Compositional Optimization and Safety Assessment of a Hydrogel Patch as a Transcutaneous Immunization Device

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The development of a simple, easy-to-use, and non-invasive vaccination system is in high demand. For transcutaneous immunization (TCI), we previously developed a hydrogel patch formulation that accelerates the penetration of an antigen (Ag) through the stratum corneum (SC) and effectively elicits Ag-specific immune responses. The present studies were performed to optimize the composition and assess the safety of the patch formulation. A hydrogel patch with a water content ratio of 5% more effectively induced an immune response compared to patches with a different composition, suggesting that the moisture content of the hydrogel patch formulation has optimal ratio for SC hydration to promote Ag penetration through the SC. TCI using a hydrogel patch induced few local and systemic adverse reactions. Based on these results, we are now advancing translational research to evaluate the safety and efficacy of our novel TCI system in humans.

Key words transcutaneous immunization; hydrogel patch; composition; skin hydration; safety

Transcutaneous immunization (TCI) is a simple, easy-to-use, and non-invasive vaccination method instead of conventional injection system.^{1,2} Langerhans cells (LCs) are the target cells for antigen (Ag) delivery in TCI.^{3–7} The top layer of the skin, the stratum corneum (SC), however, acts as a physical barrier to substance penetration.⁸ Thus, simply painting an Ag solution onto the skin surface does not allow for Ag delivery to the LCs. To overcome this issue, we developed a hydrogel patch as a TCI device that promotes Ag penetration through the SC.⁹ In TCI using a hydrogel patch, Ags are delivered to LCs and Ag-specific immunoglobulin (Ig) G production is induced. The hydrogel patch formulation forms a concentrated Ag layer on its surface, suggesting that the high Ag concentration gradient generated between the patch and the skin tissue by application of the hydrogel patch is critical for producing the driving force necessary to accelerate passive diffusion and distribute the Ags.

Little is known, however, about the optimal composition of the hydrogel patch for the enhancement of Ag penetration through the SC. Reducing the dose of Ag applied to the hydrogel patch is important for production efficiency, and exploring the most appropriate composition for the hydrogel patch is a significant matter for practical application of TCI system using a hydrogel patch. We hypothesized that one of the factors in the hydrogel patch promoting Ag penetration through SC was the induction of SC hydration by hydrogel patch application. In this study, we prepared various hydrogel patches with a different water content to obtain information about the impact of the water content of the hydrogel patch by comparing the Ag-specific IgG production induced by TCI using these patches.

We also assessed the safety of TCI using a hydrogel patch, which is crucial for the clinical use of this vaccination system. In the TCI system, it is feared that skin irritation, such as contact hypersensitivity, might be induced by frequent application of a patch formulation. In the present study, we evaluated adverse reactions at the patch application site and systemic toxicity induced by a hydrogel patch containing ex-

cess tetanus and diphtheria toxoids. These studies were performed to obtain the information necessary to proceed to clinical trials of TCI using a hydrogel patch.

MATERIALS AND METHODS

Antigens Ovalbumin (OVA, Sigma-Aldrich Inc., St. Louis, MO, U.S.A.) was used as a model Ag to evaluate the optimal patch formulation. Tetanus and diphtheria toxoids (kindly provided by The Research Foundation for Microbial Diseases of Osaka University, Suita, Japan) were used as practical Ags to evaluate the toxicity of the hydrogel patch formulation.

Animals Male and female hairless rats (5 weeks old), female ddY mice (7 weeks old), and male Wistar rats (5 weeks old) were purchased from SLC Inc. (Hamamatsu, Japan). Animals were handled in accordance with the Osaka University guidelines for experimental animal welfare. The research protocols described in this report were reviewed and approved by the Animal Care and Use Committee of Osaka University.

Patch Formulation for TCI. Four Types of Patches Were Prepared Using the Following Methods (i) Hydrogel Patch (Water Content Ratio; 5%): The hydrophilic adhesive matrix comprised crosslinked HiPASTM acrylic adhesive (CosMED Pharmaceutical Co., Ltd., Japan), octyldodecyl lactate, glycerin, and sodium hyaluronate. The coating solution was prepared by dissolving the above components in an ethyl acetate/acetone/water mixture, and the matrix was prepared by casting the above mixture solution on a polyester release liner using a knife coater. The solution was allowed to stand at room temperature for 30 min, and then oven-dried at 80 °C for 15 min to remove residual organic solvents. The dried film was then laminated onto a polyester film.

