Title
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Potency of whole virus particle and split virion vaccines using dissolving microneedle against
challenges of H1N1 and H5N1 influenza viruses in mice

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Abstract

Transdermal vaccination using a microneedle (MN) confers enhanced immunity compared with subcutaneous (SC) vaccination. Here we developed a novel dissolving MN patch for the influenza vaccine. The potencies of split virion and whole virus particle (WVP) vaccines prepared from A/Puerto Rico/8/1934 (H1N1) and A/duck/Hokkaido/Vac-3/2007 (H5N1), respectively, were evaluated. MN vaccination induced higher neutralizing antibody responses than SC vaccination in mice. Moreover, MN vaccination with a lower dose of antigens conferred protective immunity against lethal challenges of influenza viruses than SC vaccination in mice. These results suggest that the WVP vaccines administered using MN are an effective combination for influenza vaccine to be further validated in humans.

Abbreviations

APCs, antigen-presenting cells; ANOVA, analysis of variance; EID₅₀, 50% egg infectious dose; MN, microneedle; HA, hemagglutinin; MLD₅₀, 50% mouse lethal dose; PET, polyethylene terephthalate; PFU, plaque forming units; SC, subcutaneous; SV, split virion; TD, transdermal; TCID₅₀, 50% tissue culture infectious dose; WVP, whole virus particle

Keywords

vaccine; influenza; dissolving microneedle; transdermal vaccination; whole virus particle; split virion
**Introduction**

A split virion (SV) vaccine is widely used for current seasonal influenza vaccination in humans and is administered intramuscularly [1]. However, the efficacy of SV is highly controversial since the current vaccination method would not be the best combination of antigen and administration route to induce antibody responses [2]. An inactivated whole virus particle (WVP) vaccine has been reported to induce stronger antibody responses than an SV vaccine in animal studies [3–6]. Although WVP vaccines were discontinued in the 1990s due to a problem with their reactogenicity [7], WVP already has been an attractive formulation as a pandemic vaccine because a WVP vaccine is more immunogenic than an SV vaccine in individuals who have not been exposed to vaccine antigens before [8]. Thus, WVP is the recommended formulation for pandemic vaccines against H5N1 influenza viruses in Japan, and majority of the population in Japan is expected to be immunologically naïve to these viruses. Therefore, these two formulations, WVP and SV antigens, must be directly compared head to head to develop better seasonal and pandemic influenza vaccines in the future.

The epidermis and dermis contain a large population of resident antigen-presenting cells (APCs) and are considered to be active immune tissues [9]. Recently, various vaccination methods targeting these tissues have been developed and occasionally demonstrated to be better than subcutaneous (SC) injections, e.g., powder injection, electroporation, sonoporation, jet injection, mini needle injection, and microneedle (MN) injection [10–16]. MN injection has been considered the most promising method because it is simple and less invasive; it delivers vaccine antigens
directly into the skin without skin permeabilization to overcome the barrier function of the skin.

Thus, transdermal (TD) administration by MN could be considered to replace the current vaccination method.

Intradermal influenza vaccination with a metal mini needle of 1.5 mm height has already been approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) [15]. Previous studies have proved that a metal MN patch coated with WVP or SV vaccines induced antibody responses higher than or equivalent to those induced by SC administration in animals [17]. A dissolving MN using a hydrophilic biopolymer has been developed because it need no disposal, could be self-administered, would have good stability and shelf life [18–22]. Previous studies have demonstrated that TD vaccination in combination with SV vaccines prepared from seasonal influenza viruses using a dissolving biopolymer needle induced higher antibody responses than SC vaccination in humans [18].

These results suggest that the combination of WVP vaccines and dissolving MN will potentially provide highly potent vaccination; however, no comparative studies of vaccine formulations enclosed in dissolving MN have been conducted to date. In the present study, we developed a novel patched vaccine with dissolving MN. Inactivated WVP and SV prepared from H1N1 or H5N1 influenza viruses were enclosed in this MN. The immunogenicity and protective effect of WVP were compared with those of SV in MN vaccination in mice to determine a suitable vaccine formulation for MN.
Materials and Methods

Viruses and cells

Influenza viruses, A/Puerto Rico/8/1934 (H1N1) [PR8 (H1N1)], A/Hong Kong/483/1997 (H5N1) [HK483 (H5N1)], and A/duck/Hokkaido/Vac-3/2007 (H5N1) [Vac-3 (H5N1)], generated from two nonpathogenic avian influenza viruses, which is antigenically similar to HK483 (H5N1) [23,24], were used in the present study. All viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 36–48 h, and the infectious allantoic fluids were collected. Virus stocks were stored at −80°C until use.

