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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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18 **Abstract**

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

28

29 **Abbreviations**

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

34

35 **Keywords**

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

37 **Introduction**

38 A split virion (SV) vaccine is widely used for current seasonal influenza vaccination in humans
39 and is administered intramuscularly [1]. However, the efficacy of SV is highly controversial since
40 the current vaccination method would not be the best combination of antigen and administration
41 route to induce antibody responses [2]. An inactivated whole virus particle (WVP) vaccine has
42 been reported to induce stronger antibody responses than an SV vaccine in animal studies [3–6].
43 Although WVP vaccines were discontinued in the 1990s due to a problem with their reactogenicity
44 [7], WVP already has been an attractive formulation as a pandemic vaccine because a WVP vaccine
45 is more immunogenic than an SV vaccine in individuals who have not been exposed to vaccine
46 antigens before [8]. Thus, WVP is the recommended formulation for pandemic vaccines against
47 H5N1 influenza viruses in Japan, and majority of the population in Japan is expected to be
48 immunologically naïve to these viruses. Therefore, these two formulations, WVP and SV antigens,
49 must be directly compared head to head to develop better seasonal and pandemic influenza vaccines
50 in the future.

51 The epidermis and dermis contain a large population of resident antigen-presenting cells (APCs)
52 and are considered to be active immune tissues [9]. Recently, various vaccination methods
53 targeting these tissues have been developed and occasionally demonstrated to be better than
54 subcutaneous (SC) injections, e.g., powder injection, electroporation, sonoporation, jet injection,
55 mini needle injection, and microneedle (MN) injection [10–16]. MN injection has been considered
56 the most promising method because it is simple and less invasive; it delivers vaccine antigens

57 directly into the skin without skin permeabilization to overcome the barrier function of the skin.
58 Thus, transdermal (TD) administration by MN could be considered to replace the current vaccination
59 method.

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

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

76 **Materials and Methods**

77 **Viruses and cells**

78 Influenza viruses, A/Puerto Rico/8/1934 (H1N1) [PR8 (H1N1)], A/Hong Kong/483/1997
79 (H5N1) [HK483 (H5N1)], and A/duck/Hokkaido/Vac-3/2007 (H5N1) [Vac-3 (H5N1)], generated
80 from two nonpathogenic avian influenza viruses, which is antigenically similar to HK483 (H5N1)
81 [23,24], were used in the present study. All viruses were propagated in 10-day-old embryonated
82 chicken eggs at 35°C for 36–48 h, and the infectious allantoic fluids were collected. Virus stocks
83 were stored at –80°C until use.

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

87

88 **Vaccine antigen preparation**

89 PR8 (H1N1) and Vac-3 (H5N1) were inoculated into the allantoic cavities of 10-day-old
90 embryonated chicken eggs and propagated at 35°C for 48 h. The viruses in the allantoic fluids were
91 purified by differential centrifugation and sedimentation through a sucrose gradient in accordance
92 with the study of Kida *et al.* [25]. The total protein concentration was measured using the BCA
93 Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). SV of each strain was prepared
94 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.

101

102 **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

114 aluminum bag, and stored at 4°C until use.

115

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

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

128

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

130 Each of the WVP or SV vaccines of Vac-3 (H5N1) (0.01, 0.05, or 0.25 µg of HA protein) and
131 PBS were administered to the mice as described above. The mice were administered these vaccines
132 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 MLD₅₀ (10^{2.3} EID₅₀) 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.

139

140 **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.

148

149 **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 (TCID₅₀) of virus were mixed and

152 incubated for 1 h at room temperature. This mixture was then inoculated onto MDCK cells in
153 96-well tissue culture plates and incubated for 1 h at 35°C. The cells were then washed with PBS
154 and incubated in MEM containing 5 µg/ml acetylated trypsin for 3 days at 35°C. The cytopathic
155 effect was observed, and neutralization titers were expressed as reciprocals of the highest dilution of
156 serum sample that showed 50% neutralization.

157

158 **Statistical analysis**

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

164

165 **Ethics statement**

166 All experiments involving animals were authorized by the Institutional Animal Care and Use
167 Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval number:
168 15-0063), and all experiments were performed according to the guidelines of the committee. All
169 applicable international, national, and/or institutional guidelines for the care and use of animals were
170 followed. The Graduate School of Veterinary Medicine, Hokkaido University, has been accredited

171 by the Association for Assessment and Accreditation of Laboratory Animal Care International
172 (AALAC International) since 2007.

