Journal of Controlled Release 160 (2012) 495–501

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Transcutaneous immunization using a dissolving microneedle array protects against tetanus, diphtheria, malaria, and influenza

Kazuhiko Matsuo [a], Sachiko Hirobe [a], Yayoi Yokota [a], Yurika Ayabe [a], Masashi Seto [a], Ying-Shu Quan [b], Fumio Kamiyama [b], Takahiro Tougan [c], Toshihiro Horii [c], Yohei Mukai [a], Naoki Okada [a,*], Shinsaku Nakagawa [a,**]

[a] Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0781, Japan
[b] CosMED Pharmaceutical Co. Ltd., 32 Kawanishi-cho, Minami-ku, Kyoto 601-8014, Japan
[c] Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 26 August 2011
Accepted 1 April 2012
Available online 11 April 2012

Keywords:
Dissolving microneedle array
Transcutaneous immunization
Tetanus
Diphtheria
Malaria
Influenza

ABSTRACT

Transcutaneous immunization (TCI) is an attractive alternative vaccination route compared to the commonly used injection systems. We previously developed a dissolving microneedle array for use as a TCI device, and reported that TCI with the dissolving microneedle array induced an immune response against model antigens. In the present study, we investigated the vaccination efficacy against tetanus and diphtheria, malaria, and influenza using this vaccination system. Our TCI system induced substantial increases in toxoid-specific IgG levels and toxin-neutralizing antibody titer and induced the production of anti-SE36 IgG, which could bind to malaria parasite. On influenza HA vaccination, robust antibody production was elicited in mice that provided complete protection against a subsequent influenza virus challenge. These findings demonstrate that TCI using a dissolving microneedle array can elicit large immune responses against infectious diseases. Based on these results, we are now preparing translational research for human clinical trials.

1. Introduction

Global epidemics of emerging infectious diseases such as highly pathogenic avian influenza [1,2] and the threatening reemergence of infectious diseases like malaria [3,4] are a major concern, emphasizing the importance of vaccination. Vaccination is the only fundamental prophylaxis against illness and death from infectious disease. The availability of novel vaccination methods is urgently needed at the outset of a pandemic or bioterrorism attack [5,6], because several problems are associated with conventional injectable vaccination systems, such as pain, requirement for medical personnel or techniques, needle-related disease or injuries, and storage and transportation issues.

Transcutaneous immunization (TCI) is a novel method that holds great potential for increasing the compliance and efficacy of future global vaccination programs [7,8]. A number of alternative strategies to delivering Antigens (Ags) into the skin, such as electroporation [9], iontophoresis [10] and jet injector [11], are under investigation and some are undergoing clinical studies. Such approaches, however, require large-size and specific equipment to use for vaccination, or are accompanied by pain similar to standard injection systems. Some microneedle array designs have been developed [12], but most are made from metal or stainless steel, and some needles on microneedle arrays may fracture and remain in the skin, which is a safety issue. In comparison with these methods, dissolving microneedles offer several potential advantages [13]. First, this system is minimally invasive and has no need for needle disposal because the microneedles dissolve in the skin's interstitial fluid. These features might allay patient fears of needles and syringes, and also eliminate the risks of biohazardous sharps. Second, this system is simple and easy-to-use for vaccination. The use of a disposable array is suitable for self-administration by the patient. Third, this TCI system will likely cost much less than an injection system or other TCI system because the other methods require a cold chain for storage and transportation, such as TCI using a gauze patch that must be saturated with Ag solution just before application. In addition, clinical waste is eliminated because our microneedle array dissolves in the skin's interstitial fluid.

We developed dissolving microneedle arrays, called MicroHyala (MH), as a TCI device [14]. TCI using the MH induces immune responses against ovalbumin and adenovirus vector, with an efficacy comparable to that of injected vaccination systems [14]. In the present study, we investigated the vaccination efficacy of our TCI system for practical infectious disease models, which is essential for validating

Abbreviations: Ag, antigen; APC, antigen-presenting cell; BSA, bovine serum albumin; CT, cholera toxin; DT, diphtheria toxoid; HA, hemagglutinin; HI, hemagglutination inhibition; IDI, intradermal immunization; IMI, intramuscular immunization; MH, MicroHyala; PBS, phosphate-buffered saline; RBC, red blood cell; SCI, subcutaneous immunization; TCI, transcutaneous immunization; TT, tetanus toxoid.

* Corresponding author. Tel./fax: +81 6 6879 8176.
** Corresponding author. Tel.: +81 6 6879 8175; fax: +81 6 6879 8179.
E-mail addresses: okada@phs.osaka-u.ac.jp (N. Okada), nakagawa@phs.osaka-u.ac.jp (S. Nakagawa).

0168-3659/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2012.04.001

the use of this TCI system. We examined the potential advantage of the TCI system using tetanus toxoid (TT), diphtheria toxoid (DT), SE36, and influenza hemagglutinin (HA) as Ags. In addition, we compared the vaccination efficacy of our TCI system to that of conventional immunization systems, such as subcutaneous immunization (SCI), intradermal immunization (IDI), intramuscular immunization (IMI), and intranasal immunization (INI), which is a recently introduced method of administrating attenuated influenza vaccine via the nasal cavity. Our findings revealed that our TCI system induced an immune response that provides excellent protection against infectious diseases. Based on these results, we are continuing our studies to facilitate the application of this vaccination system using MH for clinical use as a simple, easy-to-use, and effective vaccination method to provide infectious disease prophylaxis.

2. Materials and methods

2.1. Animals

Female BALB/c mice (6 weeks old), ICR mice (6 weeks old), and hairless rats (5 weeks old) were purchased from SLC Inc. (Hamamatsu, Japan). All animals were specific pathogen free and maintained in the experimental animal facility at the Osaka University. The experiments were conducted in accordance with the guidelines provided by the Animal Care and Use Committee of Osaka University. Our permit number for this study is "douyaku23-3-0".

2.2. Ags

TT, DT, SE36 [15], seasonal trivalent influenza HA from each of A/Brisbane/59/2007(H1N1), A/Uruguay/716/2007(H3N2), and B/Florida/4/2006, which were the influenza Ags recommended for inclusion for the 2007–2008 season, and influenza HA from mouse-adopted influenza virus A/PR/8/34 were kindly provided by the Research Foundation for Microbial Diseases of Osaka University, Suita, Japan.

2.3. MH fabrication

Sodium hyaluronate was dissolved in distilled water, and then Ags were added and uniformly mixed. The aqueous solution was cast into micromolds and dried in a desiccator at room temperature. MHs were obtained by separating them from the molds. MHs were either 300 μm (MH300) or 800 μm (MH800) high, and contained over 200 microneedles/cm^2. To form the microneedle patch system, arrays with an area of 0.8 cm^2 were fixed onto an adhesive film with a surface area of 2.3 cm^2.

2.4. Vaccination protocol

2.4.1. TT/DT vaccination

Combined TT and DT-containing MH300 (1 or 3 μg each) and MH800 (0.1, 1, or 10 μg each) were applied onto the back skin of hairless rats for 6 h. Control SCI groups were subcutaneously immunized with combined TT and DT (10 μg each). These procedures were repeated five times at 2-week intervals. At the indicated periods, sera were collected from the rats to determine the toxoid-specific IgG titer, and sera collected 2 weeks after last vaccination were assayed for analysis of the IgG subclass and the passive challenge experiment described below.

2.4.2. SE36 vaccination

SE36-containing MH300 (0.1 or 2 μg) and MH800 (0.1 or 2 μg) were applied onto the shaved back skin of BALB/c mice for 6 h. In the SCI group, BALB/c mice were subcutaneously injected with SE36 (0.1 or 5 μg). These procedures were repeated four times at 2-week intervals. At the indicated periods, sera were collected from the mice to determine the toxoid-specific IgG titer, and sera collected 2 weeks after the last vaccination were assayed for analysis of IgG subclass and immunofluorescence assay as described below.

2.4.3. Influenza HA vaccination

For the seasonal trivalent influenza vaccination, three influenza HA-containing MH800 (0.2 μg each) were prepared. The MHs were applied to the shaved back skin of BALB/c for 6 h. For the control groups, BALB/c mice were treated by either IMI or IDI with three HAs (0.2 μg each) alone or with alum (100 μg), or by INI with three HAs (0.2 μg each) alone or with 10 μg cholera toxin (CT). These procedures were repeated twice at 4-week intervals. At the indicated periods, sera were collected from the mice to determine the HA-specific IgG titer. Sera collected 2 weeks after the last vaccination were assayed for analysis of IgG subclass and HI titer measurement.

For the live influenza virus challenge experiment, HA from mouse-adopted A/PR8/34 (0.4 μg)-containing MH800 was used. Control IMI or INI groups were treated with intramuscular injection of HA (0.4 μg) and intranasal application of HA (0.4 μg), respectively, plus CT (10 μg). The non-immunized group received Ag-free MH800. These procedures were repeated twice at 4-week intervals. At the indicated periods, sera were collected from the mice to determine the HA-specific IgG titer. Sera collected 2 weeks after the last vaccination were assayed for analysis of IgG subclass and HI titer measurement.

2.5. Antibody titer measurement

Serum was collected from animals at the indicated time-points, and the Ag-specific IgG or IgG subclass titer was determined by ELISA following previously described protocols [16]. End-point titers of Ag-specific antibody were expressed as the reciprocal $\log_2$ of the last dilution that showed 0.1 absorbance units after subtracting the background.

2.6. Passive challenge experiment for tetanus toxin

Neutralization activity of test sera collected from hairless rats that had been vaccinated five times with TT and DT was evaluated following previously described protocols [16]. Two weeks after the final vaccination, test sera were collected from hairless rats that had been vaccinated five times with TT and DT. ICR mice were injected subcutaneously with a 50-μl mixture of 25-μl test serum diluted at 1/1, 1/10, 1/100, 1/1000, and 25-μl solution containing 20 ng tetanus toxin (Sigma-Aldrich, Inc., St. Louis, MO) after incubation at 37 °C for 1 h. Mice were monitored for survival every 3 h for 96 h.

2.7. Immunofluorescence assays

Sera collected 2 weeks after the last vaccination of SE36 were assayed for analysis of immunofluorescence assay as described below. Percoll-purified red blood cells (RBC) infected by trophozoites and schizonts were prepared following the previously described protocol [17]. The RBCs were fixed with 3% paraformaldehyde/phosphate-buffered saline (PBS) for 20 min at 4 °C, centrifuged at 1870 *g* for 10 min at 4 °C, and resuspended in 3% bovine serum albumin (BSA; Sigma-Aldrich)/PBS. The fixed RBCs were dropped onto slide glass and dried at room temperature for 10 min. Then, 1% Triton X-100 in PBS was added, the plates were placed at room temperature for 1 h and then washed with PBS three times. The RBCs were blocked with 3% BSA/PBS for 1 h at room temperature. After washing twice with PBS, serum diluted 1:100 with 3% BSA/PBS was added to RBCs and incubated for 1 h at room temperature. The RBCs were washed five times with PBS and Alexa Fluor 488-conjugated anti-mouse IgG antibody (20 μg/ml; Invitrogen, Carlsbad, CA) was added. After 1 h incubation at room temperature, the RBCs were washed with PBS five times and mounted with Prolong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen).

2.8. Hemagglutination inhibition (HI) titer measurement

In influenza vaccination, sera collected 2 weeks after the last vaccination were assayed for analysis of HI titer measurement following established protocols. Briefly, sera were treated with receptor-destroying enzyme (DENKA SEIKEN Co., Ltd., Tokyo, Japan) at 37 °C overnight according to the manufacturer's instructions. After treatment, 25-μl aliquots of 2-fold serially diluted serum were incubated with 25 μl of 4 HA units of each HA at 37 °C for 1 h, followed by incubation with 50 μl of 0.5% chicken RBC (Nippon Biotest Laboratories Inc., Tokyo, Japan) at 25 °C for 45 min. The HI titer was defined as the reciprocal of the highest serum dilution that inhibited hemagglutination.