MDCK cells were grown in Minimum Essential Medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% calf serum. The cells were used for plaque assays and serum neutralization tests.

Vaccine antigen preparation

PR8 (H1N1) and Vac-3 (H5N1) were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs and propagated at 35°C for 48 h. The viruses in the allantoic fluids were purified by differential centrifugation and sedimentation through a sucrose gradient in accordance with the study of Kida et al. [25]. The total protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). SV of each strain was prepared by the ether split method [26]. In brief, purified viruses were disrupted with 0.05% Tween 80 and
an equal volume of diethyl ether for 30 min at room temperature. The water phase was collected after centrifugation for 30 min at 3,500 g. The ether dissolved in the water phase was removed by ultracentrifugation. The abundance of hemagglutinin (HA) protein was calculated from the intensity ratio of HA protein to total protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified viruses were inactivated with 0.2%–0.3% formalin at 4°C for 7–14 days, and the formalin was removed by ultracentrifugation.

**Hydrogel patch formulation and fabrication of dissolving MN**

MN patches were produced in a clean room to prevent contamination by small particles, which can adversely affect the manufacturing process (Federal Standard 209D Class 1000). Hydroxyethyl starch 70000 (Fresenius Kabi, Bad Homburg, Germany) was dissolved in distilled water and mixed with the vaccine. The aqueous solution was cast into a micromold and dried at 23°C. The vaccine content in all MN patches was confirmed by weighing the solution. This was followed by coating the polyethylene terephthalate (PET) base with an aqueous solution containing chondroitin sulfate (Maruha Nichiro, Tokyo, Japan), attaching to a micromold, and drying at 35°C. After drying, the PET base containing MN was separated from the micromold. The shape of MN was confirmed using a digital microscope (VHX-5000; Keyence, Osaka, Japan). The needle length was approximately 430 μm. The MN patch was further dried using a desiccant to achieve water content below 5%. The final MN patch was placed in a plastic case, packed with a desiccant into a sealed
aluminum bag, and stored at 4°C until use.

### Potency test of PR8 (H1N1)-based vaccines in mice

Each of the WVP or SV vaccines of PR8 (H1N1) (0.01, 0.05, or 0.25 µg of HA protein) was administered by MN to twelve 8-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) under anesthesia as follows: The dissolving MN was patched on the dorsal midline for 5 min after shaving. WVP and SV vaccines were also subcutaneously injected into 12 other mice. Phosphate-buffered saline (PBS) was administered by MN or SC injection to control mice. Four weeks later, sera of the mice were collected and the mice were challenged with 10 times of 50% mouse lethal dose (MLD50) [10^{4.5} plaque-forming units (PFU)] of PR8 (H1N1) by intranasal inoculation under anesthesia. Six mice from each group were sacrificed 3 days post-challenge and their lungs were collected. Virus titers in the lung homogenates were measured by a plaque assay using MDCK cells. The remaining six mice from each group were housed until 14 days post-challenge to measure the survival rate.

### Potency test of Vac-3 (H5N1)-based vaccines in mice

Each of the WVP or SV vaccines of Vac-3 (H5N1) (0.01, 0.05, or 0.25 µg of HA protein) and PBS were administered to the mice as described above. The mice were administered these vaccines twice at an interval of 4 weeks. Four weeks after the first administration, sera of the mice were
collected to measure the serum neutralization titer. Sera were again collected 4 weeks after the second administration, and the mice were then challenged with 30 MLD$_{50}$ ($10^{2.3}$ EID$_{50}$) of HK483 (H5N1) by intranasal inoculation under anesthesia. At 3 days post-challenge, six mice from each group were sacrificed and their lungs were collected. Virus titers in the lung homogenates were measured by a plaque assay using MDCK cells. The remaining six mice from each group were housed for 14 days to measure the survival rate.

**Plaque assay**

Ten-fold dilutions of mouse lung homogenates obtained using PBS were inoculated onto confluent monolayers of MDCK cells and incubated for 1 h at 35°C. Unbound viruses were removed, and the cells were washed with PBS. The cells were then overlaid with MEM containing 1% Bacto-agar (Becton Dickinson, New Jersey, USA) and 5 µg/ml acetylated trypsin (Sigma Aldrich, Missouri, USA). After incubation for 48 h at 35°C, the cells were stained with 0.005% neutral red. After incubation for 24 h at 35°C, the number of plaques was counted. PFU were calculated as the product of the reciprocal value of the highest virus dilution and the number of plaques in the dilution.

