However, with microneedle pretreatment, CT did stimulate the production of toxin-neutralizing antibodies. Serum samples from the group with CT and microneedle pretreatment contained similar toxin-neutralizing capacity compared to that of the DT-alum injection group ($p>0.05$).

CT-specific IgG titers were also checked in this context to determine whether CT itself or its subunits were transported across the skin (Fig. 6). CT is a very immunogenic antigen and the IgG titer reached very high level after prime independent of microneedle array treatment and remained at a plateau. This demonstrates that CT or its subunits were transported across the *stratum corneum* even in the absence of microneedle array treatment.

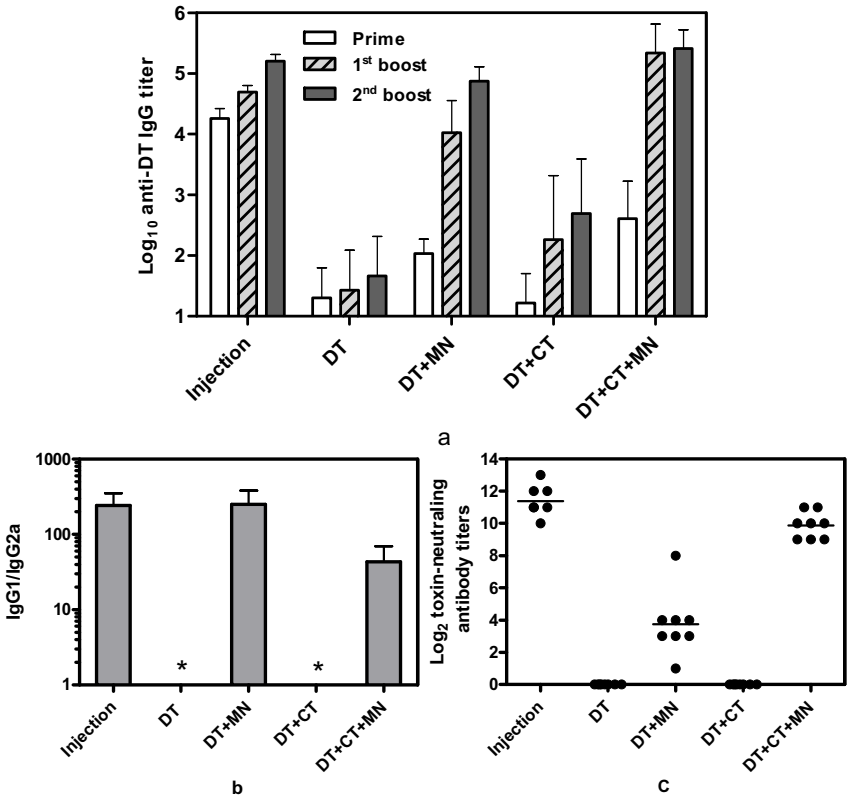

Figure 5. Immune responses in mice (n=8) after DT TCI with microneedle array (300 μm-long solid, 4×4) pretreatment on day 0, 21 and 42. (a) DT-specific serum IgG titer measured on day 20, 41, and 56. Non-responders were given an arbitrary value of 1. (b) DT-specific serum IgG1 and IgG2a titers determined on day 56. Ratios of IgG1/IgG2a titers of individual mouse were shown as mean + SD (*IgG2a titers below detection limit). (c) Neutralizing antibody titers of individual mouse, as measured with Vero cell test. Data shown are the dilution factor of the most diluted serum sample which protects Vero cells to survive incubation with diphtheria toxin. (MN=microneedle array pretreatment)

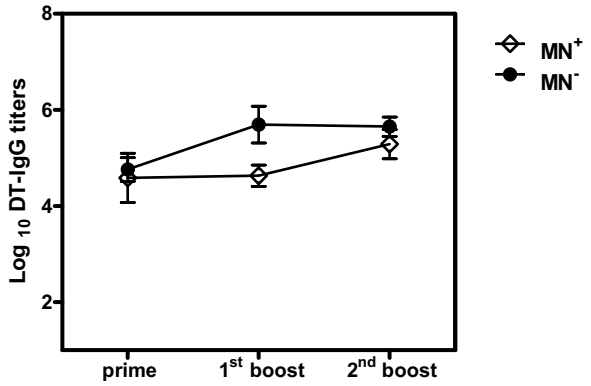

Figure 6. CT-specific serum IgG responses in mice (n=8) following the immunization by 100 μg of DT and CT on day 0, 21 and 42. Antibodies were measured on day 20, 41, and 56 and data displayed as mean ± SD. (MN=microneedle array pretreatment)

