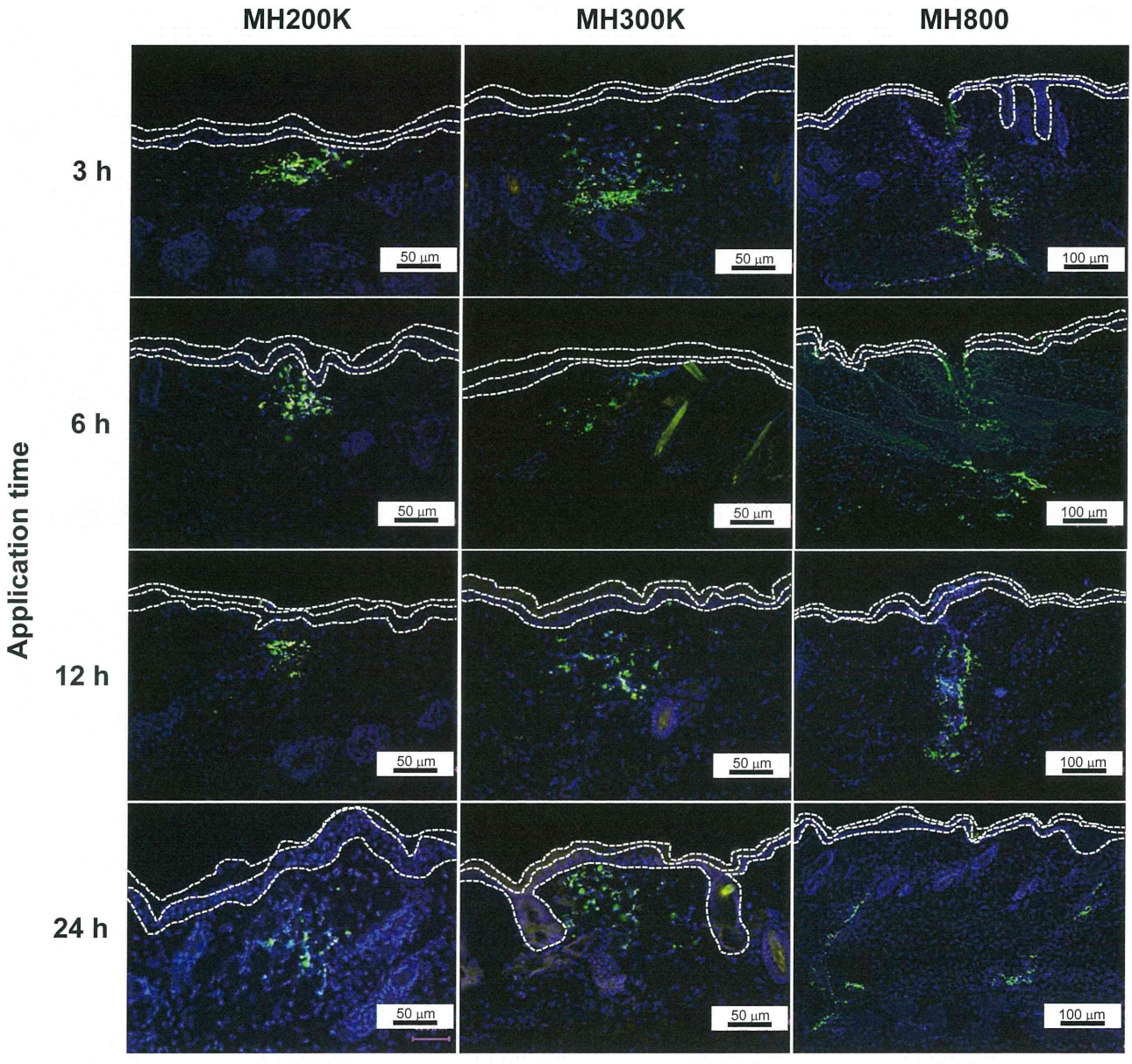


Fig. 14 Histological section of the back skin of ICR mice treated with F-OVA-loaded MH200K, MH300K, or MH800 for 3, 6, 12, or 24 h. After removal of the MH patch, the skin was harvested and frozen. Frozen sections (8-μm-thick) were photographed using a fluorescence microscope. The resulting needle track crossed the epidermis and into the superficial dermis, revealing that F-OVA was delivered (green spot) to both these layers. The nucleus was counterstained using DAPI (blue). The white dotted lines indicate the surfaces of the stratum corneum, epidermis, and superficial dermis, respectively, from top to bottom.