(ii) Hydrophobic Adhesive Patch (Water Content Ratio; 0%): The hydrophobic adhesive matrix consists of crosslinked MASCOS 10TM acrylic adhesive (CosMED Pharmaceutical Co., Ltd., Japan) and isopropyl myristate. The coating solution was prepared by dissolving the above

components in ethyl acetate, and the matrix was prepared by casting the above mixture solution on a polyester release liner using a knife coater. The solution was allowed to stand at room temperature for 30 min, and subsequently oven-dried at 80 °C for 15 min to remove residual organic solvents. The dried film was then laminated onto polyester film.

(iii) **Water-Soluble Adhesive Patch (Water Content Ratio; 66%):** The water-soluble adhesive solution comprised sodium acrylate polymer and gelatin, glycerin, and water (8 g:1 g:25 g:56 g). A crosslinker solution was prepared separately by dissolving 0.5 g dihydroxyaluminum aminoacetate in 10 g of 5% sodium tartrate solution. After mixing the crosslinker and adhesive solutions, the matrix was prepared by casting the above mixture solution onto a polyester release liner using a knife coater. The film was then laminated onto polyester non-woven fabric and allowed to stand at room temperature overnight.

Fifty microliters of the Ag solution was dropped onto the surface of each patch. The patches were applied soon after water absorption or, in some cases, after storage at 37 °C for 8 h, and at 4 °C or 25 °C for 1 month or 6 month.

(iv) **Dry Gauze and Wet Gauze Patches:** Fifty microliters of the Ag solution was dropped onto gauze. For TCI, the Ag-containing gauze was applied either before drying (wet gauze patch) or after drying (dry gauze patch).

Vaccination Protocol and Antibody Titer Measurement

Each patch formulation (2 cm²) containing 100 µg OVA was applied to the auricle of ddY mice for 24 h. The patches were covered with wound management film (BIOCLUSIVE; Johnson & Johnson Medical, Ltd., Tokyo, Japan) to allow for better skin adherence. The procedure was repeated 2 weeks later. As a positive control, an intradermal immunization group comprised mice that were intradermally injected with 100 µg OVA using the same procedure. Two weeks after the final vaccination, sera were collected from the mice and the OVA-specific antibody titer was determined using an enzyme-linked immunosorbent assay (ELISA). End-point titers of OVA-specific antibody are expressed as the reciprocal log₂ of the last dilution that showed 0.1 absorbance units after subtracting the background, as described previously.^{9,10)}

Measurement of Skin Surface Impedance after Hydrogel Patch Application The abdominal skin of Wistar rats was shaved the day before the experiment. The hydrogel patch (water content ratio; 5%) or filter paper patch containing OVA (100 µg/2 cm² each) was applied to the shaved skin. Twenty-four hours later, the patches were removed and the skin surface impedance between the patch application area and the non-application skin area was measured within 30 s using a Pocket Tester (CDM-03D; Custom Inc., Kanagawa, Japan). The impedance of the skin is defined as the resistance measurement between two electrodes in electrical contact with the epidermis.¹¹⁾

Histopathologic Image of Human Skin after Application of the Hydrogel Patch A hydrogel patch (1 cm²) was applied to human skin in the donor component of a Frantz cell system for 24 h. As a control, phosphate-buffered saline (PBS) was applied to human skin. The human skin was removed, fixed in 10% neutral formalin buffer, and embedded in paraffin. The histopathologic changes of human skin at the application site were examined using hematoxylin and eosin (HE)-stained specimens.

Release Test of Ag Contained on Hydrogel Patch OVA-containing hydrogel patch (200 µg/2 cm²) was prepared. These patches were sealed off and preserved at 4 or 25 °C for 7, 35, or 105 d. After preservation, OVA-containing patches were agitated and sonicated in deionized water. Amount of OVA in obtained supernatant was detected by gel filtration chromatography. Indicated data express as average of two.

In Vivo Toxicity Evaluation of Ag-Containing Hydrogel Patch Four types of hydrogel patches (water content ratio; 5%) containing combined tetanus and diphtheria toxoids in various doses (0, 50, 200, or 400 µg each/cm²) were applied to the back skin of individual hairless rat for 24 h four times (Fig. 3A). The patches were covered with wound management film to allow for better skin adherence. As a control group, wound management film alone was applied to the back skin of hairless rats.