2.9. Influenza virus challenge

The live influenza virus challenge was conducted at Japan Biological Science (Osaka, Japan). Two weeks after the last vaccination, mice were challenged by intranasal instillation of 5×10^6 PFU of mouse-adapted influenza virus A/PR/8/34. After that, mice were monitored for weight loss and signs of illness on a daily basis and evaluated as described in Supplementary Table 1. Six days after the challenge, the lungs were collected to determine the degree of lung consolidation based on the following scale: 1, no consolidation; 2, ≤1/3 lobe; 3, >1/3 to ≤1/2 lobe; 4, >1/2 to ≤2/3 lobe; and 5, >2/3 lobe, to measure the virus plaque using a plaque assay system, and to perform a histopathologic assessment by hematoxylin and eosin staining. Inflammation level of bronchial pneumonia and interstitial pneumonia was scored according to severity, as follows: 0, none; 1, very slight; 2, mild; 3, moderate; and 4, severe. In addition, the pneumonia was categorized as bronchopneumonia or interstitial pneumonia as follows: bronchopneumonia; hypertrophy of bronchus mucous epithelium, hyperplasia of bronchus mucous epithelium, denaturation or necrosis of bronchus mucous epithelium, and infiltration with mononuclear cells and polymorphonuclear neutrophil leukocyte of bronchial submucosa. Interstitial pneumonia; focal atelectic lung or alveolus of the lung, focal hydrops or alveolus of the lung, focal bleed or alveolus of the lung, infiltration with mononuclear cells and polymorphonuclear neutrophil leukocyte of alveolar septum and infiltration with mononuclear cells and polymorphonuclear neutrophil leukocyte of alveolus of the lung.

3. Results

3.1. Efficacy of TT/DT vaccination

We examined the vaccination efficacy of our TCI system for TT and DT as typical clinical vaccine Ags. Hairless rats vaccinated by TCI using MH800 had an increased anti-TT IgG titer after the first vaccination at a low Ag dose, which was comparable to that of the SCI groups (Fig. 1A, B). The anti-DT IgG titer increased following vaccination using either MH800 or SCI, and this effect was dose-dependent. In the TCI group using MH300, the anti-TT or DT IgG titer increased with the increasing number of vaccinations, and achieved maximum levels equivalent to the MH800 and SCI groups (Fig. 1C, D). With the MH800, the anti-DT IgG titer was dose-dependent, suggesting that TT had high antigenicity. To evaluate the Th1/Th2 immune balance in our TCI system, we analyzed the toxoid-specific IgG subclass (Fig. 1E, F). Among the rat IgG subclasses, IgG1 and IgG2a are classified as Th2-dependent, and IgG2c as Th1-dependent [18]. SCI group induced IgG1, IgG2a, and IgG2c. On the other hand, TCI using MH300 or MH800 induced mainly IgG1 and IgG2a, but not IgG2c. Thus, our TCI system elicited a Th2-type immune response rather than a Th1-type. In a passive-challenge experiment, all mice treated with serum from non-immunized rats died (Table 1). On the other hand, 4 of 4 test sera (1/100 dilution) from rats vaccinated by TCI using MH800 with combined TT and DT (10 μg each) neutralized the tetanus toxin, and this activity was also observed in TCI group using MH800 (0.1 or 1 μg each). In the TCI group using MH300, 1 of 4 test sera (1/100 dilution), or 2 of 4 test sera (1/10 dilution) neutralized the tetanus toxin. These results indicated that our TCI system using each MH type induced toxoid-specific neutralization antibody and blocked the development of infection.

3.2. Efficacy of SE36 vaccination

We examined whether anti-SE36 IgG was produced by our TCI system. In mice vaccinated by TCI using MH800, anti-SE36 IgG increased in a dose-dependent manner equivalent to that in SCI group (Fig. 2A). TCI using MH300 containing 2 μg SE36 induced SE36-specific IgG production equivalent to TCI using MH800 and SCI, but achieving the maximum IgG

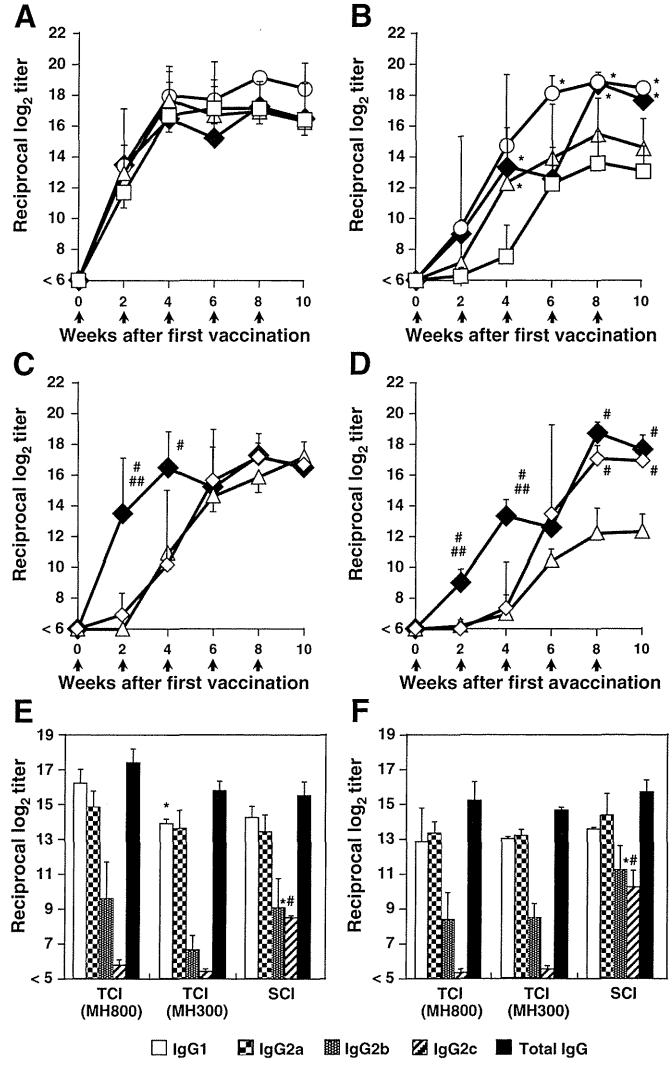


Fig. 1. Toxoid-specific IgG antibody responses after TCI. Combined TT and DT-containing (A,B) MH800 (0.1 mg; £, 1 mg; r, or 10 mg; • each), or (C,D) MH300 (1 mg; r or 3 mg; ¯ each) were applied to the back skin of hairless rats for 6 h five times at 2-week intervals. As a control SCI group (u), hairless rats were injected with combined TT and DT (10 mg each). At the indicated points, sera collected from these hairless rats were assayed for the IgG titer to TT (A, C) or DT (B, D) by ELISA. Two weeks after the final vaccination, sera collected from rats transcutaneously vaccinated using MH800 containing both TT and DT (10 mg each) or MH300 (3 mg each), and immunized subcutaneously with combined TT and DT (10 mg each) were assayed for TT (E) or DT (F)-specific IgG subclass (IgG1, IgG2a, IgG2b, or IgG2c) titer by ELISA. Data are expressed as mean ± SE of results from 3-4 rats. Arrowhead indicates vaccination points. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for multiple comparisons. (A, B) *; $p < 0.01$ versus TCI (MH800/0.1 mg) group. (C,D) #; $p < 0.01$ versus TCI (MH300/1 mg) group. ##; $p < 0.01$ versus TCI (MH300/3 mg) group. (E,F) *; $p < 0.01$ versus TCI (MH800) group. #; $p < 0.01$ versus TCI (MH300) group.

Table 1
Passive-challenge experiment of mice with tetanus toxin.[a]

Vaccination			Survival ratio		
Route	Device	Toxoid (μg/site)	1/1[b]	1/10[b]	1/100[b]
TCI	MH800	10 μg each	4/4	4/4	4/4
TCI	MH800	1 μg each	3/3	3/3	2/3
TCI	MH800	0.1 μg each	4/4	4/4	2/4
TCI	MH300	3 μg each	4/4	4/4	1/4
TCI	MH300	1 μg each	4/4	2/4	0/4
SCI	Injection	10 μg each	4/4	4/4	3/4

[a] Test sera were collected 2 weeks after the final vaccination from hairless rats that were vaccinated 5 times at 2-week intervals. The mice were injected subcutaneously with a 50-μl mixture of the indicated dilutions of test sera and 20 ng tetanus toxin after incubating the mixture at 37 °C for 1 h.
[b] Dilution ratio of test sera.

titer level required additional vaccinations. Next, we investigated whether the anti-SE36 IgG antibody binds to the malaria parasite. An immunofluorescence assay in the TCI and SCI groups detected green fluorescence derived from SE36-specific IgG in the same region of the blue fluorescence produced by the malaria parasite, suggesting that the induced antibody binds to the malaria parasite (Fig. 3). Because other groups reported that IgG2a, IgG2b, and IgG3 among the IgG subclasses have important roles in the antiproliferative effect of malaria parasites [19], we analyzed the anti-SE36 IgG subclass. In TCI using MH300 containing 2 μg SE36 and MH800 containing 0.1 or 2 μg SE36, IgG1 was the main subclass, and IgG2a, IgG2b, and IgG3 were also produced (Fig. 2B). These results demonstrated that TCI using MH could induce an acquired immune response and provide adequate protection against malaria.

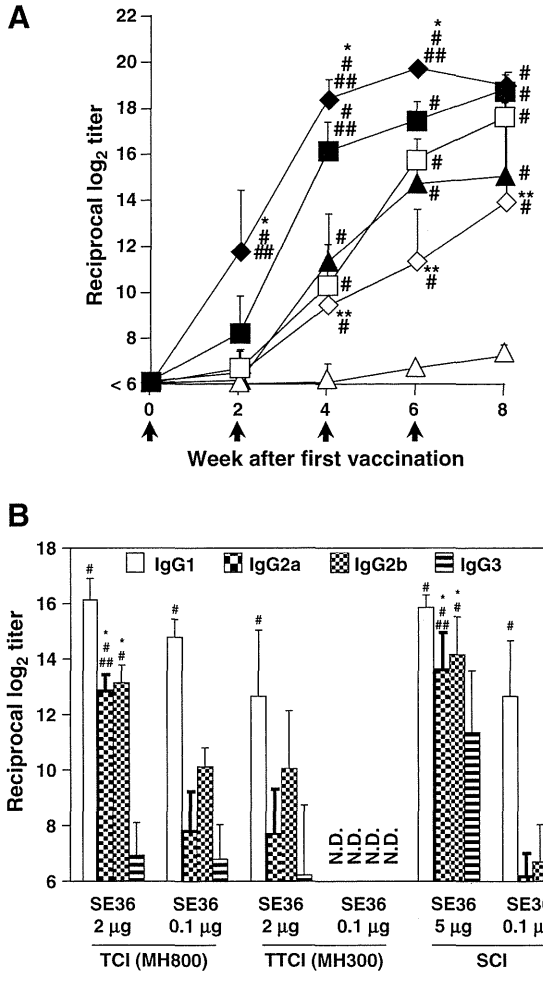


Fig. 2. SE36-specific IgG antibody responses after TCI. SE36-encapsulated MH800 (0.1 mg; £, or 2 mg; ¢) or MH300 (0.1 mg; r, or 2 mg; p) was applied to back skin of BALB/c mice for 6 h four times at 2-week intervals. As a control group, BALB/c mice were subcutaneously injected with SE36 (0.1 mg; ¯, or 5 mg; ¿). (A) At the indicated points, sera collected from these mice were assayed for the IgG titer to SE36 by ELISA. (B) Two weeks after the final treatment, sera collected from these mice were assayed for SE36-specific IgG subclass (IgG1, IgG2a, IgG2b, or IgG3) titer by ELISA. Data are expressed as mean ± SE of results from 5 mice. Arrowhead indicates vaccination points. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for multiple comparisons. *; $p < 0.01$ versus TCI (MH800/0.1 mg) group. **; $p < 0.01$ versus TCI (MH800/2 mg) group. #; $p < 0.01$ versus TCI (MH300/0.1 mg) group. ##; $p < 0.01$ versus TCI (MH300/2 mg) group.