Dermal influenza vaccination

HI assay determines the titer of functional antibodies, by measuring the inhibition of red blood cell agglutination by influenza virus. In the influenza TCI study, non-adjuvanted vaccine on intact skin induced low HI titers. Different from DT TCI, no improvement was achieved by the application of any microneedle arrays (Fig. 7a). However, when CT was co-administrated as adjuvant, it drastically increased the immune response to influenza vaccine, independent of microneedle pretreatment. The presence of CT resulted in higher HI titers than in the absence of CT, around 8 fold after prime, 32 fold after the 1^{st} boost and 8 fold after the 2^{nd} boost, respectively. A similar trend was observed in IgG subtype titers as compared to HI titers (Fig. 7b & 7c). No differences in HI and IgG subtype titers were indicated among the groups with treatment of different microneedle arrays and the group without treatment.

Discussion

The objective of this study was to evaluate microneedle array treatment in TCI of diphtheria toxoid and influenza vaccine as a potential alternative method for injection. The benefit from using microneedle arrays are: i) increase in antigen transport across the skin barrier due to the created conduits, ii) increased targeting to DCs in the dermis as the conduits can reach the upper part of the dermis [19] and/or iii) activation of the LCs and DCs, which result in an increased immune response. Recently, microneedle arrays have attracted considerable attention in the application of dermal vaccination. Studies including dermal administration of influenza vaccine, plasmid DNA encoding hepatitis B surface antigen and recombinant protective antigen of *Bacillus anthracis* resulted in immune responses at least as strong as those generated by subcutaneous or intramuscular injection [16, 28, 29]. Influenza intradermal vaccination using hollow microneedle has shown dose-sparing and been proven to be safe in human clinical trials [30]. Further application of microneedle array in dermal vaccination and transdermal drug delivery is promising.

When using microneedle arrays, the microneedles should pierce the skin reproducibly without any pain sensation. Recently, skin irritation, pain sensation and skin barrier function were examined using microneedle arrays with a length varying between 200 and 550 μm *in vivo* in human volunteers using the same electric applicator as used in this study. The microneedle

arrays caused only minimal pain sensation and skin irritation, while a reduction in skin barrier function was observed [31]. These properties make the microneedle array and its applicator an excellent candidate delivery device for TCI.