**Neutralization test**

The serum neutralization test was performed in accordance with the study of Sakabe et al. [27]. Test sera and 100 times of 50% tissue culture infectious dose (TCID$_{50}$) of virus were mixed and
incubated for 1 h at room temperature. This mixture was then inoculated onto MDCK cells in 96-well tissue culture plates and incubated for 1 h at 35°C. The cells were then washed with PBS and incubated in MEM containing 5 μg/ml acetylated trypsin for 3 days at 35°C. The cytopathic effect was observed, and neutralization titers were expressed as reciprocals of the highest dilution of serum sample that showed 50% neutralization.

Statistical analysis

Student’s t test was used to analyze the difference between the two groups, and one-way analysis of variance (ANOVA) was used to analyze the difference among multiple groups [28]. The Kaplan–Meier method with a log-rank test was applied to compare survival curves. P value was calculated using PRISM software (GraphPad Software, California, USA), and P < 0.05 was considered significant.

Ethics statement

All experiments involving animals were authorized by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval number: 15-0063), and all experiments were performed according to the guidelines of the committee. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The Graduate School of Veterinary Medicine, Hokkaido University, has been accredited
by the Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC International) since 2007.

Results

Dissolving kinetics of a novel developed MN in mouse skin

The MN patches were designed for the efficient delivery of antigens into mouse skin (Fig. 1A). Evans blue was encapsulated in the MN patches as a marker instead of vaccine antigens to facilitate imaging (Fig. 1B). All the components had dissolved in the skin after 5 min (Fig. 1C). To characterize the dissolution kinetics of these MN patches, they were inserted into the mouse skin and monitored over time (Fig. 1D). MN has sufficient capacity to dissolve in 3 min, thereby ensuring optimal use of the dissolving MN patch in administration.

Antibody responses of mice vaccinated by MN to PR8 (H1N1)

Eight-week-old female BALB/c mice were vaccinated with WVP or SV prepared from PR8 (H1N1) by MN or SC injection. Four weeks after vaccination, sera of the mice were collected and the neutralizing antibody titers were measured (Table 1). In MN vaccination groups, antibody responses were observed in mice vaccinated with 0.05 µg of WVP and SV and with the lowest dose of WVP (0.01 µg). The maximum neutralization titer was 1:1,280 in mice with the highest dose of WVP. In addition, when SVs were administered by MN, antibody responses were not detected in
mice with the lowest dose, and the maximum neutralization titer was 1:320 at the highest dose.

Antibody response was not detected in the SC vaccination groups except for the ones vaccinated with 0.05 and 0.25 µg of WVP (Table 1).

Protection of mice vaccinated by MN against challenge with PR8 (H1N1)

Four weeks after prime immunization, the mice were challenged with 10 MLD50 of PR8 (H1N1) by intranasal inoculation. In the PBS control group, all the mice died within the observation period of 14 days (Fig. 2). All the mice vaccinated by MN with 0.05 µg and 0.25 µg of WVP and SV (Fig. 2B, C) survived for 14 days, whereas a slight body weight loss was observed in the group of mice vaccinated with 0.05 µg of SV (Fig. 2E, F). In addition, all the mice vaccinated by MN with the lowest dose (0.01 µg) of WVP survived for 14 days without any body weight loss (Fig. 2A, D). In the SC administration groups, the survival rate of the group of mice vaccinated with 0.25 µg of WVP was 100% (Fig. 2C), while those of mice vaccinated with 0.05 µg of WVP or SV were 33% and 0%, respectively (Fig. 2B). The virus titers in the lungs of mice vaccinated by MN decreased in a dose-dependent manner (Table 1). In particular, no virus was detected in the lungs of mice vaccinated with the highest dose of WVP. These results demonstrate that the MN patch induced immunity to reduce virus replication in the lungs against the lethal challenge with the H1N1 influenza virus in mice. WVP vaccines in MN showed the highest potency to reduce the impact of virus challenge.
Antibody responses of mice vaccinated by MN against Vac-3 (H5N1)

Eight-week-old female BALB/c mice were vaccinated with WVP or SV of Vac-3 (H5N1) twice at an interval of 4 weeks. Four weeks after each vaccination, sera of the mice were collected and the serum neutralization antibody titers were measured. No antibody response was observed in both MN and SC vaccination groups after the first vaccination, except in one mouse vaccinated by MN with 0.25 µg of WVP (Table 2). Four weeks after the second vaccination, a higher antibody response was observed at the lowest dose (0.01 µg) of WVP and SV in the MN vaccination groups than in the SC vaccination groups. The maximum neutralization titer reached 1:2,560 (WVP) and 1:1,280 (SV) at the highest dose in the MN vaccination group. Thus, the MN patch induced a higher immune response than SC vaccination against the H5N1 influenza virus in mice. Similar to the results of the H1N1 influenza virus, WVP enclosed in MN had the maximum immunogenecity.