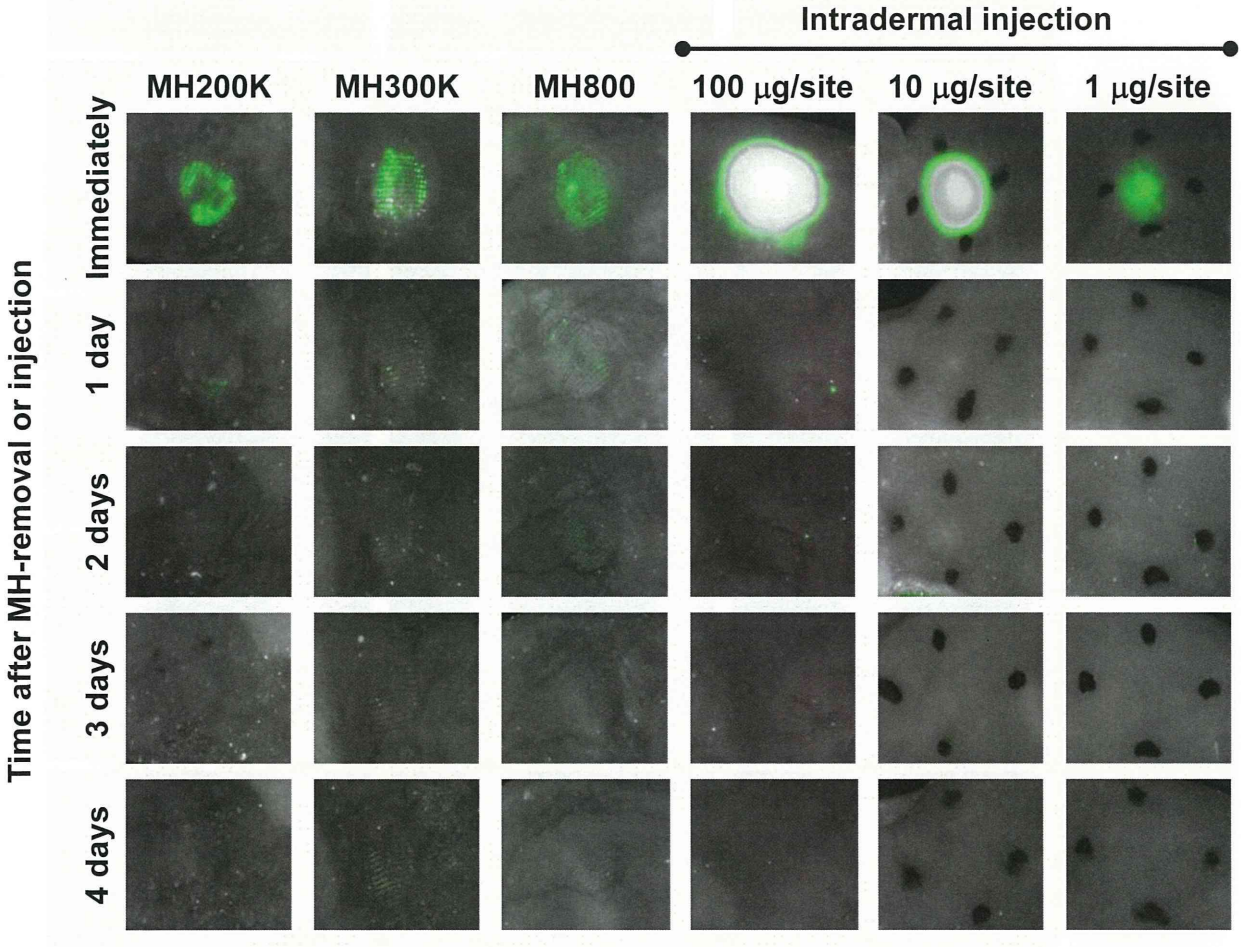


Fig. 15 Fluorescent images of mouse skin applied with MH containing FD4. ICR mice were applied with MH200K, MH300K, or MH800 containing FD4 on back skin for 1 h. Control groups were intradermally injected with FD4 at 1, 10, or 100 μg/site. At the indicated time, the MH-applied sites or the injected sites were photographed by using a fluorescence imager (Maestro EX) with 455-490 nm blue filter.