173

174 **Results**

175 **Dissolving kinetics of a novel developed MN in mouse skin**

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

182

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

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

190 mice with the lowest dose, and the maximum neutralization titer was 1:320 at the highest dose.
191 Antibody response was not detected in the SC vaccination groups except for the ones vaccinated
192 with 0.05 and 0.25 µg of WVP (Table 1).

193

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

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

209

210 **Antibody responses of mice vaccinated by MN against Vac-3 (H5N1)**

211 Eight-week-old female BALB/c mice were vaccinated with WVP or SV of Vac-3 (H5N1) twice
212 at an interval of 4 weeks. Four weeks after each vaccination, sera of the mice were collected and
213 the serum neutralization antibody titers were measured. No antibody response was observed in
214 both MN and SC vaccination groups after the first vaccination, except in one mouse vaccinated by
215 MN with 0.25 µg of WVP (Table 2). Four weeks after the second vaccination, a higher antibody
216 response was observed at the lowest dose (0.01 µg) of WVP and SV in the MN vaccination groups
217 than in the SC vaccination groups. The maximum neutralization titer reached 1:2,560 (WVP) and
218 1:1,280 (SV) at the highest dose in the MN vaccination group. Thus, the MN patch induced a
219 higher immune response than SC vaccination against the H5N1 influenza virus in mice. Similar to
220 the results of the H1N1 influenza virus, WVP enclosed in MN had the maximum immunogenicity.

221

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

224 Four weeks after the second immunization, the mice were challenged with 30 MLD₅₀ of HK483
225 (H5N1) by intranasal inoculation. In the non-vaccinated group, all the mice died within the
226 observation period of 14 days (Fig. 3). All the mice vaccinated by MN with 0.05 and 0.25 µg of
227 WVP survived for 14 days (Fig. 3B, C), and no body weight loss was observed in them (Fig. 3E, F).

228 In particular, the survival rate of mice vaccinated by MN with 0.01 µg of WVP was 100% without
229 body weight loss (Fig. 3A, D). In contrast, the survival rates were 0% in the groups of mice
230 subcutaneously vaccinated with 0.01 µg of WVP or SV (Fig. 3B). The virus titers in the lungs of
231 mice vaccinated by MN were suppressed in a dose-dependent manner. The virus titers in the lungs
232 in the SC vaccination groups were comparable to those in the control group (Table 2). These results
233 indicate that MN vaccination also induced higher immunity to reduce virus replication in the lungs
234 against the challenge with the H5N1 influenza virus in mice than SC vaccination. Again, MN with
235 WVP induced the highest protective immunity against challenge with the H5N1 influenza virus.

236

237 **Discussion**

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

246 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

266 using a lower dose of antigens.

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

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

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290

291 **Conflict of interest**

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

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382 **Figure legends**

383 **Fig. 1**

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

389

390 **Fig. 2**

391 Survival rates and body weight changes of mice vaccinated with PR8 (H1N1) after challenge with
392 homologous virus. The 8-week-old BALB/c mice were vaccinated by microneedle (MN) or
393 subcutaneously (s.c.) with 0.01 µg (A, D), 0.05 µg (B, E), or 0.25 µg (C, F) of whole virus particle
394 (WVP) or split virion (SV), respectively. The vaccinated mice were challenged with 10 MLD₅₀ of
395 PR8 (H1N1). *, $p < 0.05$ versus the group of mice injected with PBS.

396

397 **Fig. 3**

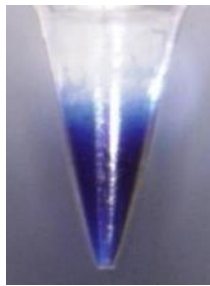
398 Survival rates and body weight changes of mice vaccinated with Vac-3 (H5N1) after challenge with
399 HK483 (H5N1). The 8-week-old BALB/c mice were vaccinated by microneedle (MN) or
400 subcutaneously (s.c.) with 0.01 µg (A, D), 0.05 µg (B, E), or 0.25 µg (C, F) of whole virus particle

401 (WVP) or split virion (SV) respectively. The vaccinated mice were challenged with 30 MLD₅₀ of
402 HK483 (H5N1). *, $p < 0.05$ versus the group of mice injected with PBS.