Protection of mice vaccinated with Vac-3 (H5N1) vaccines against challenge with HK483 (H5N1)

Four weeks after the second immunization, the mice were challenged with 30 MLD$_{50}$ of HK483 (H5N1) by intranasal inoculation. In the non-vaccinated group, all the mice died within the observation period of 14 days (Fig. 3). All the mice vaccinated by MN with 0.05 and 0.25 µg of WVP survived for 14 days (Fig. 3B, C), and no body weight loss was observed in them (Fig. 3E, F).
In particular, the survival rate of mice vaccinated by MN with 0.01 µg of WVP was 100% without body weight loss (Fig. 3A, D). In contrast, the survival rates were 0% in the groups of mice subcutaneously vaccinated with 0.01 µg of WVP or SV (Fig. 3B). The virus titers in the lungs of mice vaccinated by MN were suppressed in a dose-dependent manner. The virus titers in the lungs in the SC vaccination groups were comparable to those in the control group (Table 2). These results indicate that MN vaccination also induced higher immunity to reduce virus replication in the lungs against the challenge with the H5N1 influenza virus in mice than SC vaccination. Again, MN with WVP induced the highest protective immunity against challenge with the H5N1 influenza virus.

Discussion

In the present study, WVP and SV induced high neutralizing antibody responses and conferred protective immunity against lethal challenge at a lower dose of antigens in our dissolving MN vaccination than SC injection in mice. In addition, WVP induced a higher neutralization antibody response than SV in MN vaccination. Previously, the immunogenicity and protective effect of these antigens using MN have been independently studied in mice and humans [16–20]. To the best of our knowledge, this is the first study to compare the vaccine efficacy using MN in mice. Our results clearly indicated that WVP is a more suitable antigen for TD vaccination for treating influenza than SV.

The number of APCs initiating adaptive immune responses, such as Langerhans cells and
dermal dendritic cells in the epidermis and dermis, was higher than that in SC tissues or muscles [9,29], suggesting that TD vaccination induces an antibody response to a lower dose of antigens than SC vaccination. Consistent with these findings, MN vaccination with WVP and SV prepared from H1 and H5 influenza viruses showed higher potency than SC vaccination. In particular, in the group of mice vaccinated by MN with 0.25 µg of WVP prepared from PR8 (H1N1), virus titers in the lungs were found to be under the detection limit. Interestingly, 0.4 µg of HA protein of PR8 (H1N1) in previously developed patches was insufficient to induce protective immunity against lethal challenge with influenza virus infection [16]. These results demonstrated that our patched vaccine induced protective immunity at a lower dose than in previously developed approaches in mice.

In MN vaccination, single immunization with the vaccine prepared from PR8 (H1N1) is sufficiently potent in mice. On the other hand, MN vaccination with Vac-3 (H5N1) requires two immunizations to induce a detectable antibody response. In agreement with the result of MN vaccination, single SC vaccination with the highest dose of Vac-3 (H5N1) did not induce an antibody response, whereas that with PR8 (H1N1) induced a high antibody response in mice. A previous study suggested that some H5N1 vaccine candidates had low immunogenicity in mice [30]. These results indicate that the immunogenicity of Vac-3 (H5N1) is lower than that of PR8 (H1N1) in mice. However, 0.1 µg of total HA protein of Vac-3 (H5N1) is sufficient to confer protective immunity to mice. Thus, this MN overcomes the low immunogenicity of Vac-3 (H5N1) by two immunizations
using a lower dose of antigens.

It is clear that compared with SC vaccination, MN vaccination conferred protective immunity to mice against lethal challenges of H1N1 and H5N1 influenza viruses. In TD administration, the WVP vaccine prepared from PR8 (H1N1) and Vac-3 (H5N1) conferred protective immunity to mice at the lowest dose (0.01 µg). In addition, SV prepared from Vac-3 (H5N1), the immunogenicity of which is expected to be lower than that of PR8 (H1N1), conferred protective immunity at the same dose. The mice vaccinated with Vac-3 (H5N1) received two doses of vaccine, whereas the mice vaccinated with PR8 (H1N1) received only one. We estimated that booster doses of SV prepared from Vac-3 (H5N1) conferred higher protective immunity to mice than the primary dose of SV prepared from PR8 (H1N1). It may be suggested that two or several doses of vaccine are more important for vaccine efficacy than the immunogenicity of antigens in TD administration in mice.