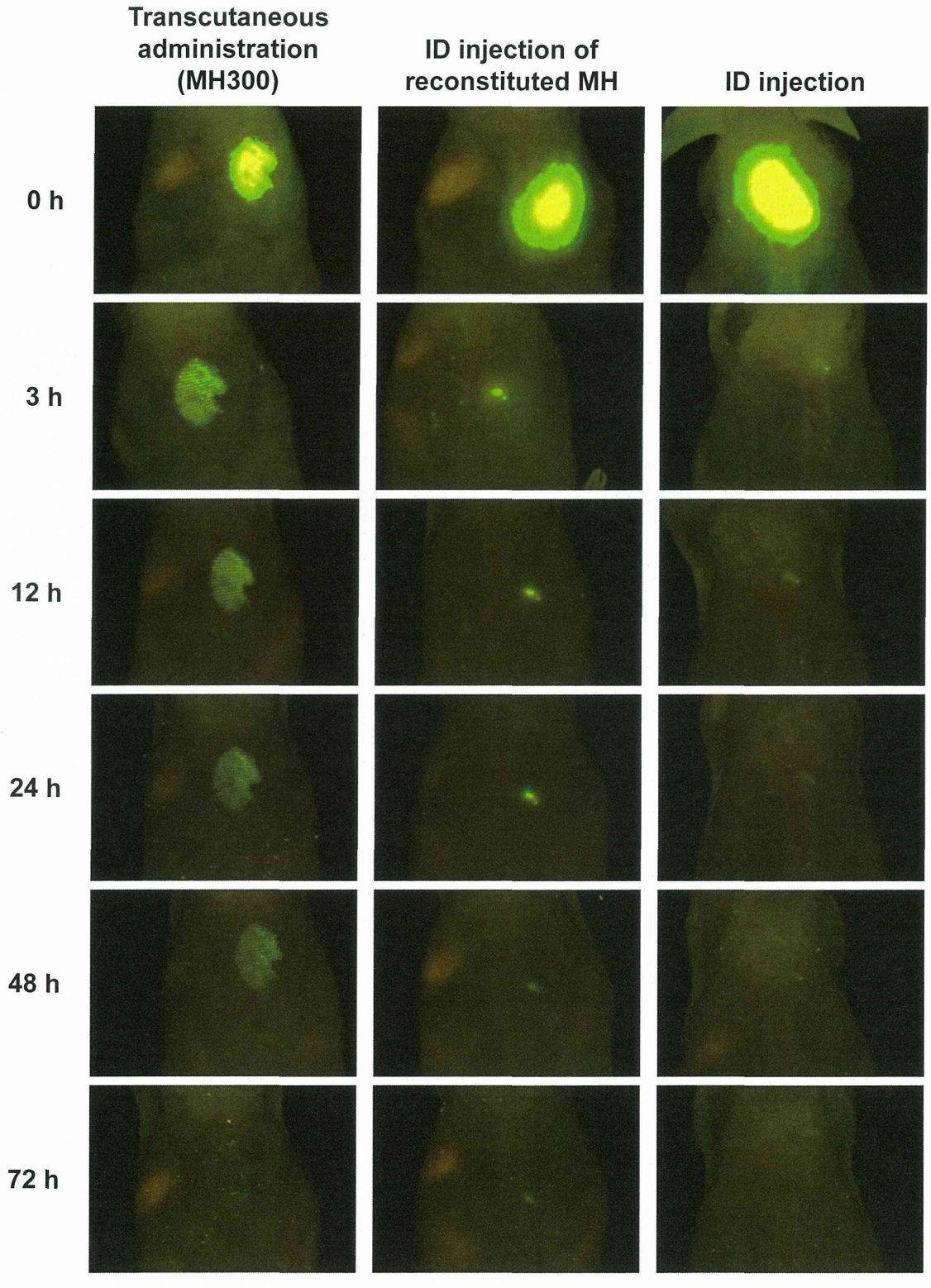


Fig. 16 Kinetics of antigen deposition after F–OVA administration to HR-1 hairless mice via MH300 patch delivery, intradermal (ID) injection of reconstituted F–OVA, or ID injection of phosphate-buffered saline containing F–OVA. Representative in vivo fluorescence imaging for antigen deposition from 4 mice for each group. Images were captured by the CRi Maestro EX system at the indicated time point after administration. Here, time 0 means the time of MH300 removal after 1-h application.