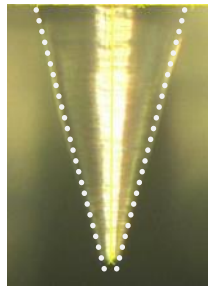
A



B



C

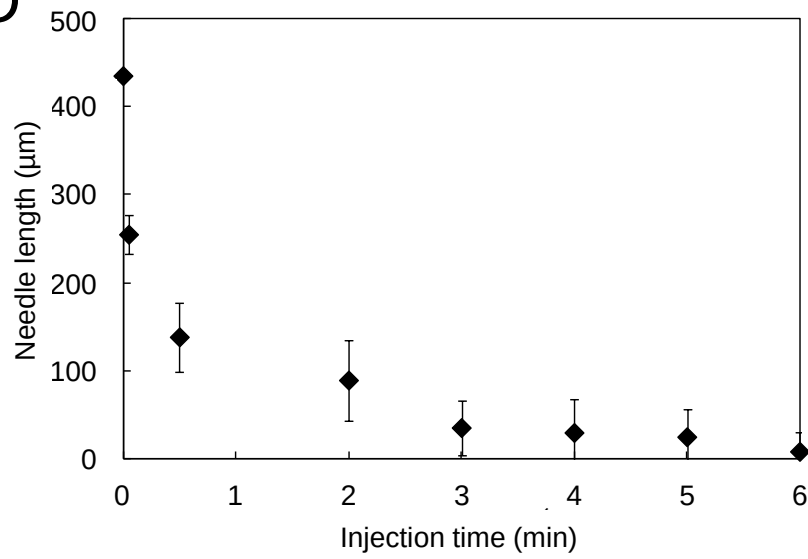


Before injection



After injection

D

Fig.1 Nakatsukasa *et al.*

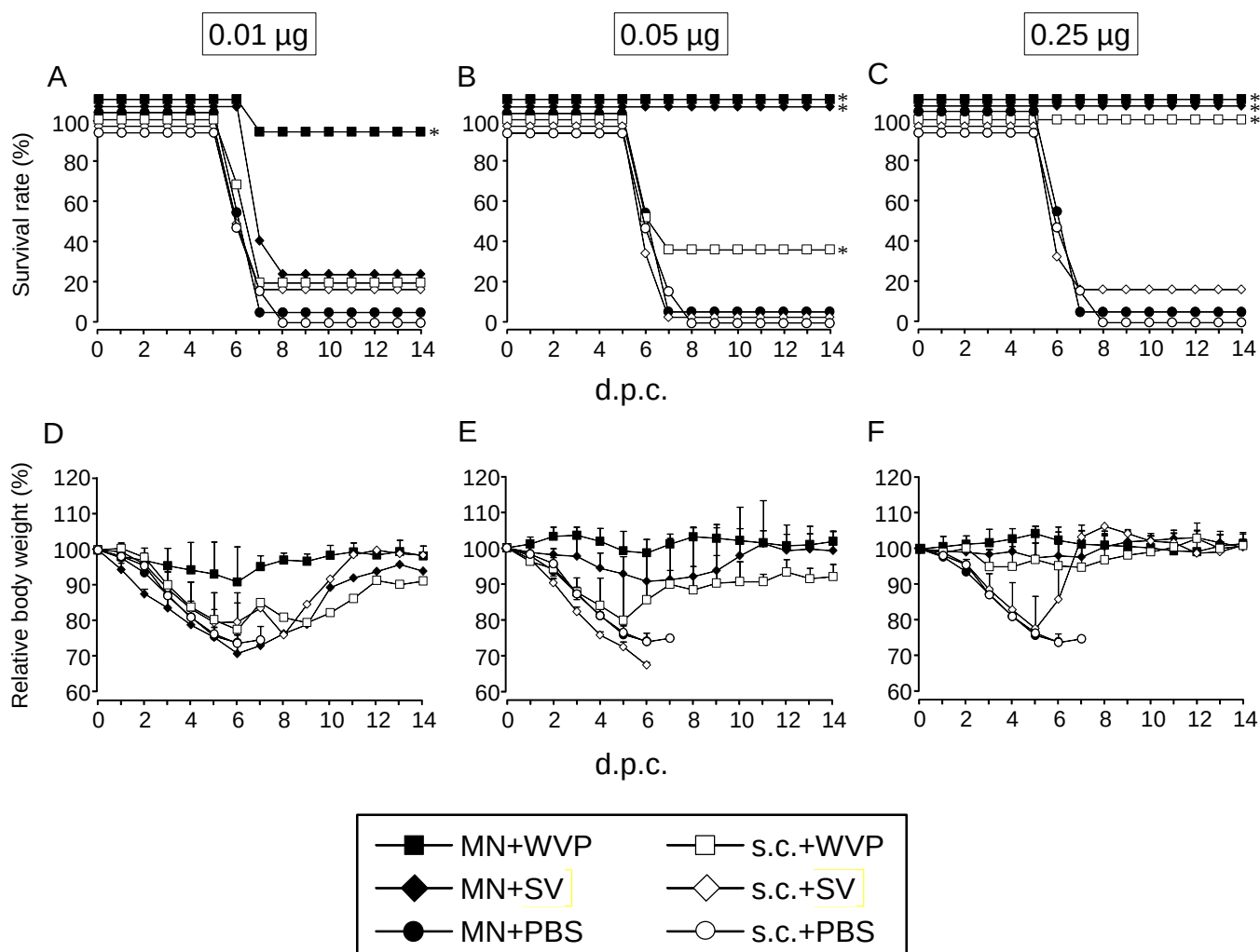


Fig.2 Nakatsukasa *et al.*