Body weight was monitored during this time. On day 23, the test sites were observed immediately after removal of the hydrogel patch and scored for signs of erythema or edema according to the Draize dermal scoring criteria.¹²⁾ This scale is used to assess irritation as follows: 0, no erythema or edema; 1, very slight erythema and/or barely perceptible edema; 2, well-defined erythema and/or slight edema; 3, moderate to severe erythema or moderate edema; and 4, severe erythema and/or edema. Also, on day 23, serum, skin, liver, kidney, spleen, and lymph nodes were collected from hairless rats, and then weights of harvested liver, kidney, and spleen were measured. Each tissue (skin, liver, kidney, spleen, and lymph node) was fixed in 10% neutral-buffered formalin at pH 7.4 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and embedded in paraffin. Sections (5 µm) were prepared for HE staining. Histopathologic examinations were performed at the Applied Medical Research Laboratory (Osaka, Japan). Inflammation level was scored according to the severity of the inflammatory cell infiltration: 0, none; 1, rare; 2, mild; 3, moderate; 4, severe. Scoring was performed by an investigator masked to the treatment condition to avoid bias. In addition, a commercial kit was used for quantitative colorimetric determination of glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and creatinine (CRE) (Transaminase-CII-Test-Wako and Creatinine-test-Wako, respectively; all purchased from Wako Pure Chemical Industries) from rat serum.

RESULTS AND DISCUSSION

Ag-Specific IgG Production Induced by TCI Using Various Patches We previously revealed that the hydrogel patch was an effective vaccine device for TCI.^{9,10)} Other groups have reported that TCI using gauze patch as the TCI device also induces Ag-specific immune responses.¹³⁾ We compared the immune response induced by TCI using either dry gauze patch, wet gauze patch, or the hydrogel patch. TCI using wet gauze patch, but not dry gauze patch, induced an OVA-specific immune response (Fig. 1A), suggesting that SC hydration caused by application of wet gauze patch promoted Ag penetration through the SC. TCI using the hydrogel patch, however, induced a larger immune response. We previously revealed that the hydrogel patch formulation forms a concentrated Ag layer on its surface, and hypothesized that

the high Ag concentration gradient generated between the patch and the skin tissue by application of the hydrogel patch is critical for producing the driving force necessary to accelerate passive diffusion and distribution of the Ags.⁹⁾ Because the hydrogel patch is an airtight structure as compared with a wet gauze patch, we speculated that application of the hydrogel patch would restrict water evaporation from the skin, increase the hydration of the SC, and thus promote Ag penetration through the SC, which would more efficiently induce

Ag-specific IgG production.

Therefore, to explore the most appropriate water content ratio of the patch formulation, which might determine the degree of SC hydration, we prepared three types of patch formulations with different water content ratios (0%; hydrophobic adhesive patch, 5%; hydrogel patch, and 66%; water-soluble adhesive patch). Mice vaccinated using a hydrogel patch (water content ratio: 5%) had the highest serum anti-OVA IgG titer (Fig. 1B), suggesting that there is an optimal water content level for the hydrogel patch to induce Ag penetration into the skin. Generally, it appeared that the amount of Ag penetrating the SC would increase if the SC were more hydrated. A patch formulation with too much water might impede distribution of the antigenic proteins into the skin, because the SC comprises mainly lipophilic components. Therefore, a hydrogel patch with a water content ratio of 5%, which induced the largest immune response, was used in the subsequent experiments.

Hydration and Humectation of Skin after Hydrogel Patch Application To analyze the hydration effect of SC caused by Ag-containing hydrogel patch application, we measured skin surface impedance after patch application. Surface impedance of skin with application of the hydrogel patch was far lower than that of skin without the patch (Table 1), suggesting that the SC of skin applied with the hydrogel patch was hydrated. In addition, SC swelling was observed in resected human skin following 24 h-application of the hydrogel patch, but not by the application of phosphate-buffered saline (Fig. 2). Thus, hydrogel patch application caused hydration and swelling of SC. In hydrated SC, intercellular gaps are loose and substances are more likely to penetrate into the skin.^{14,15)} Therefore, by this mechanism, TCI using a hydrogel patch could accelerate Ag penetration through the SC and induce an effective immune response.