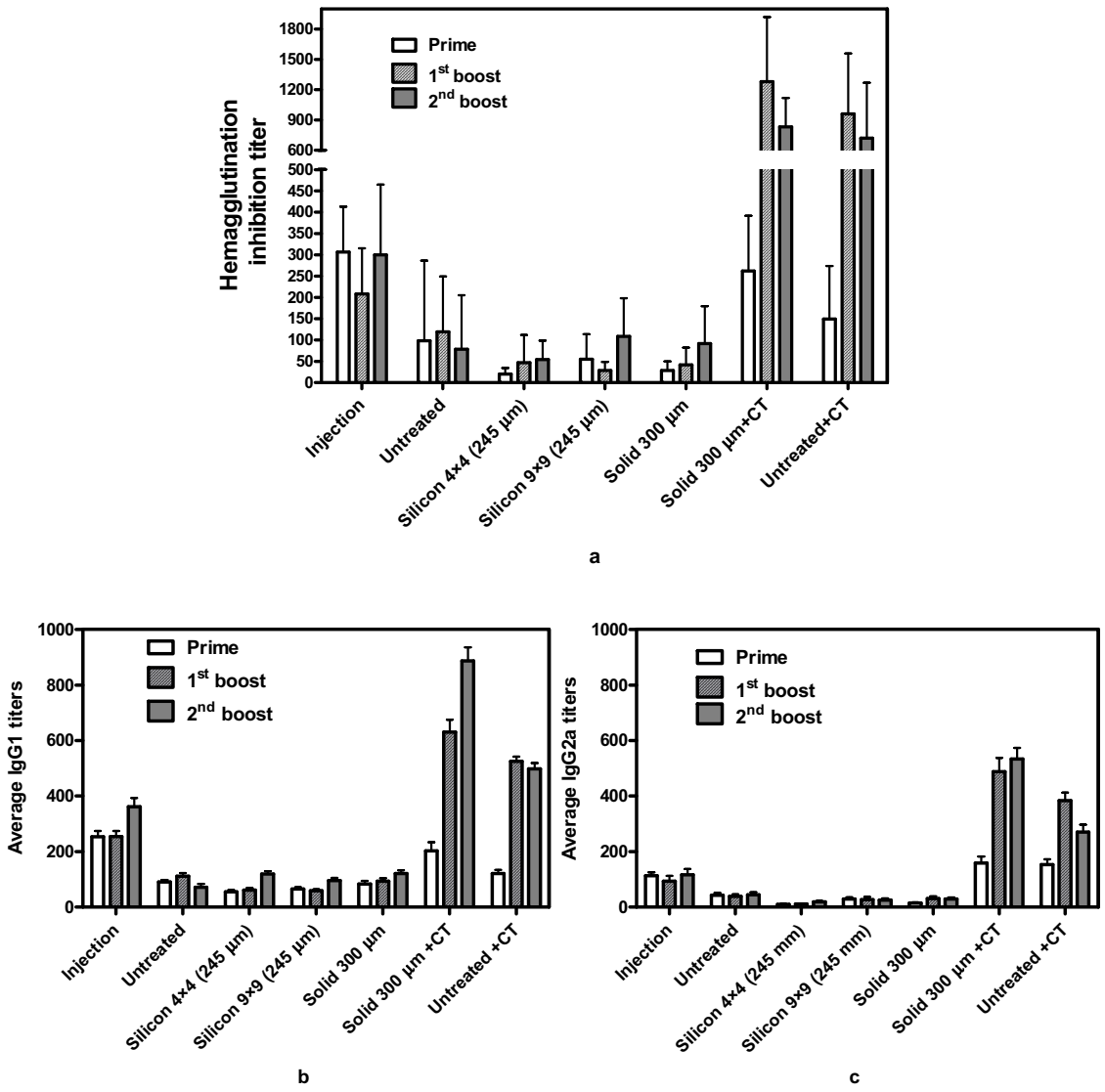

Figure 7. Immune responses in mice (n=6) after influenza TCI with microneedle arrays pretreatment on day 0, 21 and 42. (a) Hemagglutination inhibition was tested against influenza virus (H3N2) on day 20, 41 and 56 using Turkey red blood cells. Data displayed as the dilution factor of the most diluted serum sample that inhibits influenza agglutination (mean + SD), non-responding sera were assigned an arbitrary titer of 5, half of the detection limit. (b) & (c) Serum IgG1 & IgG2a titers against influenza subunit antigen (H3N2) were measured with ELISA on day 20, 41 and 56 and shown as mean + SD.

Piercing mouse skin

The first part of this study was to examine and validate the *in vivo* piercing capacity of various microneedle arrays (the assembled, solid and silicon hollow arrays with different needle lengths) using both the handheld and the electric applicators. The low TEWL values measured before microneedle array treatment but after shaving indicated that the shaving and the 70% ethanol wipe did not increase the permeability of the skin and the integrity was well preserved (Fig. 3 and 4). With the handheld applicator, piercing was only possible with 550 μm and longer microneedles. By using the newly designed electric applicator and driving microneedle arrays at the speed of 3 m/s, 300 μm and even 245 μm long microneedles pierced mouse skin *in vitro* and *in vivo* in a reproducible manner, similar as observed in human skin *in vitro* [19]. This is an important observation considering that mouse skin is much more flexible than human skin and demonstrates that piercing with an optimal velocity effectively counteracts the elasticity of the mouse skin.