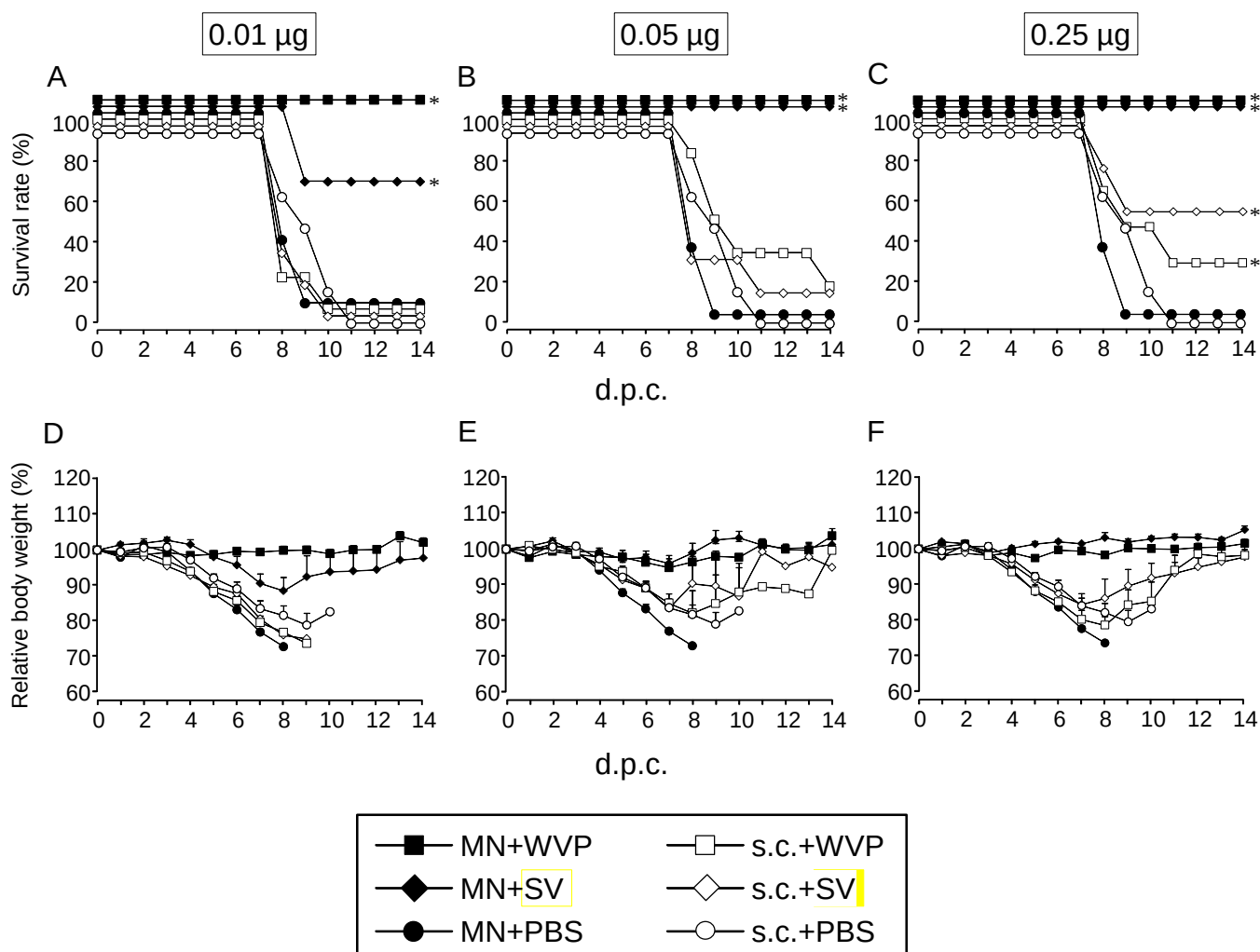


Fig.3 Nakatsukasa *et al.*

Table 1. Neutralizing antibody titers of mice injected with the vaccine and virus titers in the lungs after challenge against PR8 (H1N1)