In the present study, we revealed that the influenza vaccine prepared from H1 and H5 influenza viruses using dissolving MN showed higher immunogenicity in mice. Moreover, the MN vaccination conferred protective immunity to mice against influenza virus infection at a lower dose than the SC vaccination. Considering practical application to humans, our dissolving MN has a sufficient potential to enclose the conventional dose (15 µg of HA protein) of quadrivalent influenza vaccine (data not shown). Moreover, vaccination using this MN should induce an effective antibody response at a lower dose than the conventional dose [31]. Thus, vaccine immunogenicity using this MN should be evaluated in non-primate and human clinical trials.
Acknowledgments

We would like to thank Dr. K.F. Shortridge from Hong Kong University for providing A/Hong Kong/483/1997 (H5N1). We would also like to thank technical staff of the Pharmaceutical & Healthcare Research Laboratories, FUJIFILM Corporation. Finally, we thank Mr. Taniguchi, Shionogi & Co., Ltd., for his technical support in the animal experiments.

Conflict of interest

Y. Sakoda received research funding from FUJIFILM Corporation (Tokyo, Japan).
References


Figure legends

**Fig. 1**
Dissolution kinetics of developed microneedle (MN). Dissolving MN patch (A). Digital microscope picture of MN fabricated with Evans blue instead of vaccine antigens (B). MN dissolution in mouse skin (C). Left, before immunization; right, after immunization in the skin. Broken line indicates the overall shape of MN. Dissolving MN delivery efficiency to mice in vivo (D).

**Fig. 2**
Survival rates and body weight changes of mice vaccinated with PR8 (H1N1) after challenge with homologous virus. The 8-week-old BALB/c mice were vaccinated by microneedle (MN) or subcutaneously (s.c.) with 0.01 µg (A, D), 0.05 µg (B, E), or 0.25 µg (C, F) of whole virus particle (WVP) or split virion (SV), respectively. The vaccinated mice were challenged with 10 MLD$_{50}$ of PR8 (H1N1). *, $p < 0.05$ versus the group of mice injected with PBS.

**Fig. 3**
Survival rates and body weight changes of mice vaccinated with Vac-3 (H5N1) after challenge with HK483 (H5N1). The 8-week-old BALB/c mice were vaccinated by microneedle (MN) or subcutaneously (s.c.) with 0.01 µg (A, D), 0.05 µg (B, E), or 0.25 µg (C, F) of whole virus particle
(WVP) or split virion (SV) respectively. The vaccinated mice were challenged with 30 MLD$_{50}$ of HK483 (H5N1). *, $p < 0.05$ versus the group of mice injected with PBS.
Fig. 1 Nakatsukasa et al.

A

B

C

D

**Before injection**

**After injection**

Needle remaining length after injection (µm)

Injection time (min)

Needle length (µm)

Injection time (min)

**Fig.1** Nakatsukasa et al.
Fig. 2 Nakatsukasa et al.

Survival rate (%) vs. Relative body weight (%)

A. 0.01 µg
B. 0.05 µg
C. 0.25 µg

D-F: Further details on survival rates and relative body weights at different time points (d.p.c.).