Pharmaceutical Stability of the Hydrogel Patch Formulation To investigate the stability of the Ag-containing hydrogel patch, we compared the production of Ag-specific IgG

Fig. 1. The Comparison of Ag-Specific IgG Antibody Production Induced by TCI Using Various Patches

(A) Dry gauze patch, wet gauze patch, or a hydrogel patch, (B) the three types of hydrogel patches with a different water content ratio (0%, 5%, or 66%), containing 100 μ g OVA, were prepared. These patches were applied to the auricle skin of mice for 24 h. As a control group, mice were intradermally injected with 100 μ g OVA. These procedures were performed twice with a 2-week interval. Two weeks after the final vaccination, the serum was collected from these mice and assayed for the OVA-specific IgG titer by ELISA. The results are expressed as means $\pm$ S.E. of 3 to 7 mice. N.D.; not detectable, TCI; transcutaneous immunization, IDI; intradermal immunization.

Table 1. Impedance of Skin Surface on Rat Abdomen

	Skin surface impedance (M Ω ·cm)
Intact skin	4.15
Hydrogel patch-applied skin	0.01
Filter paper-applied skin	5.80

Fig. 2. Histopathologic Image of Human Skin after Application of the Hydrogel Patch

A hydrogel patch was applied to human skin in the donor component of a Frantz cell system for 24 h. As a control, PBS was applied to human skin. The human skin was removed, fixed in 10% neutral formalin buffer, and embedded in paraffin. The histopathologic changes of human skin at the application site were examined using HE-stained specimens.

Fig. 3. Evaluation about Long-Term Storage of Ag-Containing Hydrogel Patch

Mice were vaccinated with the two types of hydrogel patches containing 100 μ g OVA, which were used for TCI immediately after preparation or after 8 h storage at 37 °C (A), or four types of hydrogel patch containing 100 μ g OVA, which were stored for 1 or 6 months at 4 °C (□) or 25 °C (■) (B). As a control group, mice were intradermally injected with 100 μ g OVA. These procedures were performed twice with a 2-week interval. Two weeks after the final vaccination, the serum was collected from these mice and assayed for the OVA-specific IgG titer by ELISA. The results are expressed as means $\pm$ S.E. of 3 to 8 mice. TCI; transcutaneous immunization, IDI; intradermal immunization.

antibody induced by application of hydrogel patch immediately after preparation or after storing the patch for 8 h at 37 °C. TCI using a hydrogel patch stored for 8 h at 37 °C induced an immune response equivalent to that induced by a hydrogel patch used immediately after preparation (Fig. 3A), suggesting that the Ag contained on the hydrogel patch was relatively stable as vaccine-Ag for 8 h. Next, to examine whether Ag-containing hydrogel patch formulation could be preserved under room temperature, we compared the Ag-specific IgG production induced by TCI using hydrogel patch stored under 4 °C and 25 °C for a long period. TCI using a hydrogel patch stored at 25 °C induced an immune response, comparable to that at 4 °C for 1 or 6 months (Fig. 3B). Furthermore, OVA contained on the hydrogel patch kept stable for at least 3 months at 25 °C after preparation as well as at 4 °C (Table 2). These results suggested that Ag-containing hydrogel patch formulation could keep at room temperature without cold chain. This is where our TCI system using a hydrogel patch has advantage over injection vaccine system which must require the use of the cold chain.

Based on these results, TCI using a hydrogel patch formulation (water content ratio; 5%) could induce an Ag-specific immune response most effectively when Ag penetration through the SC is enhanced by hydrating the skin. For practical application, the establishment of a manufacturing method

Table 2. Release Test of Antigenic Protein Contained on Hydrogel Patch

Storage temperature (°C)	Ratio of release amount to contained amount (%)		
	7 ^{a)}	35 ^{a)}	105 ^{a)}
4	108	115	107
25	112	110	105

a) Storage period (Days).

of the hydrogel patch that follows Good Manufacturing Practice is essential and is currently underway.

Toxicity Assessment of TCI Using a Toxoid-Containing Hydrogel Patch For the development of clinical trials, it is crucial to evaluate the side effects caused by application of the Ag-containing hydrogel patch in an animal model. We investigated local irritation on the skin and systemic adverse reactions in hairless rats after applying hydrogel patches containing combined tetanus and diphtheria toxoids with various doses per patch unit area according to the schedule shown in Fig. 4A.