3.3. Efficacy of influenza HA vaccination

We compared the immune response induced by TCI using MH800 with that of the IMI, IDI, or INI groups. The results of the anti-HA IgG titer measurement (Fig. 4A–C) indicate that the IgG production profile in the TCI group was similar to that of IMI groups with or without alum as an adjuvant. The INI mice with HA alone produced a much lower anti-HA titer than the other groups. The IgG titer of the IDI mice with HA alone was also quite low. On the other hand, the anti-HA IgG titer increased in mice vaccinated by INI with HA plus CT or IDI with HA plus alum. Moreover, a sufficiently high IgG titer was still detected 16 weeks after the last vaccination, suggesting that the anti-HA IgG induced by the TCI system would remain effective throughout the duration of the flu season. In addition, we analyzed the HA-specific IgG subclass. Among the mouse IgG subclasses, IgG1 is classified as Th2-dependent, and IgG2a as Th1-dependent [20,21]. TCI with HAs had a similar IgG subclass pattern as the IMI and IDI groups, whereas the INI group had greater induction of the Th1-type IgG subclass (IgG2a) than the other vaccinated groups (Fig. 4D–F). We then analyzed the HI titer of sera as the serological measure of

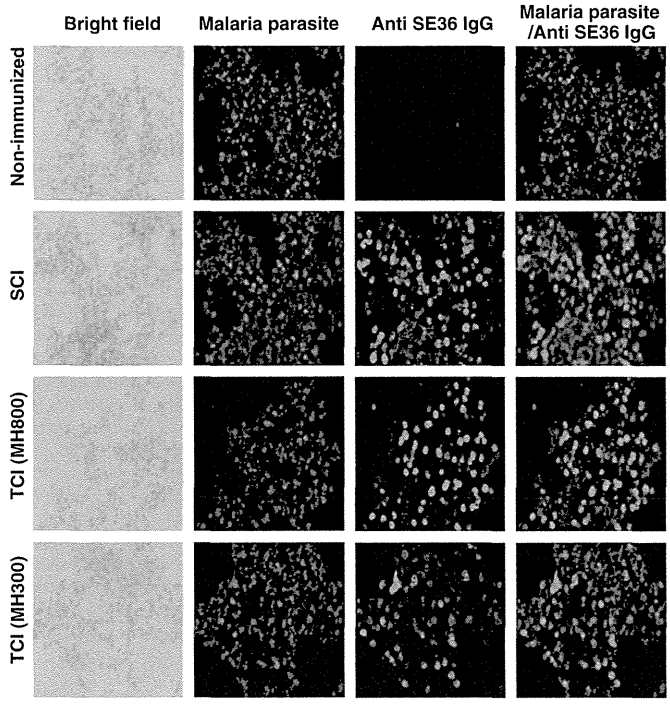


Fig. 3. Fluorescence microscopic analysis of antibody-binding activity. BALB/c mice were transcutaneously vaccinated with SE36 (2 μg/MH300) or (2 μg/MH800) for 6 h four times at 2-week intervals. A control group was subcutaneously injected with SE36 (5 μg) four times at 2-week intervals. Two weeks after the final treatment, sera were collected from these mice. Trophozoite- and schizont (CDC1)-infected erythrocytes were identified in the sera. Anti-SE36 antibody binding to CDC1-infected erythrocytes was stained with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody. The nucleus was counterstained using 4′,6-diamidino-2-phenylindole, and then CDC1-infected erythrocytes were photographed under a fluorescence microscope. TCI; transcutaneous immunization, SCI; subcutaneous immunization.

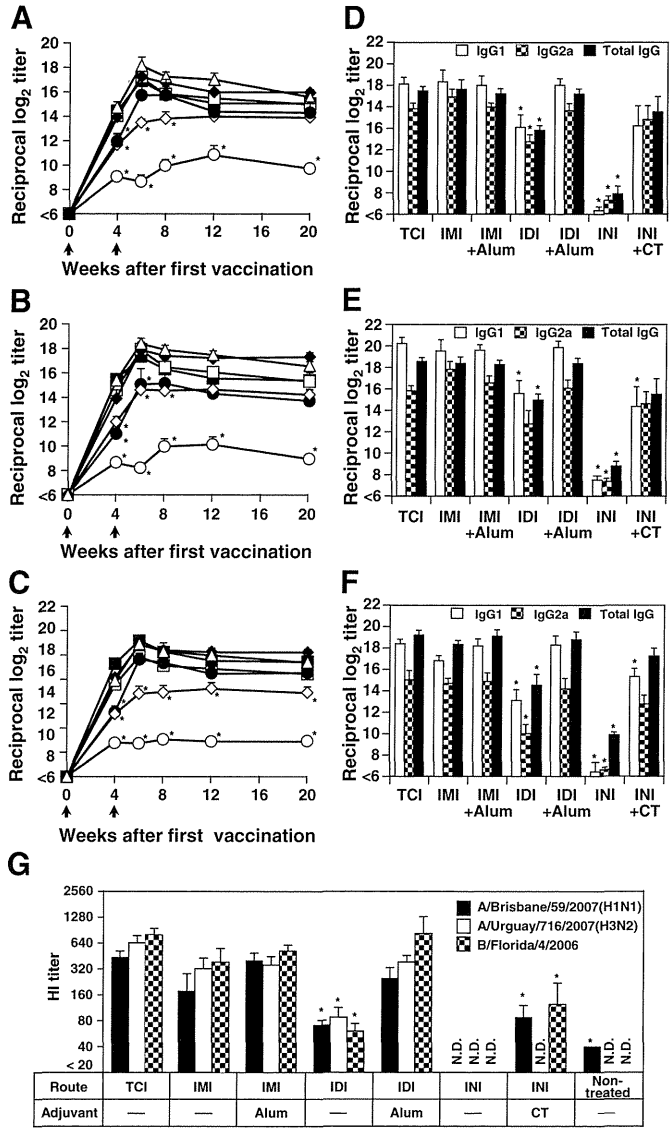


Fig. 4. Anti-HA immune responses in BALB/c mice after TCI. BALB/c mice were transcutaneously vaccinated with trivalent seasonal influenza HA from [(A,D) A/Brisbane/59/2007 (H1N1), (B,E) A/Uruguay/716/2007 (H3N2), and (C,F) B/Florida/4/2006] (0.2 mg each; r) for 6 h twice at 4-week intervals. Control groups were treated with intramuscular (£), intradermal (¯), or intranasal (•) application of HAs (each 0.2 mg) twice at 4-week intervals. Another control group was treated with intramuscular injection of HA Ags (each 0.2 mg) with alum (100 mg) (¢), intradermal application of HA Ags (each 0.2 mg) combined with alum (100 mg) (¿), or intranasal application of HA Ags (each 0.2 mg) with CT (10 mg) (•) twice at 4-week intervals. (A-C) At the indicated points, sera collected from these mice were assayed to determine the HA-specific IgG titer by ELISA. (D-F) Sera collected 2 weeks after the last treatment were assayed for HA-specific IgG subclass (IgG1 and IgG2a) by ELISA. (G) Two weeks after the final treatment, sera collected from these mice were assayed for the HI titer. HI activity expressed as the highest dilution that resulted in complete inhibition of hemagglutination. Data are expressed as mean ± SE of results from 5-7 mice. INI; intranasal immunization, IDI; intradermal immunization, N.D.; not detectable. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for multiple comparisons. *; $p < 0.01$ versus TCI (MH800) group.

functional antibodies. HI activity of TCI group was comparable to that in the IMI and IDI groups (combined with alum), and the levels appeared to correlate with the levels of anti-HA IgG antibody response (Fig. 4G). On the other hand, the IDI group (without alum) and INI group had lower HI activity than the TCI group. INI (with CT) induced high HA-specific IgA production which has a critical role in protecting against infection, whereas little anti-HA IgA was detected in the other vaccination groups, suggesting that our TCI system could not induce an HA-specific IgA response to the same extent as the conventional injection system (Supplementary Fig. 1). The results presented above indicate that TCI using MH induced strong antibody responses with significant HI titers.

In virus challenge experiment, TCI elicited HA-specific functional IgG antibody production equivalent to that in the IMI and INI groups (Fig. 5A–C), whereas little anti-HA IgA antibody was detected in the TCI and IMI groups (Supplementary Fig. 2). After A/PR/8/34 influenza virus challenge, control mice treated with Ag-free MH800 (MH800/placebo)

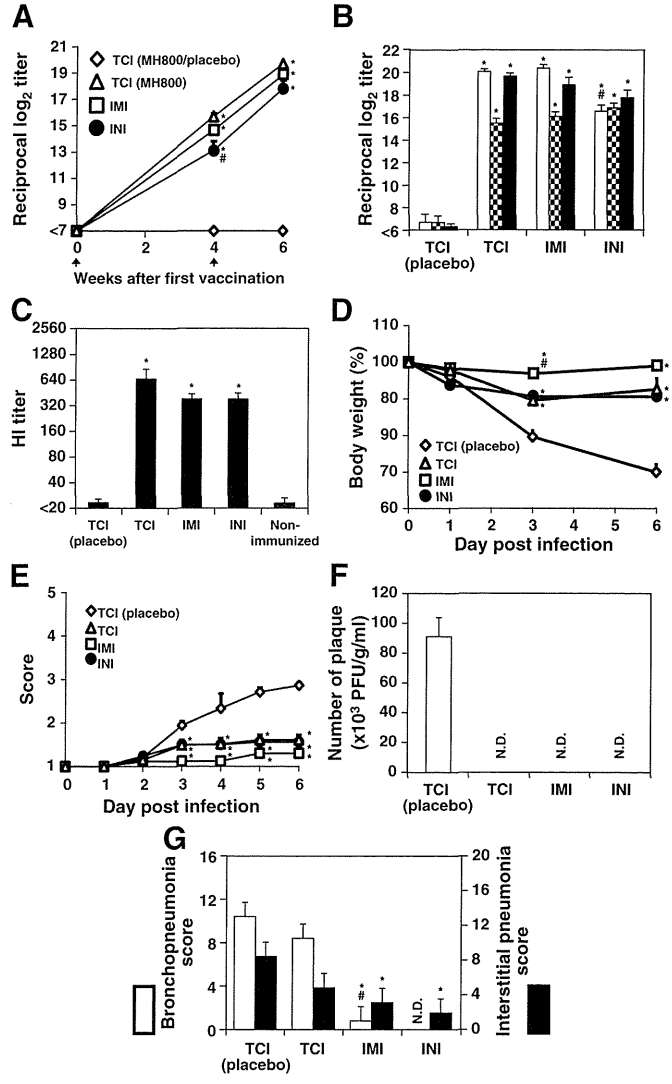


Fig. 5. Protection of vaccinated mice against influenza virus challenge. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 mg) for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 mg), or intranasal application of HA (0.4 mg) combined with CT (10 mg) twice at 4-week intervals. These mice were each infected intranasally with 6×10^5 PFU of the A/PR/8/34(H1N1) virus. (A) At the indicated points, sera collected from these mice were assayed for the HA-specific IgG titer by ELISA. (B,C) Two weeks after the final treatment, sera collected from these mice were assayed for (B) anti-HA IgG subclass (IgG1 and IgG2a) and (C) the HI titer. HI activity is expressed as the highest dilution that resulted in complete inhibition of hemagglutination. Data are expressed as mean ± SE of results from 13 mice. (D) Body weight was measured each day and is presented as a percentage of the original weight before infection (day 0). (E) The performance status of the mice was scored every day. (F) Six days after infection, the lungs were collected from these mice and number of viruses in the lung homogenate was determined using a plaque assay system. (G) The degree of inflammation of the lung was scored as follows: 0, none; 1, very slight; 2, mild; 3, moderate; and 4, severe. Pathologic findings were classified into bronchopneumonia and interstitial pneumonia. (D-G) Data are expressed as mean ± SE of results from 10 mice. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for multiple comparisons. *; $p < 0.01$ versus TCI (MH800placebo) group. #; $p < 0.01$ versus TCI (MH800) group.