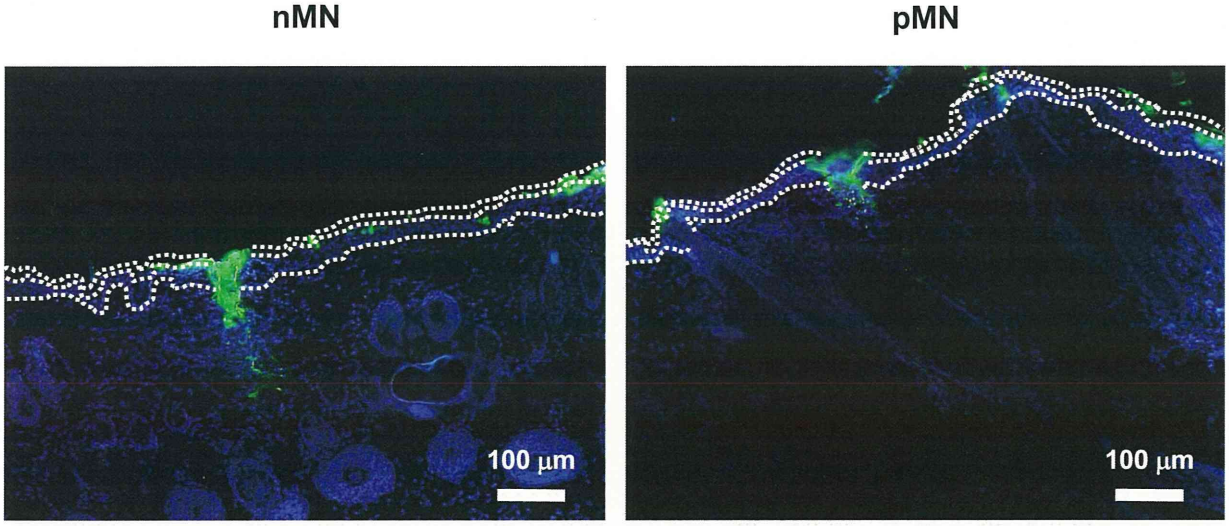


Fig. 17 Histological section of the back skin of ICR mice administrated transcutaneously with F-OVA. ICR mice were transcutaneously administrated with 25 μg F-OVA by using puncturing method. Six hours later, the skin was harvested and frozen. Frozen sections (8-μm-thick) were photographed using a fluorescence microscope. The resulting needle track crossed the epidermis and into the superficial dermis, revealing that F-OVA was delivered (green spot) to both these layers. The nucleus was counterstained using DAPI (blue). The white dotted lines indicate the surfaces of the stratum corneum, epidermis, and superficial dermis, respectively, from top to bottom.

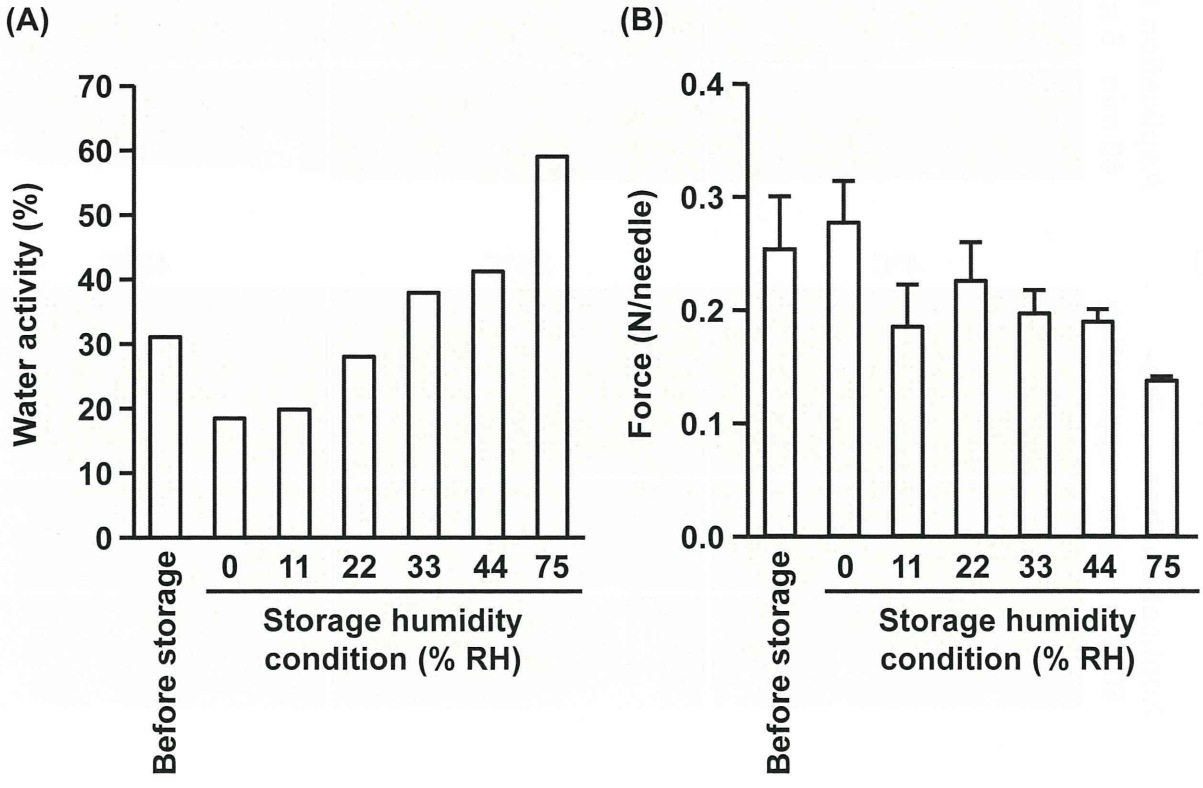


Fig. 18 Water activity and mechanical failure force for placebo MH800. (A) Water activity of MH800 after storage under various humidity conditions. MH800 was placed under 0, 11, 22, 33, 44, or 75% humidity conditions for 1 week. The water activity was measured using a water activity analyzer. As a control, the water activity before storage was also measured. (B) Mechanical failure force of MH800 as a function of water activity. The force required to fracture 55 microneedles was measured using a TA-XT plus texture analyzer. Data represent the average ± SD of 3 measurements each.