To examine local toxicity, we evaluated erythema and edema at the patch application site using Draize scoring after the final application of the hydrogel patches (Fig. 4B). No edema was observed in any rats (data not shown) and in some rats, slight erythema that quickly resolved was observed on the skin applied with the hydrogel patch. Wound management film alone, which is used in clinical practice, however, also induced slight erythema. There were no differences in the degree of erythema between hydrogel patches containing various doses of toxoids (0, 50, 200, 400 μ g/cm²). These results suggest that the toxoid dose contained on the hydrogel patch did not relate to the degree of erythema, and that physical irritation by the adhesive patch or tape application itself caused this erythema. The erythema disappeared within 1 h after removal of each toxoid-containing hydrogel patch, indicating that the degree of skin irritation caused by the hydrogel patch application was a very mild and temporary reaction. In the histopathologic observation, the inflammation score was low in most rats, but moderate inflammatory cell infiltration was observed in some rats with application of the toxoid-containing hydrogel patch as well as with application of the wound management film (Fig. 4C). This phenomenon might also be a result of physical irritation by the adhesive patch or tape application, as described above. These results of the local toxicity test were similar between males and females. Thus, the toxoid-containing hydrogel patch caused little irritation and inflammation to the skin. Furthermore, we estimate that the infiltration of lymphocytes induced by application of the Ag-containing hydrogel patch promoted the activation of immunocompetent cells in the skin.

We then investigated the systemic side effects caused by TCI using a toxoid-containing hydrogel patch. The body weight change in rats with application of the toxoid-containing hydrogel patch followed a pattern similar to that of rats with application of the wound management film alone (Fig. 4D). There were also no significant changes in serum GOT, GPT, or CRE levels in rats with the toxoid-containing hydrogel patch compared to the control group (Fig. 4E). Moreover, there was no difference in organ weights between the two

Fig. 4. Local and Systemic Toxicity by TCI Using a Toxoid-Containing Hydrogel Patch

(A) The experimental schedule. A hydrogel patch containing various doses of combined tetanus and diphtheria toxoids (0, 50, 200, or 400 μg each/ cm^2) was applied to hairless rats for 24 h, four times. Each experiment was conducted at the indicated time points. Numbers in parentheses indicate experimental number (B)—(G). (B) The degree of erythema on the skin of the hairless rats was evaluated by Draize scoring: 0, no erythema or edema; 1, very slight erythema and/or barely perceptible edema; 2, well-defined erythema and/or slight edema; 3, moderate to severe erythema or moderate edema; and 4, severe erythema and/or edema. Mean value is indicated by the bar. (C) The histopathologic score was determined using HE stained specimens. Lymphocyte infiltration was expressed as follows: 0, no; 1, rare; 2, mild; 3, moderate; 4, severe. Mean value is indicated by the bar. (D) Body weight of hairless rats with application of the hydrogel patch or wound management film alone was monitored during the experiment. (E) Serum levels of GOT, GPT, or CRE were measured in hairless rats with application of the toxoid-containing hydrogel patch (■) or wound management film alone (□). (F) Weight of liver, kidney, and spleen harvested from hairless rats with application of the hydrogel patch (■) or wound management film alone (□) was measured. (G) Histopathologic scores of liver, kidney, spleen, and lymph node harvested from hairless rats with application of the hydrogel patch or wound management film alone were assigned based on HE-stained sections. Histopathologic changes were expressed as follows: 0, no; 1, rare; 2, mild; 3, moderate; 4, severe. Mean value is indicated by the bar. Data are expressed as mean $\pm$ S.E. of results from 8 rats (D), (E), (F).

groups (Fig. 4F). These results of body weight monitoring, biochemical test, and organ weight measurement were similar to untreated control group in males and females and all were within the normal range (data not shown). The histopathologic scores of liver, kidney, spleen, and lymph nodes after application of the toxoid-containing hydrogel patch were within 0 to 1, and there were no toxic changes in any groups (Fig. 4G). In histopathological analysis on spleen, the score of some male rats was higher than the others, but it may just happened that way, but not sex difference. Thus, the systemic toxicity examinations indicated that application of the toxoid-containing hydrogel patch induced no systemic side effects.

Taken together, these findings indicate that TCI with toxoid-containing hydrogel patch is a non-invasive vaccination method without the local and systemic adverse effects associated with immunization. Clinical trials in healthy volunteers are now being planned.

CONCLUSION

In the present study, we determined the optimal water content ratio for the hydrogel patch formulation, and demonstrated the safety and stability of our original TCI system using the hydrogel patch in an animal model. The efficacy of TCI using an Ag-containing hydrogel patch formulation was previously demonstrated to provide effective protection against infection. Thus, this effective, easy to use, and noninvasive TCI system could play an important role in providing effective vaccination instead of injection. We are now planning translational research in humans to assess the safety and efficacy of TCI using a hydrogel patch containing tetanus and diphtheria toxoids.

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