Table 2
Degree of lung consolidation.

Vaccination	Lung weight (g; mean ± SE)		Consolidation (/tested mice)				Score (mean ± SE)[a]	
	L[b]	R[b]	L[b]	R[b]	R&L[b]	No	L[b]	R[b]
TCI (placebo)	0.154 ± 0.007	0.243 ± 0.022	1	0	9	0	4.2 ± 0.2	3.4 ± 0.5
TCI	0.068 ± 0.007	0.142 ± 0.013	0	1	0	9	1.0 ± 0.0	1.1 ± 0.1
IMI	0.059 ± 0.003	0.123 ± 0.005	0	0	0	10	1.0 ± 0.0	1.0 ± 0.0
INI	0.057 ± 0.002	0.117 ± 0.003	0	0	0	10	1.0 ± 0.0	1.0 ± 0.0

[a] Degree of consolidation: 1; no consolidation, 2; ≤1/3 lobe, 3; >1/3 to ≤1/2 lobe, 4; >1/2 to ≤2/3 lobe, and 5; >2/3 lobe.
[b] L; left lobe, R; right lobe, R&L; right and left lobes.

succumbed; they lost 30% of their body weight and showed worsening of symptoms (Fig. 5D, E). In contrast, TCI group mice showed no remarkable weight loss or other symptoms of illness, similar to the IMI and INI groups. In MH800/placebo group mice, influenza virus was detected at 10^5 plaque forming units (PFU)/g/ml, but the virus titer in the lungs of the TCI group was below the detection limit, demonstrating that our TCI system provided protection equal to that of IMI and INI (Fig. 5F). The weight of lungs harvested from mice vaccinated by TCI was equal to that of the IMI and INI groups. In the macroscopic findings, consolidation was not observed in 9/10 of the TCI group, 10/10 of the IMI group, and 10/10 of the INI group (Table 2). On the other hand, in the non-immunized group, lung weight was much greater than in the other groups and consolidation was detected in all of the mice. Based on our evaluation of the degree of bronchial pneumonia and interstitial pneumonia, the TCI group had a tendency toward a lower degree of lung inflammation compared with the non-immunized group, and the inflammation score in the IMI and INI groups was significantly lower (Fig. 5G). On the basis of these results, our TCI system confers protective immunity as effectively as IMI and INI administrations.

4. Discussion

We developed a novel TCI system using dissolving microneedle arrays, comprising MHs. This study demonstrated the vaccination efficacy of our TCI system against tetanus and diphtheria, malaria, and influenza as practical infection models.

TCI using MH without adjuvant induced effective immune responses as well as IMI, IDI, and SCI, indicating that this TCI system can replace the conventional injection system. INI system is attractive because it can induce the mucosal immune responses based on IgA production. Unfortunately, in this study, our TCI system did not induce the HA-specific IgA. Other reports showed that TCI system combining with some adjuvants such as CT, heat-labile toxin, or CpG could induce mucosal immune responses [22,23]. Therefore, we are currently exploring adjuvant candidates for our TCI system to improve our TCI system such as induction of mucosal immunity or cellular immunity.

In a previous report [24] on TCI using a hydrogel patch containing TT and DT, 100 μg each of TT and DT was needed to induce the maximum effect. Our TCI system using MH induced the same maximal effect with a lower Ag dose; 1/100–1/1000 as compared with that of a hydrogel patch, suggesting that the TCI system using MH is superior to that using a hydrogel patch in terms of Ag dose reduction. We also investigated whether TCI using a hydrogel patch containing SE36 induced SE36-specific IgG antibody production, and the results showed that TCI using a hydrogel patch with or without adjuvant did not induce anti-SE36 IgG production (Supplementary Fig. 3). Moreover, TCI using MH effectively induced immune responses against particulate Ags, like influenza HA. Based on these results, TCI using MH represents a significant advance over previous TCI approaches.

There were difference in strength of Ag-specific antibody production induced by TCI using MH300 and MH800 in TT/DT or SE36 vaccination. This distinct immune response seemed to be due to differences in the Ag-presenting cell (APC) subset involved in Ag capture. Additionally, based on the IgG subclass analysis, TCI using MH induced a greater Th2-dominant immune response than the injection system in TT/DT vaccination, but almost the same IgG subclass pattern as the injection system in influenza vaccination. The detailed mechanism underlying the immune responses or Th1/Th2 balance is unknown. APCs are thought to have an important role in controlling the characteristics of immune responses, and therefore we are now investigating the characteristics of the immune response in Lang-DTR-EGFP mice [25] or CD11c-DTR-EGFP mice [26], in which the Langerhans cells or CD11c-positive cells can be depleted by intraperitoneal injection of diphtheria toxin to study the role of APCs in our TCI system. Evaluation of the impact of our TCI using MH300 and MH800 on the strength of immune responses and Th1/Th2 balance in animals will help to define the characteristics of the TCI system, and to determine additional applications of our TCI system.

5. Conclusion

The findings of our present study indicate that our TCI system using MH induced effective immune responses against infectious diseases. For clinical application of this TCI system, we are planning translational studies and manufacturing methods for the TCI formulation must also be established. We expect that our vaccine delivery technology will lead to the launch of a TCI system that is efficacious, easy-to-use, cost-effective, and widely acceptable to the public.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2012.04.001.

Acknowledgment

This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), by Health Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare, and by Grant-in-Aid for Scientific Research (B) (24390041) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

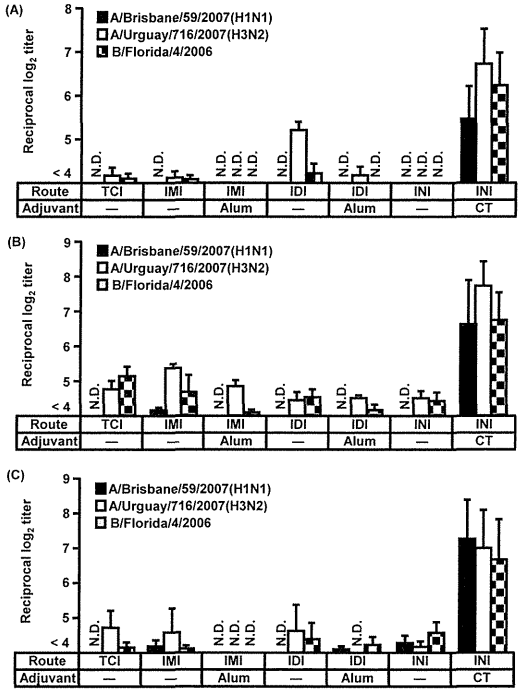
[1] J. Cohen, M. Enserink, Swine flu. After delays WHO agree: the 2009 pandemic has begun, Science 324 (2009) 1496–1497.
[2] C. Fraser, C.A. Donnelly, S. Cauchemez, W.P. Hanage, M.D. Van Kerkhove, T.D. Hollingsworth, J. Griffin, R.F. Baggaley, H.E. Jenkins, E.J. Lyons, T. Jombart, W.R. Hinsley, N.C. Grassly, F. Balloux, A.C. Ghani, N.M. Ferguson, A. Rambaut, O.G. Pybus, H. Lopez-Gatell, C.M. Alpuche-Aranda, I.B. Chapela, E.P. Zavala, D.M. Guevara, F. Checchi, E. Garcia, S. Hugonnet, C. Roth, Pandemic potential of a strain of influenza A (H1N1): early findings, Science 324 (2009) 1557–1561.
[3] A.J. Tatem, D.L. Smith, P.W. Gething, C.W. Kabaria, R.W. Snow, S.I. Hay, Ranking of elimination feasibility between malaria-endemic countries, Lancet 376 (2010) 1579–1591.
[4] R. Romi, D. Boccolini, S. D'Amato, C. Cenci, M. Peragallo, F. D'Ancona, M.G. Pompa, G. Majori, Incidence of malaria and risk factors in Italian travelers to malaria endemic countries, Travel Med. Infect. Dis. 8 (2010) 144–154.
[5] E.L. Giudice, J.D. Campbell, Needle-free vaccine delivery, Adv. Drug Deliv. Rev. 58 (2006) 68–89.
[6] N. Azad, Y. Rojanasakul, Vaccine delivery—current trends and future, Curr. Drug Deliv. 3 (2006) 137–146.
[7] M.M. Levine, Can needle-free administration of vaccines become the norm in global immunization? Nat. Med. 9 (2003) 99–103.