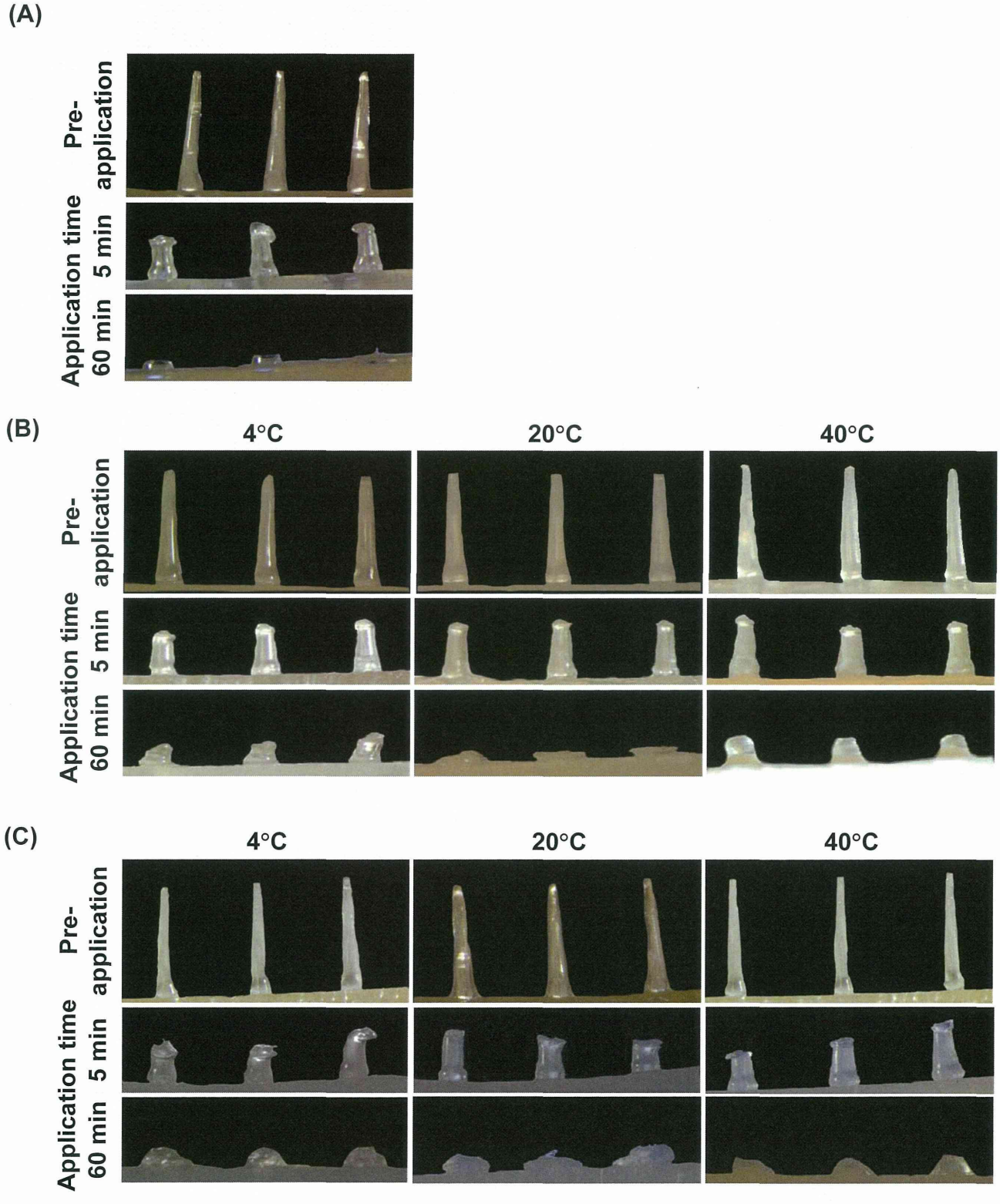


Fig. 19 Dissolution kinetics of combined TT/DT-loaded MH800 (A) before storage or after storage at 4, 25, or 40°C for (B) 6 or (C) 12 months. The MH800 were applied on the back skin of Wistar ST rats and left for 5 or 60 min. After removal of the MH800, the microneedles remaining on the MH800 were photographed using a stereoscopic microscope.