Key:
- MN+WVP
- s.c.+WVP
- MN+SV
- s.c.+SV
- MN+PBS
- s.c.+PBS

* ** *** indicate significant differences.
Fig. 3 Nakatsukasa et al.
Table 1. Neutralizing antibody titers of mice injected with the vaccine and virus titers in the lungs after challenge against PR8 (H1N1)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Administration route</th>
<th>Formulation</th>
<th>Dose of vaccine (µg)</th>
<th>Neutralizing antibody titer</th>
<th>Virus titer (Mean log PFU/g) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR8 (H1N1)</td>
<td>MN</td>
<td>WVP</td>
<td>0.01</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, 20, 20, 20, 40, 80, 80, 160</td>
<td>5.83 ± 0.63*</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>20, 40, 80, 160, 160, 160, 160, 160, 160, 320, 320, 320, 640, 1,280</td>
<td>1.81 ± 0.79*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
<td>80, 160, 160, 160, 160, 320, 320, 320, 320, 640, 640</td>
<td>0.00 ± 0.00*</td>
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<tr>
<td></td>
<td></td>
<td>SV</td>
<td>0.01</td>
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<td>7.68 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>subcutaneous injection</td>
<td>WVP</td>
<td>0.01</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20</td>
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<td>0.05</td>
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<td>0.25</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, 20, 40, 40, 40, 80, 80, 160</td>
<td>6.63 ± 0.34**</td>
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<tr>
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<td>SV</td>
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<td>8.15 ± 0.08</td>
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<tr>
<td></td>
<td>subcutaneous injection</td>
<td>-</td>
<td>0.01</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20</td>
<td>8.07 ± 0.19</td>
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<td>0.05</td>
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<td>7.89 ± 0.10</td>
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<td></td>
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<td></td>
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<td>MN</td>
<td>-</td>
<td>-</td>
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<td>7.95 ± 0.10</td>
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<td>8.07 ± 0.19</td>
</tr>
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</table>

Each of vaccine was administrated by microneedle (MN) or subcutaneously into 12 mice. Mice were challenged with 10 MLD₃₀ (10⁴.⁵ PFU) of PR8 (H1N1).

"-" indicates that no vaccine is included.

* $P < 0.05$, vs. virus titers in PBS group vaccinated transdermally.

** $P < 0.05$, vs. virus titers in PBS group vaccinated subcutaneously.

SV, split virion; WVP, whole virus particle.
### Table 2. Neutralizing antibody titers of mice injected with the vaccine and virus titers in the lungs after challenge against HK483 (H5N1)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Administration route</th>
<th>Formulation</th>
<th>Dose of vaccine (µg)</th>
<th>Neutralizing antibody titer</th>
<th>Virus titer (Mean log PFU/g) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>One injection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac-3 (H5N1)</td>
<td>MN</td>
<td>WVP</td>
<td>0.01</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ES</td>
<td>0.01</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
</tr>
<tr>
<td>subcutaneous injection</td>
<td>WVP</td>
<td></td>
<td>0.01</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
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<tr>
<td></td>
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<td>ES</td>
<td>0.01</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td>0.05</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td>0.25</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, ND</td>
<td></td>
</tr>
<tr>
<td><strong>Two injections</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac-3 (H5N1) × 2</td>
<td>MN</td>
<td>WVP</td>
<td>0.01 × 2</td>
<td>&lt;20, 20, 20, 20, 20, 20, 20, 40, 80, 160, 160, 160, 160, 320, 320</td>
<td>5.48 ± 0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05 × 2</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, 80, 80, 80, 80, 640</td>
<td>3.67 ± 0.73*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25 × 2</td>
<td>&lt;80, 80, 160, 320, 320, 320, 320, 320, 320, 640, 640, 640, 640, 1,280, 1,280</td>
<td>0.82 ± 0.48*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SV</td>
<td>0.01 × 2</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, 40, 40, 40, 40</td>
<td>5.67 ± 0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05 × 2</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, 80, 80, 160, 160, 320, 320, 1,280, 1,280</td>
<td>4.16 ± 0.43*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25 × 2</td>
<td>&lt;80, 80, 80, 80, 160, 160, 160, 160</td>
<td>1.20 ± 0.70*</td>
</tr>
<tr>
<td>subcutaneous injection</td>
<td>WVP</td>
<td></td>
<td>0.01 × 2</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, 20, 20, 20, 20, 20</td>
<td>6.26 ± 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05 × 2</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, 20, 20, 20, 20, 20</td>
<td>5.49 ± 0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25 × 2</td>
<td>&lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, 160, 320, 320, 320, 320, 320</td>
<td>4.19 ± 1.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05 × 2</td>
<td>&lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, &lt;20, 20, 20, 20, 20</td>
<td>5.98 ± 0.08</td>
</tr>
<tr>
<td></td>
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<td>0.25 × 2</td>
<td>&lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, &lt;80, 160, 320, 320, 320, 320, 320</td>
<td>5.27 ± 0.31</td>
</tr>
</tbody>
</table>

Each of vaccine was administrated by MN or subcutaneously into 12 mice. Mice were challenged with 30 MLD₅₀ (10⁻¹³ EID₅₀) of HK483 (H5N1).

"-" indicates no vaccine is included.

* P < 0.05, vs. virus titers in PBS group vaccinated transdermally.

** P < 0.05, vs. virus titers in PBS group vaccinated subcutaneously.

ND: Not done

SV, split virion; WVP, whole virus particle