[8] G.M. Glenn, T. Scharton-Kersten, C.R. Alving, Advances in vaccine delivery: transcutaneous immunisation, Expert Opin. Investig. Drugs 8 (1999) 797–805.
[9] P. Chiarella, V.M. Fazio, E. Signori, Application of electroporation in DNA vaccination protocols, Curr. Gene Ther. 10 (2010) 281–286.
[10] P. Batheja, R. Thankur, B. Michniak, Transdermal iontophoresis, Expert Opin. Drug Deliv. 3 (2006) 127–138.
[11] J. Baxter, S. Mitragotri, Needle-free liquid jet injections: mechanisms and applications, Expert Rev. Med. Devices 3 (2006) 565–574.
[12] D.V. McAllister, P.M. Wang, S.P. Davis, J.H. Park, P.J. Canatella, M.G. Allen, M.R. Prausnitz, Microfabricated needles for transdermal delivery of macromolecules and nanoparticles: fabrication methods and transport studies, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13755–13760.
[13] S.P. Sullivan, D.G. Koutsonanos, M. Del Pilar Martin, J.W. Lee, V. Zarnitsyn, S.O. Choi, N. Murthy, R.W. Compans, I. Skountzou, M.R. Prausnitz, Dissolving polymer microneedle patches for influenza vaccination, Nat. Med. 16 (2010) 915–920.
[14] K. Matsuo, Y, Yokota, Y. Zhai, Y.S. Quan, F. Kamiyama, Y. Mukai, N. Okada, S. Nakagawa, A low-invasive and effective transcutaneous immunization system using a novel dissolving microneedle array for soluble and particulate antigens, J. Control. Release (in press).
[15] T. Horii, H. Shirai, L. Jie, K.J. Ishii, N.Q. Palacpac, T. Tougan, M. Hato, N. Ohta, A. Bobogare, N. Arakaki, Y. Matsumoto, J. Namazue, T. Ishikawa, S. Ueda, M. Takahashi, Evidences of protection against blood-stage infection of *Plasmodium falciparum* by the novel protein vaccine SE36, Parasitol. Int. 59 (2010) 380–386.
[16] K. Matsuo, Y. Ishii, Y.S. Quan, F. Kamiyama, Y. Mukai, Y. Yoshioka, N. Okada, S. Nakagawa, Transcutaneous vaccination using a hydrogel patch induces effective immune responses to tetanus and diphtheria toxoid in hairless rat, J. Control. Release 149 (2011) 15–20.
[17] T. Sugiyama, K. Suzue, M. Okamoto, J. Inselburg, K. Tai, T. Horii, Production of recombinant SERA proteins of *Plasmodium falciparum* in *Escherichia coli* by using synthetic genes, Vaccine 14 (1996) 1069–1076.
[18] J. Binder, E. Graser, W.W. Hancock, B. Wasowska, M.H. Sayegh, H.D. Volk, J.W. Kupiec-Weglinski, Downregulation of intragraft IFN-gamma expression correlates with increased IgG1 alloantibody response following intrathymic immunomodulation of sensitized rat recipients, Transplantation 60 (1995) 1516–1524.
[19] N.C. Smith, L. Favila-Castillo, A. Monroy-Ostria, C. Hirunpetcharat, M.F. Good, The spleen, IgG antibody subsets and immunity to *Plasmodium berghei* in rats, Immunol. Cell Bio. 75 (1997) 318–323.
[20] F.D. Finkelman, I.M. Katona, T.R. Mosmann, R.L. Coffman, IFN-γ regulates the isotypes of Ig secreted during in vivo humoral immune responses, J. Immunol. 140 (1988) 1022–1027.
[21] S. Bergstedt-Lindqvist, H.B. Moon, U. Persson, G. Möller, C. Heusser, E. Severinson, Interleukin 4 instructs uncommitted B lymphocytes to switch to IgG1 and IgE, Eur. J. Immunol. 18 (1988) 1073–1077.
[22] I.M. Belyakov, S.A. Hammond, J.D. Ahlers, G.M. Glenn, J.A. Berzofsky, Transcutaneous immunization induces mucosal CTLs and protective immunity by migration of primed skin dendritic cells, J. Clin. Invest. 113 (2004) 998–1007.
[23] G.M. Glenn, C.P. Villar, D.C. Flver, A.L. Bourgeois, R. McKenzie, R.M. Lavker, S.A. Frech, Safety and immunogenicity of an enterotoxigenic *Escherichia coli* vaccine patch containing heat-labile toxin: use of skin pretreatment to disrupt the stratum corneum, Infect. Immun. 75 (5) (2007) 2163–2170.
[24] K. Matsuo, Y. Ishii, Y.S. Quan, F. Kamiyama, Y. Mukai, Y. Yasuo, N. Okada, S. Nakagawa, Transcutaneous vaccination using a hydrogel patch induces effective immune responses to tetanus and diphtheria toxoid in hairless rat, J. Control. Release 149 (2011) 15–20.
[25] A. Kissenpfennig, S. Henri, B. Dubois, C. Laplace-Builhé, P. Perrin, N. Romani, C.H. Tripp, P. Douillard, L. Leserman, D. Kaiserlian, S. Saeland, J. Davoust, B. Malissen, Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells, Immunity 22 (2005) 643–654.
[26] S. Jung, D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, E.G. Pamer, D.R. Littman, R.A. Lang, In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens, Immunity 17 (2002) 211–220.

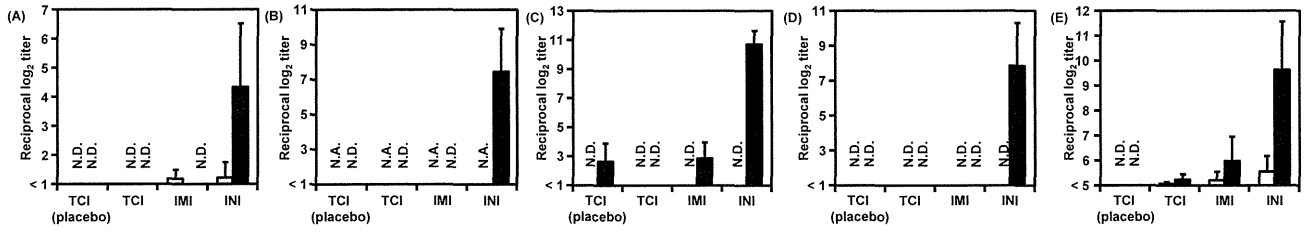
Supplementary Table 1

Score for signs of illness

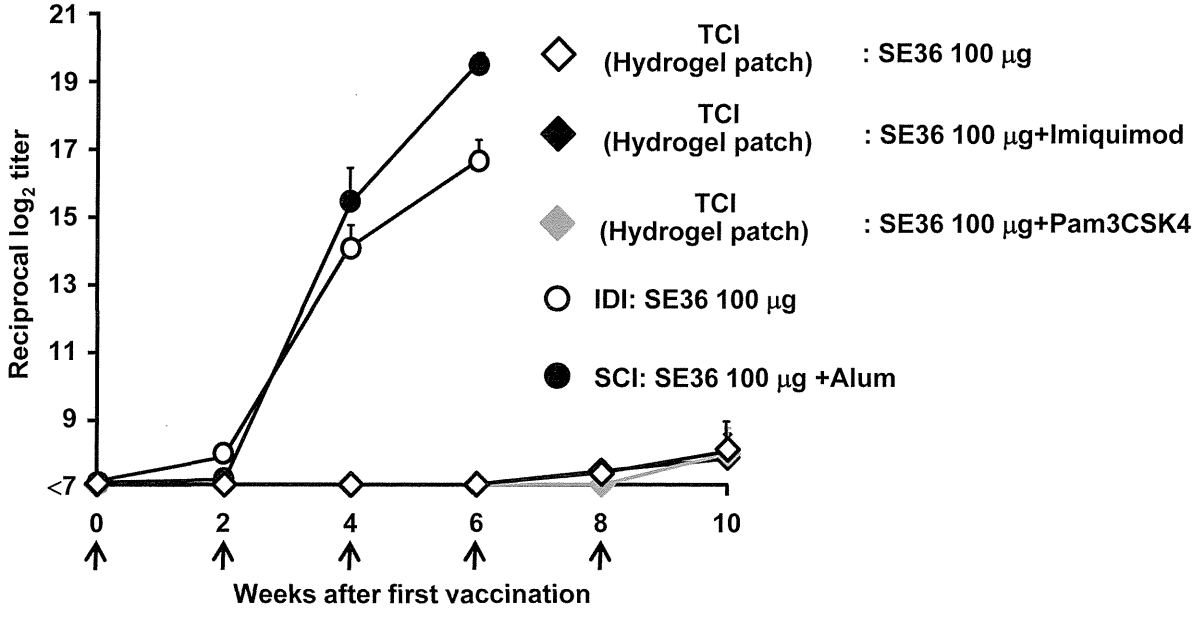
Score	Condition			
	Eye	Fur	Behavior	Others
5	Death	Death	Death	Death
4	Blepharosynechia	Very dull fur	Decrease in spontaneous behavior (Severe)	Respiration failure Cooling of body Cadaverous
3	Loss of eyelid reflex	Dull fur Piloerection (Moderate)	Decrease in spontaneous behavior (Moderate)	Irregular respirations Cadaverous
2	Closed eye	Piloerection (Mild)	Hyperkinesis	irregular respirations
1	Good	Good	Normal	Normal



Supplementary Fig. 1 Anti-HA IgA responses in BALB/c mice after transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with trivalent seasonal influenza HA antigens [A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), and B/Florida/4/2006] (0.2 μg each; △) for 6 h twice at 4-week intervals. Control groups were treated with intramuscular injection of HA antigens (0.2 μg each; □), intradermal application of HA antigens (0.2 μg each; ◇), or intranasal application of HA antigens (0.2 μg each; ○) twice at 4-week intervals. Other control groups were treated with intramuscular injection of HA antigens (0.2 μg each) combined with alum (100 μg) (■), intradermal application of HA antigens (0.2 μg each) combined with alum (100 μg) (◆), or intranasal application of HA antigens (0.2 μg each) with CT (10 μg) (●) twice at 4-week intervals. Sixteen weeks after final vaccination, nasal washes (A), saliva (B) and vaginal wash fluids (C) were collected, and at several points, fecal extracts (D) and sera (E) collected from these mice were assayed to determined HA-specific IgA titer by ELISA. Data are expressed as mean ± SE of results from 5-7 mice. TCI; transcutaneous immunization, IMI; intramuscular immunization, INI; intranasal immunization, N.D.; not detectable.



Supplementary Fig. 2 Anti-HA IgA responses in BALB/c mice after transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with influenza HA antigen of [(A/PR/8/34 (H1N1)] (0.4 μg) using MH800 for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application of antigen-free MH800, intramuscular injection of HA antigens (0.4 μg), or intranasal application of HA antigens (0.4 μg) combined with CT (10 μg) twice at 4-week intervals. Two weeks after the first vaccination/treatment (□) or two weeks after the second vaccination/treatment (■), fecal extracts (A) , nasal wash (B), vaginal wash (C), saliva (D), or serum (E) collected from these mice were assayed to determined HA-specific IgA titer by ELISA. Data are expressed as mean ± SE of results from 13 mice.



Supplementary Fig. 3 SE36-specific IgG in BALB/c after TCI using a hydrogel patch. BALB/c mice were transcutaneously vaccinated with 100 μg SE36 alone or in combination with 100 μg Imiquimod or 10 μg Pam3CSK4 using a hydrogel patch 5 times at 2-week intervals. The other mice were vaccinated with 100 μg SE36 alone or with 100 μg alum 3 times at 2-week intervals by intradermal injection. Sera collected from these mice were assayed by ELISA to determine the SE36-specific IgG titer. Data are expressed as mean ± SE of results from 4 or 5 mice. Arrow indicates vaccination point.

Journal of Controlled Release 184 (2014) 18–19

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Corrigendum

Corrigendum to "Transcutaneous immunization using a dissolving microneedle array protects against tetanus, diphtheria, malaria, and influenza" [J. Control. Release 160 (2012) 495–501]

Kazuhiko Matsuo[a], Sachiko Hirobe[a], Yayoi Yokota[a], Yurika Ayabe[a], Masashi Seto[a], Ying-Shu Quan[b], Fumio Kamiyama[b], Takahiro Tougan[c], Toshihiro Horii[c], Yohei Mukai[a], Naoki Okada[a,*], Shinsaku Nakagawa[a,**]

[a] Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0781, Japan
[b] CosMED Pharmaceutical Co. Ltd., 32 Higashikujokawanishi-cho, Minami-ku, Kyoto 601-8014, Japan
[c] Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan

The authors regret that several errors appeared in the figure legends and Acknowledgment section of the article. The correct related texts appear below.

Fig. 1 (page 497)

Toxoid-specific IgG antibody responses after TCI. Combined TT and DT-containing (A, B) MH800 (0.1 μg; □, 1 μg; △, or 10 μg; ○ each), or (C, D) MH300 (1 μg; △or 3 μg; each) was applied to the back skin of hairless rats for 6 h five times at 2-week intervals. As a control SCI group (•), hairless rats were injected with combined TT and DT (10 μg each). At the indicated points, sera collected from these hairless rats were assayed for the IgG titer to TT (A, C) or DT (B, D) by ELISA. Two weeks after the final vaccination, sera collected from rats transcutaneously vaccinated using MH800 containing both TT and DT (10 μg each) or MH300 (3 μg each), and immunized subcutaneously with combined TT and DT (10 μg each) were assayed for TT (E) or DT (F)-specific IgG subclass (IgG1, IgG2a, IgG2b, or IgG2c) titer by ELISA. Data are expressed as mean ± SE of results from 3 to 4 rats. Arrowhead indicates vaccination points. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for multiple comparisons. (A, B) *; $p < 0.01$ versus the TCI (MH800/0.1 μg) group. (C, D) #; $p < 0.01$ versus the TCI (MH300/1 μg) group. ##; $p < 0.01$ versus the TCI (MH300/3 μg) group. (E, F) *; $p < 0.01$ versus the TCI (MH800) group. #; $p < 0.01$ versus the TCI (MH300) group.