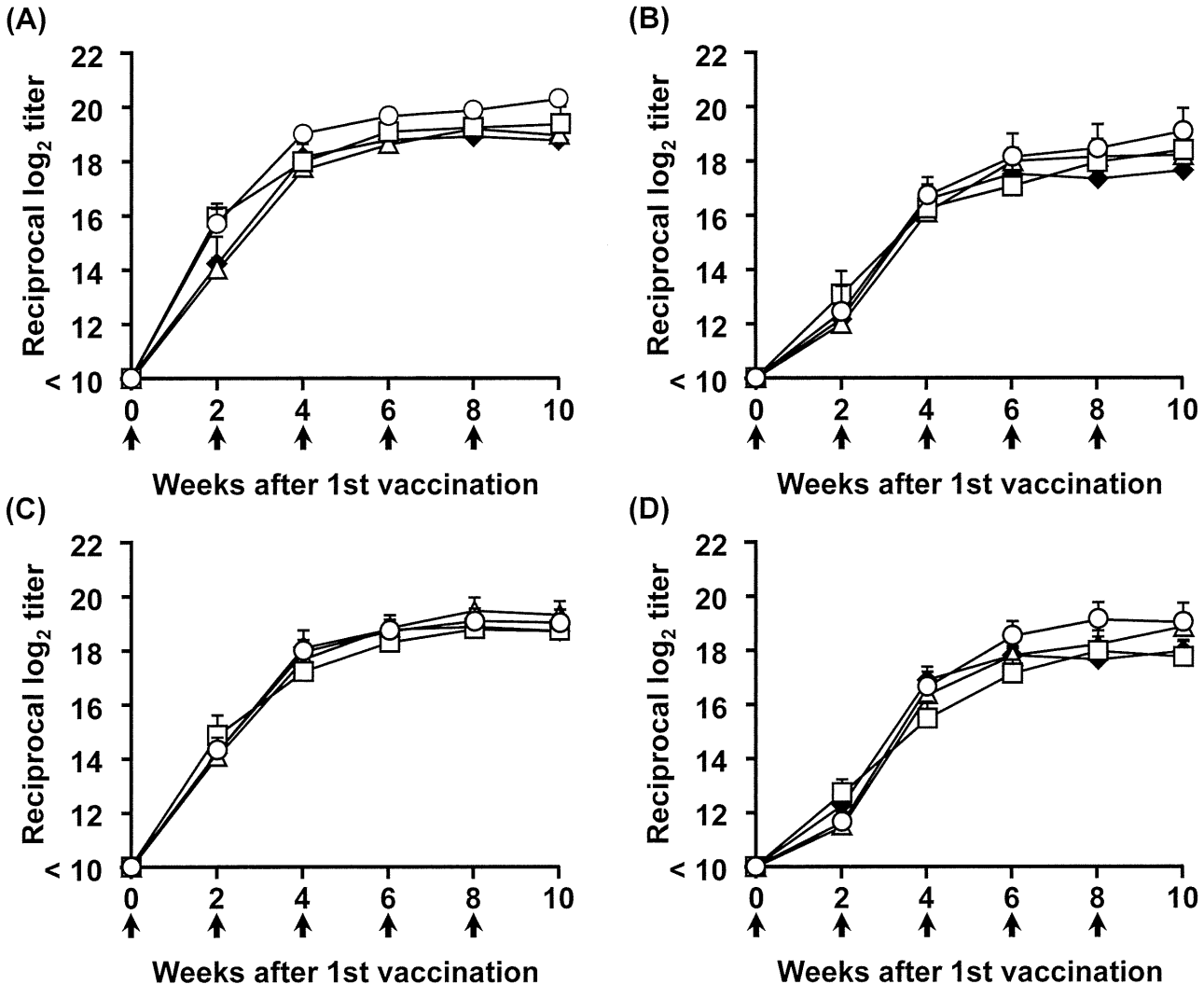


Fig. 20 OVA-specific antibody responses after vaccination using MH800 patches stored at various temperatures for 6 or 12 months. Combined TT (20 μg) and DT (10 μg)-loaded MH800 patches stored at 4 (○), 25 (□), or 40 (△) for (A, B) 6 or (C, D) 12 months were applied to the back skin of Wistar ST rats. Vaccinations were repeated 5 times every 2 weeks. Arrows indicate the vaccination timing. As a control, freshly prepared combined TT (20 μg) and DT (10 μg)-loaded MH800 patches were also applied to the back skin of Wistar ST rats under the same vaccination schedule (◆). At the indicated points, sera were collected from the rats to determine the toxoid-specific IgG titers for TT (A, C) or DT (B, D) by ELISA. Data are expressed as mean ± SE of results from 5 rats.