Vaccine	Administration route	Formulation	Dose of vaccine (µg)	Neutralizing antibody titer	Virus titer (Mean log PFU/g) ± SE
PR8 (H1N1)	MN	WVP	0.01	<20, <20, <20, <20, <20, 20, 20, 20, 40, 80, 80, 160	5.83 ± 0.63*
			0.05	20, 40, 80, 160, 160, 160, 160, 160, 320, 320, 640, 1,280	1.81 ± 0.79*
			0.25	80, 160, 160, 160, 160, 160, 320, 320, 320, 320, 640, 640	0.00 ± 0.00*
		SV	0.01	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	7.68 ± 0.14
			0.05	20, 40, 40, 40, 40, 40, 40, 40, 80, 80, 80, 160	6.91 ± 0.40
			0.25	40, 80, 80, 160, 160, 160, 160, 160, 320, 320, 320, 320	5.14 ± 0.54*
	subcutaneous injection	WVP	0.01	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	7.60 ± 0.15
			0.05	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, 80	7.42 ± 0.09
			0.25	<20, <20, <20, <20, <20, 20, 40, 40, 40, 40, 80, 160	6.63 ± 0.34**
		SV	0.01	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	8.15 ± 0.08
			0.05	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	7.89 ± 0.06
			0.25	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, 20, 80	7.63 ± 0.16
PBS	MN	-	-	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	7.89 ± 0.10
	subcutaneous injection	-	-	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	8.07 ± 0.19

Each of vaccine was administrated by microneedle (MN) or subcutaneously into 12 mice. Mice were challenged with 10 MLD₅₀ (10^{4.5} 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)

Vaccine	Administration route	Formulation	Dose of vaccine (μg)	Neutralizing antibody titer	Virus titer (Mean log PFU/g) ± SE	
One injection						
Vac-3 (H5N1)	MN	WVP	0.01	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND	
			0.05	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND	
			0.25	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, 20	ND	
		ES	0.01	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND	
			0.05	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND	
			0.25	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND	
		subcutaneous injection	WVP	0.01	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND
				0.05	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND
				0.25	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND
	ES		0.01	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND	
			0.05	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND	
			0.25	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND	
	PBS	MN	-	-	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND
		subcutaneous injection	-	-	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	ND
	Two injections					
Vac-3 (H5N1) × 2	MN	WVP	0.01×2	<20, 20, 20, 20, 20, 40, 80, 160, 160, 160, 320, 320	5.48 ± 0.23	
			0.05×2	<20, <20, <20, 20, 80, 80, 160, 320, 320, 320, 320, 640	3.67 ± 0.73*	
			0.25×2	80, 80, 160, 320, 320, 320, 640, 640, 640, 640, 1,280, 2,560	0.82 ± 0.48*	
		SV	0.01×2	<20, <20, <20, <20, <20, <20, <20, <20, 20, 40, 40, 80	5.67 ± 0.12	
			0.05×2	20, 20, 20, 40, 40, 40, 40, 40 80, 160, 160, 320	4.16 ± 0.43*	
			0.25×2	<20, 80, 80, 80, 80, 160, 160, 320, 320, 1,280, 1,280, 1,280	1.20 ± 0.70*	
		subcutaneous injection	WVP	0.01×2	<20, <20, <20, <20, <20, <20, <20, <20, <20, 20, 20, 20	6.26 ± 0.09
				0.05×2	<20, <20, <20, <20, <20, <20, <20, <20, <20, 20, 80, 80	5.49 ± 0.25
				0.25×2	<20, <20, <20, <20, <20, 80, 80, 80, 80, 160, 320, 320	4.19 ± 1.22
		SV	0.01×2	<20, <20, <20, <20, <20, <20, <20, 20, 20, 20, 20, 20	6.20 ± 0.19	
			0.05×2	<20, <20, <20, <20, <20, <20, 20, 20, 20, 20, 20, 80	5.98 ± 0.08	
			0.25×2	<20, <20, <20, <20, <20, 40, 40, 160, 320, 320, 320	5.27 ± 0.31	
	PBS × 2	MN	-	-	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	5.78 ± 0.27
		subcutaneous injection	-	-	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20, <20	5.74 ± 0.20

Each of vaccine was administrated by MN or subcutaneously into 12 mice. Mice were challenged with 30 MLD₅₀ (10^{2.3} 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