Fig. 2 (page 498)

SE36-specific IgG antibody responses after TCI. SE36-encapsulated MH800 (0.1 μg; □, or 2 μg; ■) or MH300 (0.1 μg; △, or 2 μg; ▲) was applied to the back skin of BALB/c mice for 6 h four times at 2-week intervals. As a control group, BALB/c mice were subcutaneously injected with SE36 (0.1 μg; , or 5 μg; •). (A) At the indicated points, sera collected from these mice were assayed for the IgG titer to SE36 by ELISA. (B) Two weeks after the final treatment, sera collected from these mice were assayed for SE36-specific IgG subclass (IgG1, IgG2a, IgG2b, or IgG3) titer by ELISA. Data are expressed as mean ± SE of results from 5 mice. Arrowhead indicates vaccination points. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for multiple comparisons. *; $p < 0.01$ versus the TCI (MH800/0.1 μg) group. **; $p < 0.01$ versus the TCI (MH800/2 μg) group. #; $p < 0.01$ versus the TCI (MH300/0.1 μg) group. ##; $p < 0.01$ versus the TCI (MH300/2 μg) group.

Fig. 4 (page 499)

Anti-HA immune responses in BALB/c mice after TCI. BALB/c mice were transcutaneously vaccinated with trivalent seasonal influenza HA from (A, D) A/Brisbane/59/2007 (H1N1), (B, E) A/Uruguay/716/2007 (H3N2), and (C, F) B/Florida/4/2006 (0.2 μg each; △) for 6 h twice at 4-week intervals. Control groups were treated with intramuscular (□), intradermal ( ), or intranasal (○) application of HAs (each 0.2 μg) twice at 4-week intervals. Another control group was treated with intramuscular injection of HA Ags (each 0.2 μg) with alum (100 μg) (■), intradermal application of HA Ags (each 0.2 μg) combined with alum (100 μg) (•), or intranasal application of HA Ags (each 0.2 μg) with CT (10 μg) (●) twice at 4-week intervals. (A–C) At the indicated points, sera collected from these mice were assayed to determine the HA-specific IgG titer by ELISA. (D–F) Sera collected 2 weeks after the last treatment were assayed for HA-specific IgG subclass (IgG1 and IgG2a) by ELISA. (G) Two weeks after the final treatment, sera collected from these mice were assayed for the HI titer. HI activity expressed as the highest dilution that resulted in complete inhibition of hemagglutination. Data are expressed as mean ± SE of results from 5 to 7 mice. INI; intranasal immunization, IDI; intradermal immunization, N.D.; not detectable. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for multiple comparisons. *; $p < 0.01$ versus the TCI (MH800) group.

Fig. 5 (page 499)

Protection of vaccinated mice against influenza virus challenge. BALB/c mice were transcutaneously vaccinated with HA from A/PR/8/34 (H1N1) (0.4 μg) for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 μg), or intranasal application of HA (0.4 μg) combined with CT (10 μg) twice at 4-week intervals. These mice were each

DOI of original article: http://dx.doi.org/10.1016/j.jconrel.2012.04.001.
* Corresponding author. Tel./fax: +81 6 6879 8176.
** Corresponding author. Tel.: +81 6 6879 8175; fax: +81 6 6879 8179.
E-mail addresses: okada@phs.osaka-u.ac.jp (N. Okada), nakagawa@phs.osaka-u.ac.jp (S. Nakagawa).

http://dx.doi.org/10.1016/j.jconrel.2014.04.001

infected intranasally with 6×10^5 PFU of the A/PR/8/34 (H1N1) virus. (A) At the indicated points, sera collected from these mice were assayed for the HA-specific IgG titer by ELISA. (B, C) Two weeks after the final treatment, sera collected from these mice were assayed for (B) anti-HA IgG subclass (IgG1 and IgG2a) and (C) the HI titer. HI activity is expressed as the highest dilution that resulted in complete inhibition of hemagglutination. Data are expressed as mean ± SE of results from 13 mice. (D) Body weight was measured each day and is presented as a percentage of the original weight before infection (day 0). (E) The performance status of the mice was scored every day. (F) Six days after infection, the lungs were collected from these mice and the number of viruses in the lung homogenate was determined using a plaque assay system. (G) The degree of inflammation of the lung was scored as follows: 0, none; 1, very slight; 2, mild; 3, moderate; and 4, severe. Pathologic findings were classified into bronchopneumonia and interstitial pneumonia. (D–G) Data are expressed as mean ± SE of results from 10 mice. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for multiple comparisons. *; $p < 0.01$ versus the TCI (MH800 placebo) group. #; $p < 0.01$ versus the TCI (MH800) group.

Acknowledgment (page 500)

This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), by a Health Labour Sciences Research Grant from the Ministry of Health, Labour, and Welfare, and by a Grant-in-Aid for Scientific Research (B) (24390041) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Notes

The corrections made in this Corrigendum do not affect the original conclusion.

The authors would like to apologize for any inconvenience caused.

Review

総　説

経皮ワクチンの進歩と展望*

廣部祥子**　岡田直貴**　中川晋作**

Key Words：transcutaneous vaccination, hydrogel patch, self-dissolving microneedle array, clinical study

はじめに

1980年のWHOによる天然痘根絶宣言がワクチン開発の成果であるように[1]，感染症対策においてワクチンが果たしてきた功績は輝かしいものである．しかしながら，実用化ワクチンの大半を占める注射型ワクチンは，その施行に医療技術者を必要とし，注射剤の輸送・保管に一貫した低温温度管理(コールドチェーン)が不可欠であるなど，経済的・技術的な制約によって予防接種を最も必要とする地域へのワクチン普及を停滞させている．また，新興・再興感染症の世界的流行やバイオテロリズム発生時に，ワクチンの大規模投与を迅速に実施できない点も懸念されている．

このような背景の下，医療技術者や注射針などの医療器具を必要としない簡便なワクチン手法として，「飲むワクチン」「吸うワクチン」「貼るワクチン」などの開発が精力的に行われている．その中で著者らは，皮膚に貼るだけで感染症に対する防御効果を獲得可能な経皮ワクチン製剤「貼るワクチン」の実用化を目指している．

本総説では，これまでの経皮ワクチン用デバイスの進歩を概説するとともに，著者ら独自のデバイスである親水性ゲルパッチならびに皮膚内溶解型マイクロニードルの研究成果を通して，経皮ワクチンの今後の展望を述べる．

経皮ワクチンデリバリー技術の発展

皮膚は，生体と外界を隔てる組織であり，常に異物侵入の危険に曝されている．そのため，皮膚内には，免疫監視機構において非常に重要な役割を担う抗原提示細胞(APC)が豊富に存在する(図1)．このように皮膚は，生体の防御機能が高度に発達した「免疫学的バリアー」を備えており，ワクチンのターゲットとして非常に優れた組織である．APCは，異物を認識・捕食した後に免疫誘導の場である所属リンパ節へと遊走する．そして，T細胞ならびにB細胞を抗原特異的に活性化することで，全身性の獲得免疫応答を誘導する．したがって，貼るワクチンにおいて抗原特異的な免疫応答を強力に誘導するためには，抗原を効率よくAPCの存在する生きた表皮・真皮に送達することが必要である．しかしながら，皮膚の最外層には物質透過を防ぐ物理的バリアーとなる角質層が存在するため[2]，ペプチドや蛋白質といった水溶性で高分子の抗原を単に皮膚表面に塗布するだけでは，角質層下のAPCに抗原を効率よく送達することはできない．そこで，DDS領域で開発されてきた経皮薬物デリバリー技術を経皮抗原デリバリー技術として応用することで，APCの存在する生きた表皮・真皮にまで抗原を送達しうる「経皮ワクチンシステム」の開発が行われてきた．

* The advance and prospect of transcutaneous vaccination system.

** Sachiko HIROBE, Naoki OKADA, Ph.D. & Shinsaku NAKAGAWA, Ph.D.: 大阪大学大学院薬学研究科薬剤学分野〔〒565-0871 大阪府吹田市山田丘1-6〕; Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, JAPAN

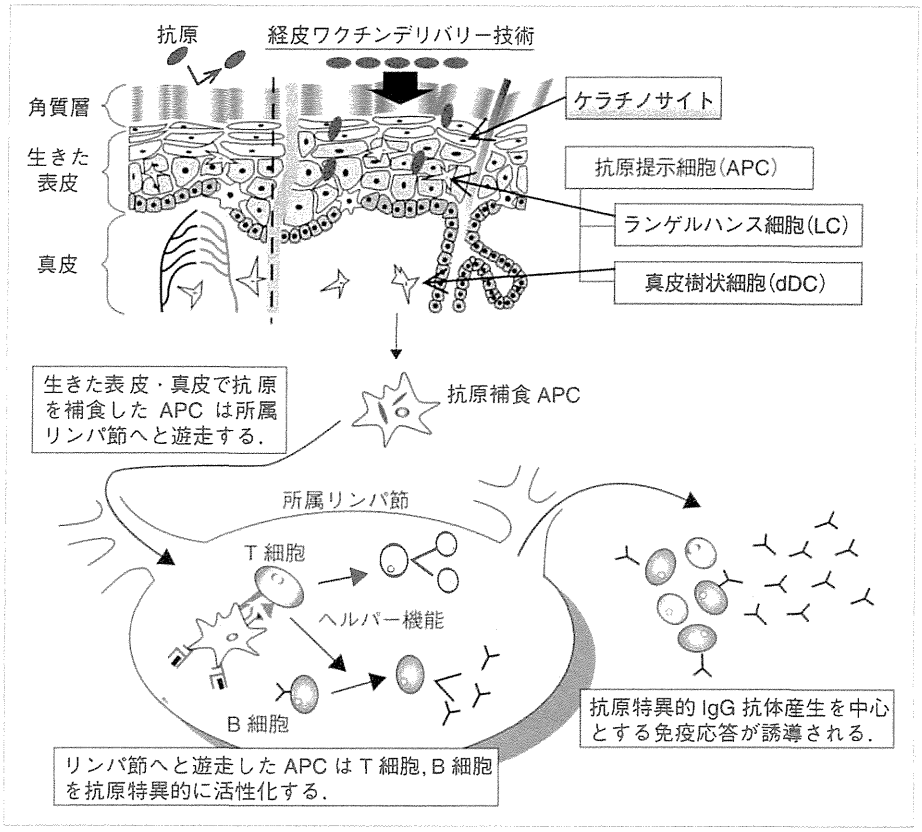


図1　経皮免疫誘導メカニズム
ケラチノサイト：各種サイトカインやケモカイン，増殖因子を分泌する．角質層下の生きた表皮の約90%を占めており，異物侵入に対する自然免疫の誘導にかかわっている．
抗原提示細胞：抗原捕食によって，抗原特異的にリンパ球を感作する．ランゲルハンス細胞(LC)は生きた表皮に，真皮樹状細胞(dDC)は真皮に常在する．

具体例としては，皮膚への電圧負荷により一時的に角質層に孔をあけるエレクトロポレーション法[3]，電流負荷による水の移動とともにイオン性の物質を生きた表皮へと送達するイオントフォレシス法[4]，空気の圧力によって抗原を注入するJet injector[5]法などがあげられる．これらの手法によって確かに抗原が角質層を透過して生きた表皮にまで送達されることが実証されており，抗原特異的IgG抗体価の上昇が認められている．しかしこれらの方法は，特殊な装置を必要とすることから簡便性に乏しく，角質層を破壊してしまうことによる二次的な炎症や感染が危惧されるために，今のところ実用化までには至っていない．