Table 10 Rat tetanus toxin challenge study

TT/DT-containing MH800		Survival ratio (survival rats/tested rats)
Storage temperature	Storage period	
4°C	6 months	5/5
	12 months	5/5
25°C	6 months	5/5
	12 months	5/5
40°C	6 months	5/5
	12 months	5/5
Unvaccinated		0/5

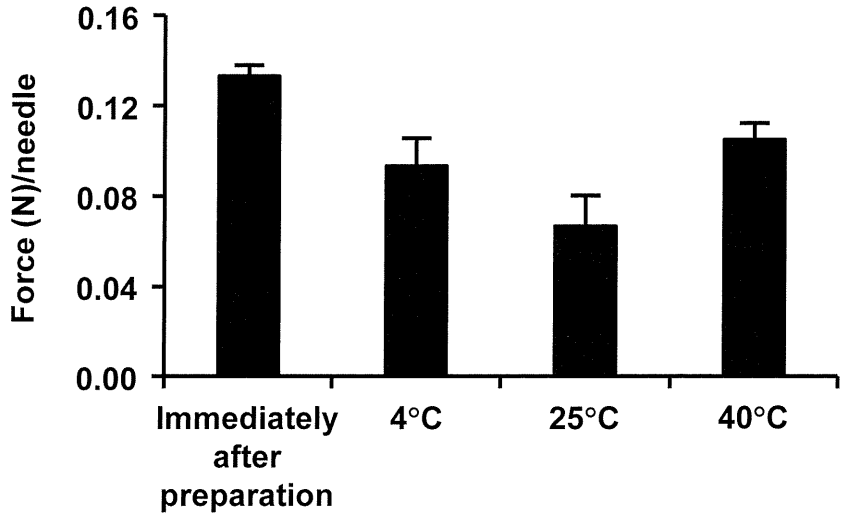


Fig. 21 Mechanical failure force of antigen-loaded MH800 before and after 6-month storage at various temperatures. MH800 loaded with influenza HA antigen were stored at 4, 25, or 40°C for 6 months. Then, the force required to fracture 55 microneedles was measured using a texture analyzer. Data represent the mean ± SD of 3 measurements each.