Comparison of dermal DT and influenza immunization

Microneedle array pretreatment

Through TCI on intact skin, neither DT nor influenza vaccine induced substantial immune responses as indicated by low level of IgG and functional antibody titers. During TCI after microneedle array pretreatment, the vaccine has to diffuse along the conduits into the skin. However, only a fraction of the total dose may enter these conduits. Microneedle array pretreatment provided major improvement as compared to plain DT applied on intact skin, and with the presence of CT, it reached similar levels of serum IgG and toxin-neutralizing antibody titers as those of the DT-alum injection group. TCI with influenza presented a different result as compared to DT. The immune responses induced by plain vaccine were very low as indicated by the HI and IgG titers when compared to CT-adjuvanted vaccine, independent of microneedle array pretreatment. Several reasons may account for this result. i) Lower potency of the subunit influenza vaccine as compared to DT; ii) Lower concentration of vaccine applied (0.1 μg/μl influenza vaccine *vs.* 1.45 μg/μl DT); iii) The aggregates formed by the hydrophobic transmembrane domains of haemagglutinin trimmers may further reduce its permeation. The relatively low immune responses induced by plain influenza vaccine in barrier-disrupted skin were also reported by Garg *et al*. who vaccinated mouse with subunit

influenza vaccine (H5N1) using the Passport™ system (a patch device creating pores by thermal ablation) [8] and Skountzou *et al*. who applied inactivated influenza virus (WIV, PR8) on tape-stripped mouse skin pretreated with 70% ethanol [32].

Adjuvanticity of CT

In the current study, the presence of CT played an important role in enhancing both immune responses. It was previously shown that CT is toxic when administered mucosally, while it does not exert any toxicity when applied transcutaneously [33]. From the results described here, the adjuvanticity of CT on dermal delivered vaccines is antigen dependent. It was shown by the Vero cell test that CT, as an adjuvant in DT dermal vaccination, failed to stimulate protective responses to diphtheria toxin when co-administered on intact skin. However, high toxin-neutralization capacity could be achieved when applying DT and CT on microneedle array pretreated skin. CT-adjuvanted influenza TCI induced substantially higher immune responses concerning HI as well as IgG subtype titers in the serum, which were much higher than those after intramuscular injection of plain vaccine. Therefore, the addition of CT may lead to dose sparing benefit for influenza TCI. Furthermore, one should be aware of the difference in efficiency of TCI when comparing the murine model with human. The mouse skin has much thinner *stratum corneum* with considerably higher density of hair follicles than human skin. The relative area of application in mice is much larger than in humans. TCI in mice, therefore, can be successful even without physical pretreatment of the skin for certain antigens. However, when moved to humans, it is more difficult to get a protective response [34]. Potent adjuvant as well as microneedle array pretreatment may be necessary to archive effective protection and dose sparing.

Antigen penetration through the skin barrier

CT is well known as a potent antigen for dermal vaccination which actually initiated the research on TCI [35]. The current study showed clearly that CT, as an antigen with MW of 84 kDa, induced high levels of CT-specific IgG titers without increasing skin permeability. The response to TCI of influenza vaccine does not rely on microneedle array pretreatment, either. In general it is stated that when molecules are larger than 500 Da, the skin penetration reduces drastically [36]. Although TCI of vaccine and adjuvant is intended for delivery to the more superficial epidermis, it is remarkable that CT and influenza

vaccine or their subunits can penetrate the *stratum corneum* different from DT. We speculate that the vaccines may diffuse through the hair follicles as it has been shown that particles up to 40 nm can diffuse through the hair follicular opening and reach epidermal LCs [37, 38]. Taking the data of influenza and DT vaccination together, microneedle array pretreatment and CT co-administration seem to involve different diffusion pathway and immunological mechanisms. The fact that CT is a more potent adjuvant for influenza subunit vaccine than for DT may be due to the properties of the individual antigen, *e.g.* pathology of the disease, antigenicity of the vaccine, charge of the vaccine molecule, particular status of the vaccine and the molecular interactions between antigen and CT. It is not fully understood and requires more investigation.

In summary, we showed in this study that TCI can be strongly improved by using microneedle arrays combined with adjuvant. For DT, the microneedle array pretreatment is essential to obtain high antibody response, which is modestly augmented further by co-administration of CT. TCI of DT and CT after microneedle treatment results in comparable protection as injection of DT-alum. In contrast, for influenza vaccine, CT is very efficient in potentiating the immune response but does so independent of microneedle array pretreatment of the skin. TCI of influenza vaccine with CT is superior to the injection method.

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