そこで，まさに皮膚に貼るだけという簡便な操作で抗原特異的な免疫応答を誘導できる粘着性パッチおよびガーゼパッチの研究開発が注目されている[6,7]．しかしながら，これらの経皮ワクチン製剤は抗原を十分に浸透させるために，角質層あるいは角質層脂質成分を部分的に除去する前処理を必要とする．さらに，ガーゼパッチを応用した経皮ワクチン製剤は，皮膚に適用する直前に抗原溶液を浸み込ませるため，簡便性に欠けており，また注射型ワクチンと同様に抗原溶液の輸送や保管にコールドチェーンを必要とするなど，開発途上国へのワクチン普及を推し進めるためにはさらなる改良を加える必要がある．

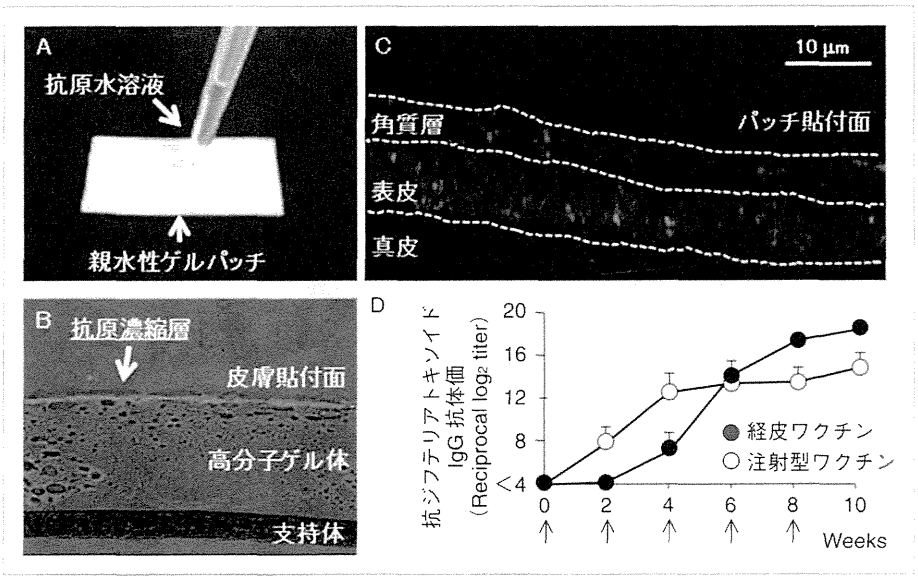


図２　親水性ゲルパッチ

A：親水性ゲルパッチ（1 cm×2 cm）への抗原蛋白質水溶液の滴下．
B：抗原蛋白質（赤色蛍光標識）水溶液を滴下して30分後の親水性ゲルパッチ断面の顕微鏡写真．
C：抗原蛋白質（赤色蛍光標識）を含む親水性ゲルパッチをマウス耳介皮膚に６時間貼付した後，パッチ適用部位の皮膚組織を摘出し，LCの蛍光免疫染色（緑色）を行った．共焦点レーザー顕微鏡で撮像した２次元データから３次元画像を構築した．
D：ヘアレスラット背部皮膚にジフテリアトキソイド100 μgを含んだ1 cm×2 cmの親水性ゲルパッチを24時間貼付した．対照群のヘアレスラットには同量のジフテリアトキソイドを背部皮下注射した．これらのワクチン投与を２週間隔で５回繰り返し（図中の↑），経時的に回収した血中の抗トキソイド抗体価をELISA法により測定した．

親水性ゲルパッチを用いた経皮ワクチン

著者らがコスメディ製薬株式会社と共同開発した親水性ゲルパッチを応用した経皮ワクチンは，皮膚の前処理をすることなく抗原特異的な抗体産生を誘導できる点で画期的である[8-11]．親水性ゲルパッチはアクリル酸エステル系粘着基剤をベースに，湿潤剤，吸収促進剤などすでに医薬品や化粧品などで用いられている安全性の高い材料を配合して作製している．また，本パッチの皮膚貼付面に抗原蛋白質水溶液を滴下すると（図2-A），水分のみが高分子ゲル体に吸収されてパッチ表面に抗原蛋白質の濃縮層が形成される（図2-B）．このように，抗原を含浸させた状態で取り扱うことができるために，輸送・保管が容易になると考えられる．抗原含有親水性ゲルパッチをマウス耳介皮膚に貼付すると，抗原は角質層を透過して表皮組織にまで到達する（図2-C）．親水性ゲルパッチによる抗原分子の角質層透過機構の一つとして，ゲルパッチの貼付により抗原が角質層に分配し，現出した皮膚内の抗原濃度勾配が単純拡散の駆動力となって抗原の生きた表皮への送達を促進させることがあげられる．また，ゲルパッチの貼付により角質層が水和することで，角質層の細胞間隙を構成する脂質二重層の構造が緩み，水溶性の高分子が角質層へと分配しやすくなることも考えられる．角質層下に送達された抗原はAPCにより捕食され，そのAPCが免疫誘導の場である所属リンパ節へと遊走し，抗原特異的免疫応答を誘導することを確認している[8]．

次に，本親水性ゲルパッチを用いて破傷風・ジフテリア感染症モデルにおける経皮ワクチンシステムの有効性について検証した（図2-D）．トキソイド含有親水性ゲルパッチの貼付により

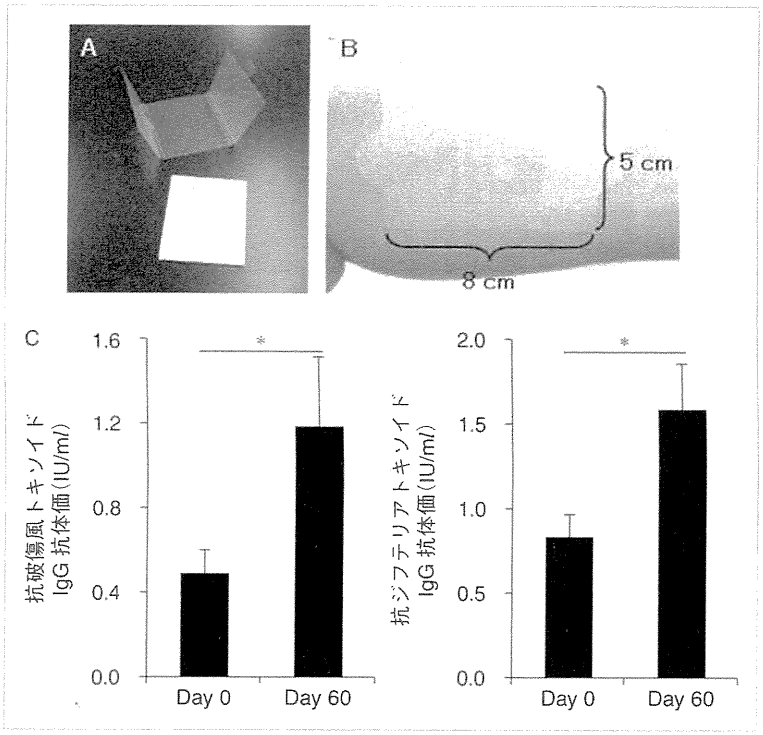


図3　親水性ゲルパッチを用いた経皮ワクチンの臨床研究
A：製品化を志向した親水性ゲルパッチの形状と包装．
B：親水性ゲルパッチのヒト上腕内側皮膚への貼付写真．
C：破傷風・ジフテリアトキソイドを各2 mg含有した5 cm×8 cmの親水性ゲルパッチをヒト上腕内側皮膚に24時間貼付した．貼付前ならびに貼付60日後に血中の抗トキソイド抗体価をELISA法により測定した．データはmean±S. E.を示した．*$P<0.01$（paired t-test）

各トキソイド特異的なIgG抗体が産生され，それらが各毒素に対する中和活性を有することが確認された[10]．同量の抗原をろ紙に浸み込ませて貼付したところ，トキソイドに対する抗体産生はまったく認められず，われわれ独自の親水性ゲルパッチはその表面上に抗原濃縮層を形成することで優れた抗原送達を可能にしていることが示唆された．また，抗原を含有したゲルパッチを貼付した皮膚局所に顕著な刺激性は認められなかったこと，各種血液検査や病理組織学的検査などにおいても異常が確認されなかったことから，本経皮ワクチン製剤の安全性が実証された[11]．

さらに著者らは，これらの結果に基づき，破傷風・ジフテリア経皮ワクチンの安全性・有効性をヒトにおいて検証する臨床研究を実施した[12]．破傷風・ジフテリアトキソイドを含有させた親水性ゲルパッチ製剤（5 cm×8 cm）を作製し（図3-A），ヒト上腕内側皮膚に24時間貼付した（図3-B）．その結果，ゲルパッチ剥離直後に貼付部位において一時的な軽微の紅斑が認められたものの，数日以内に消失した．また，各種血液検査においても顕著な変化は確認されず，ゲルパッチ製剤の貼付は全身性の副反応を誘発しないことが示された．さらに，各トキソイドに対する抗体価を測定すると，本経皮ワクチン製剤の貼付によりヒトにおいても有意に抗体価の上昇が達成されることが明らかとなった（図3-C）．このように親水性ゲルパッチはヒトにおいても安全かつ有効なワクチンデバイスであることが実証された．本邦では多くの人が，乳幼児期の破傷風・ジフテリアトキソイドワクチンの接種により抗トキソイド抗体を有している．

しかし，その抗体価は年齢を重ねるとともに低下するといわれており，皮膚に貼るだけと簡便な手法でブースト免疫を誘導できる本製剤は非常に有用な新規ワクチン製剤であるといえる．

しかしながら，これまで開発されてきたガーゼパッチはもちろんのこと，著者らの親水性ゲルパッチについても，十分な抗原を皮膚内へ送達するためには大量の抗原を必要とする．親水性ゲルパッチについて，角質層下に送達される抗原量は含浸させた量の数%程度であることを確認しており，注射に比べて抗原の利用率が低いのが現状である．そのため，簡便かつ安全なだけでなく安価でより有効な経皮ワクチン製剤の実用化に向けては，抗原の角質層透過効率に優れるゲルパッチ，ならびに免疫増強効果を図るための臨床応用可能な経皮アジュバントを開発していかなければならない．また，製剤安定性の評価，製剤製造法の樹立や品質試験法の確立など，経皮ワクチンの製品化・実用化に向けた取り組みを推進していくことが重要である．

皮膚内溶解型マイクロニードルを用いた経皮ワクチン

親水性ゲルパッチは水溶性抗原物質の角質層への分配とその後の単純拡散を増大することによってAPCへの送達効率を上昇させるため，抗原物質が水に不溶性あるいは懸濁された粒子状形態の場合には適用が困難である．ところが，注射型ワクチン製剤として実用化されている抗原の多くは，無毒・弱毒化したウイルスや細菌(BCG, 麻疹ワクチンなど)，あるいはそれら病原体由来コンポーネントの凝集体(インフルエンザHA抗原など)といった粒子状形態であるため，親水性ゲルパッチに適応可能なワクチン抗原は限定されてしまう．すなわち，経皮ワクチン製剤の適応を拡大するためには，不溶性ならびに粒子状ワクチン抗原にも対応しうる新たな経皮ワクチンデバイスが必要とされる．

そこで著者らは，微小な針により角質層に孔をあけることで物質を送達するマイクロニードル法を用いた経皮ワクチンの開発に着目した[13]．この方法は神経終末が存在する真皮の深部にまで針が到達しないことから，痛みを伴わずにワクチン投与ができる．さらに，抗原透過のバリアーとなる角質層を物理的に突破するため，さまざまなワクチン抗原に対応可能な新規経皮ワクチンデリバリー技術として期待されている．マイクロニードルという概念は1976年にGerstelとPlaceらによってはじめて報告されて以来[14]，製造技術が困難であることから費用対効果の面が問題となり開発研究は停滞していた．しかしながら，1990年代になって電子工業が発展することで微細加工技術が容易になり，現在ではさまざまなマイクロニードルの開発が進められている[15)~18)](図4)．

これまでに開発されてきたマイクロニードルは，経皮送達機構や構成材料の種類によってさまざまなタイプに分類される．第一世代のマイクロニードルはシリコンや金属(ステンレス，チタン)を構成材料としたものであり，マイクロニードルで処置した皮膚に対してワクチン抗原を塗布する(図4-A)，微小針の中空から抗原溶液を注入する(図4-B)，微小針にワクチン抗原を吸着させて経皮送達する(図4-C)，といった方法がある．これらは剛性に優れる，成形しやすい，といった利点を有しているが，微小針が生体内で折れ残り，重篤な組織傷害をひき起こす危険性が払拭できないために，実用化する上で大きな課題を抱えている．そこで近年では，第二世代マイクロニードルとして，ポリ乳酸(PLA)，ポリグリコール酸(PGA)，ポリ乳酸・グリコール酸(PLGA)といった生分解性バイオポリマーやヒアルロン酸などを利用した溶解型マイクロニードルの開発が注目されている[18)19)]．これらは生体適合性に優れる構成素材を使用し，微小針自体が溶解することによって装填あるいは吸着した抗原を皮膚内へと送達することができるといった特徴を有する(図4-D)．そのため，第一世代マイクロニードルの安全面における問題点を克服できると考えられており，臨床応用・実用化が待望されている．

著者らは共同研究を行っているコスメディ製薬株式会社が独自に開発した皮膚内溶解型マイクロニードルを経皮ワクチンへと応用することに成功している[19)20)](図5-A)．この第二世代マイクロニードルは皮膚組織成分であるヒアルロン酸を主成分

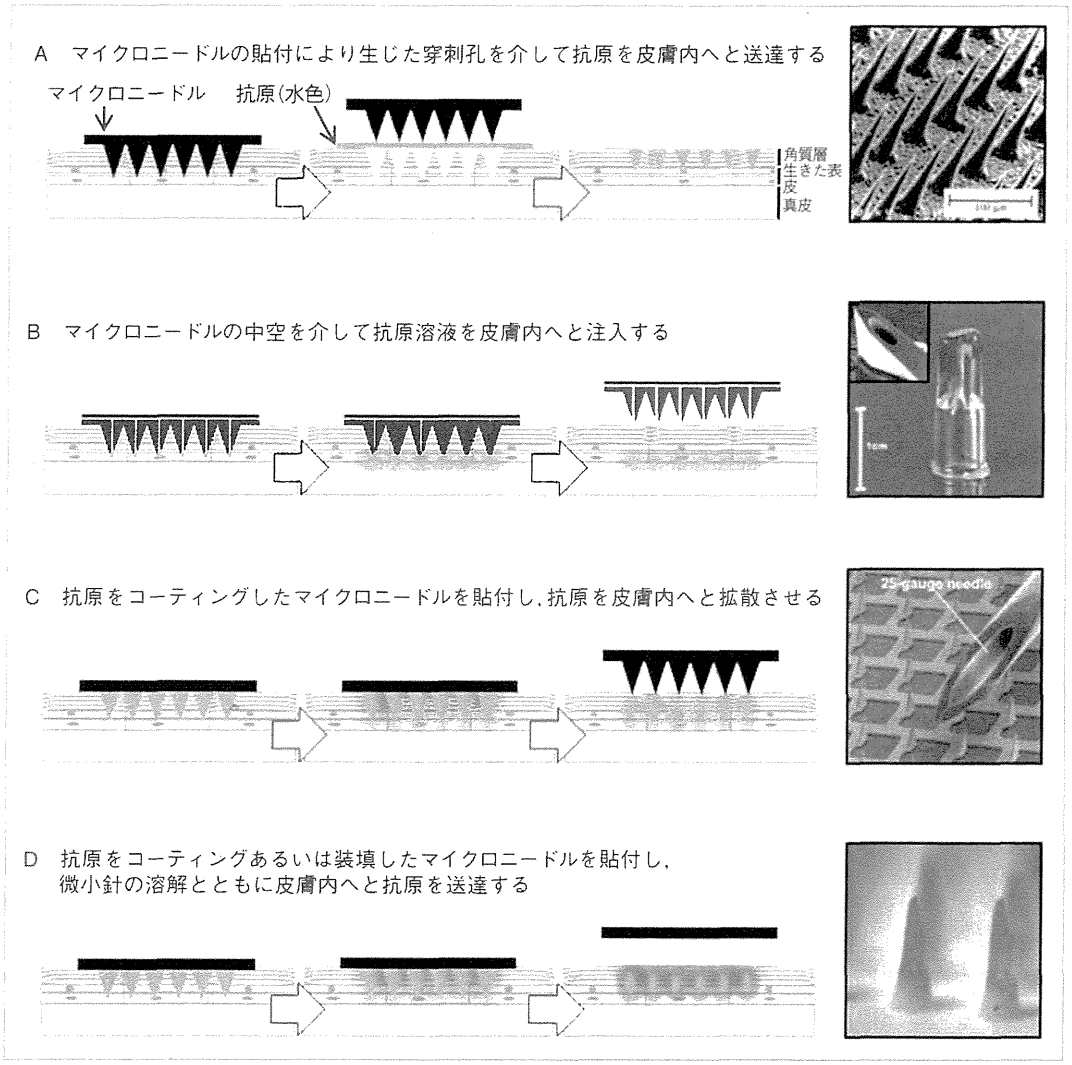


図4　マイクロニードルを用いた経皮送達機構とその例
A：シリコン製マイクロニードル[5]，B：シリコン製中空マイクロニードル(NanoPass)[6]，C：チタン製コーティング用マイクロニードル[7]，D：ポビドン主体溶解型マイクロニードル[8]．

としており，針長が200 μmのマイクロニードルは，すでに化粧品として上市・販売されていることから，医薬品のデバイスとしてもヒトへの適用が期待できる(表1)．本マイクロニードルの微小針は皮膚内へと挿入された後，水分を吸収することによって溶解し，装填した抗原を角質層下へと容易に送達するように設計されている．また，微小針の形状や長さは自由に制御することができる．実際に，さまざまな形状の抗原装填皮膚内溶解型マイクロニードルを貼付すると，針は皮膚内で溶解しており，装填物質の形状が可溶性分子，不溶性粒子にかかわらず，APCが存在する生きた表皮ならびに真皮へと物質を送達できることが確認された(図5-B)．また，針部の長さに依存して抗原を送達する部位が異なることが示され，皮膚内への物質送達においてその深度までも制御可能であることが示唆された．さらに，可溶性抗原である破傷風・ジフテリアトキソイドのみならず，粒子状抗原であるインフルエンザHA抗原を装填したマイクロニードルパッチにおいても動物皮膚に貼付することで抗原特異的抗体産生が誘導され，その抗体価は注射群よりもわずかではあるが，高値

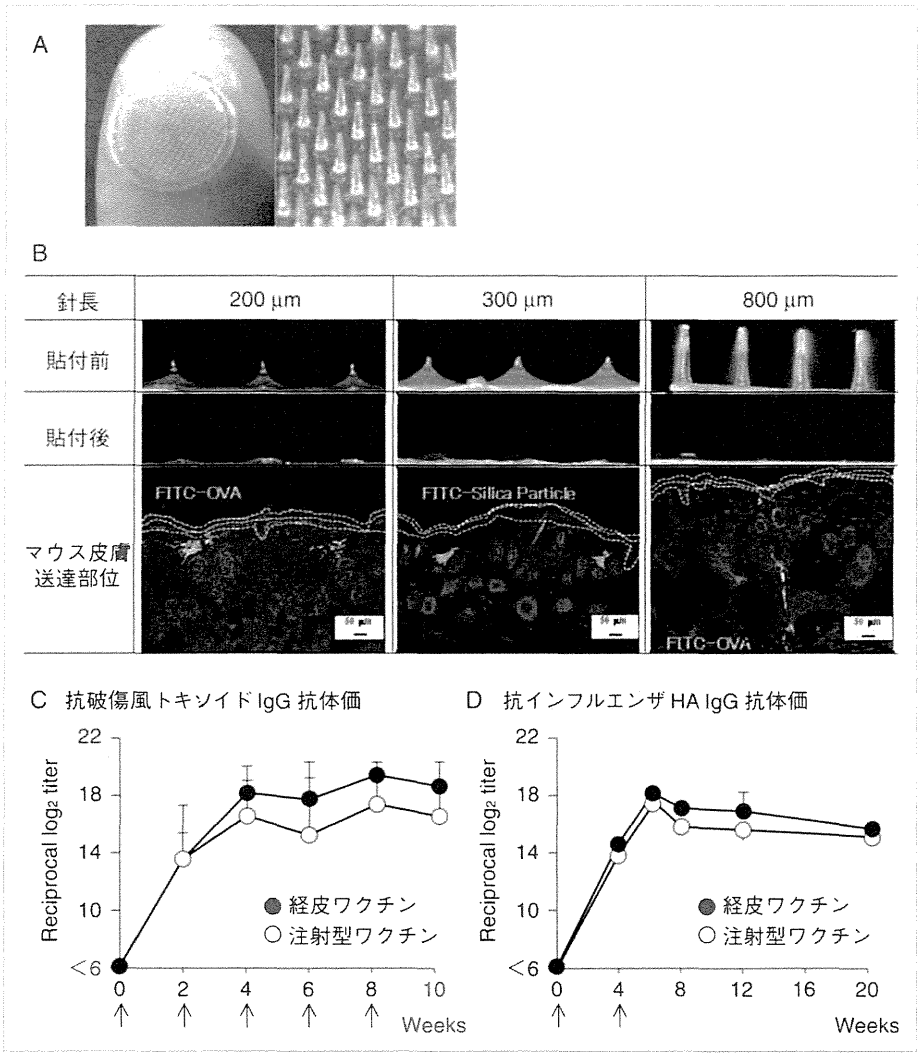


図5　皮膚内溶解型マイクロニードルを用いた経皮ワクチン

A：皮膚内溶解型マイクロニードル．面積は0.8 cm²であり，生体適合性に優れたヒアルロン酸を主成分として形成された微小な針を有する．

B：針部の長さおよび形状が異なる3種類の皮膚内溶解型マイクロニードルに蛍光標識OVAあるいは蛍光標識ナノ粒子を装填し，マウス背部皮膚に6時間貼付した．貼付部位の皮膚を回収し，凍結切片を作製後，蛍光顕微鏡により観察した．

C：破傷風トキソイドを10 μg装填した針長800 μmのマイクロニードルパッチをラット背部皮膚に6時間貼付した．対照群のラットには同量の破傷風トキソイドを背部皮下注射した．これらのワクチン投与を2週間隔で5回繰り返し（図中の↑），経時的に血中の抗トキソイド抗体価をELISA法により測定した．

D：インフルエンザHA抗原〔A/Brisbane/59/2007（H1N1）〕を0.2 μg装填した針長800 μmのマイクロニードルパッチをマウス背部皮膚に6時間貼付した．対照群のマウスには同量のインフルエンザHA抗原を大腿部筋肉内注射した．これらのワクチン投与を4週間隔で2回実施し（図中の↑），経時的に血中の抗HA抗体価をELISA法により測定した．

を示すことが明らかとなった（図5-C, D）．これらの成果に基づき，すでに著者らは本デバイスのヒトへの適用を検証する臨床研究を開始しており，抗原装填マイクロニードルパッチが皮下注射とほぼ同等の抗体産生を誘導することを確認している．現在欧米のみならず，本邦においても新規剤形で