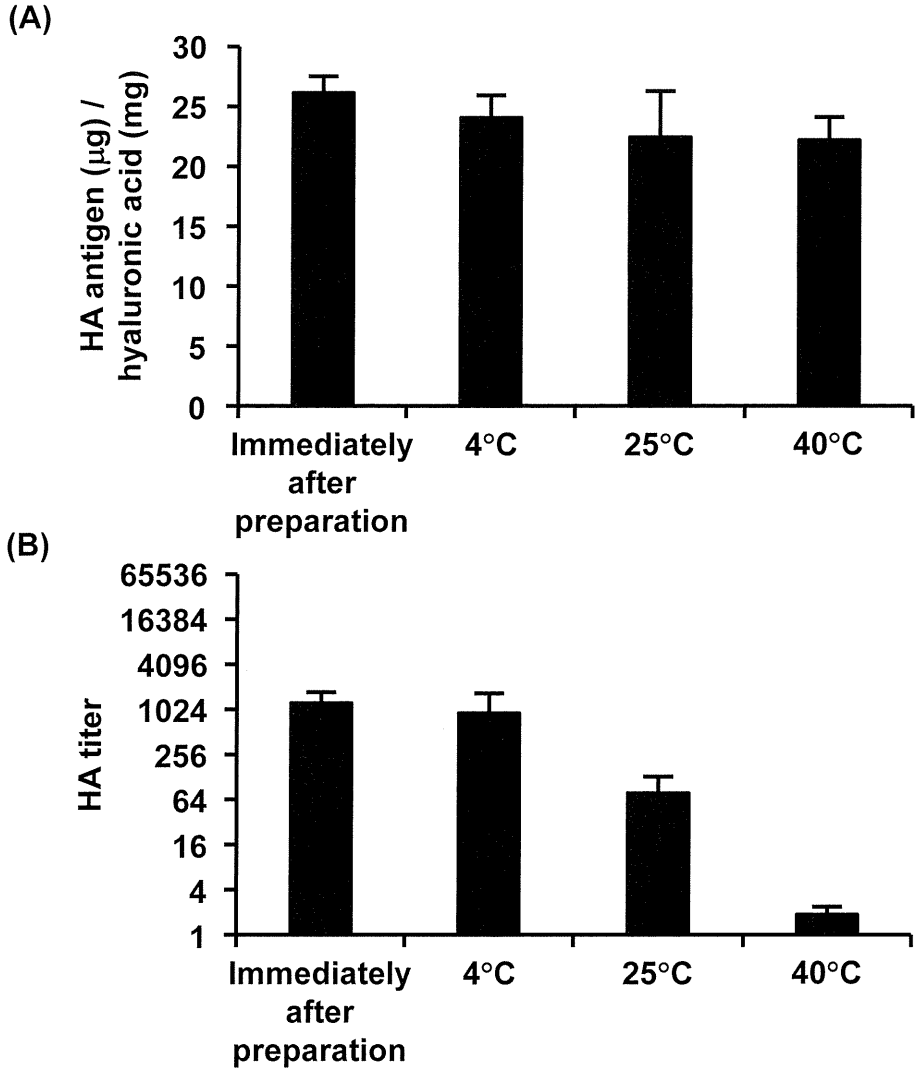


Fig. 22 Amount and titer of HA antigen loaded in MH800 before and after 6-month storage at various temperatures. (A) Amount of HA antigen and hyaluronic acid in each MH800 were determined by Lowry method and ELISA, respectively. (B) HA titer in HA antigen solution prepared from each MH800 was determined by hemagglutination assay. Data represent the mean ± SD of 5 measurements each.

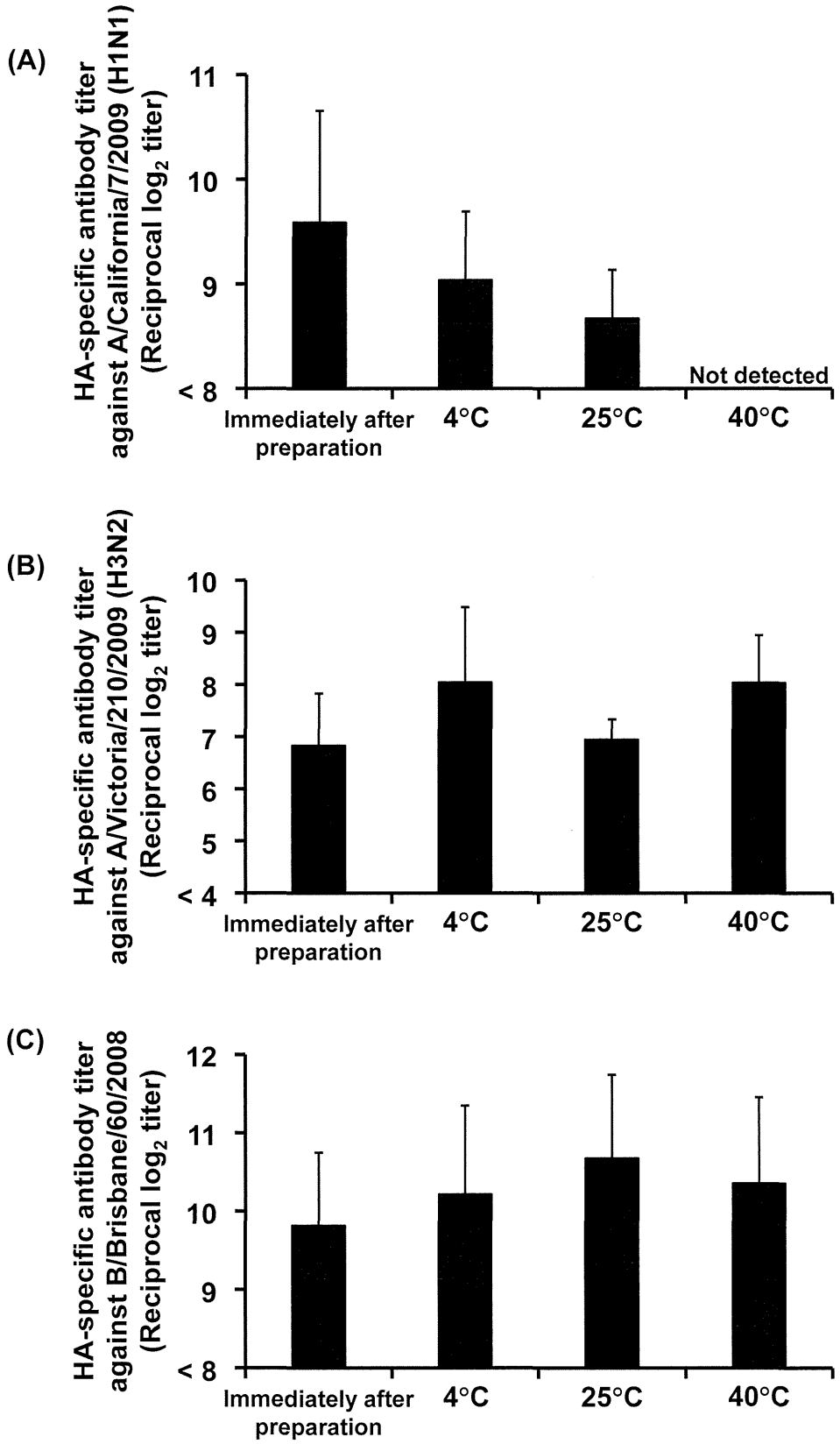
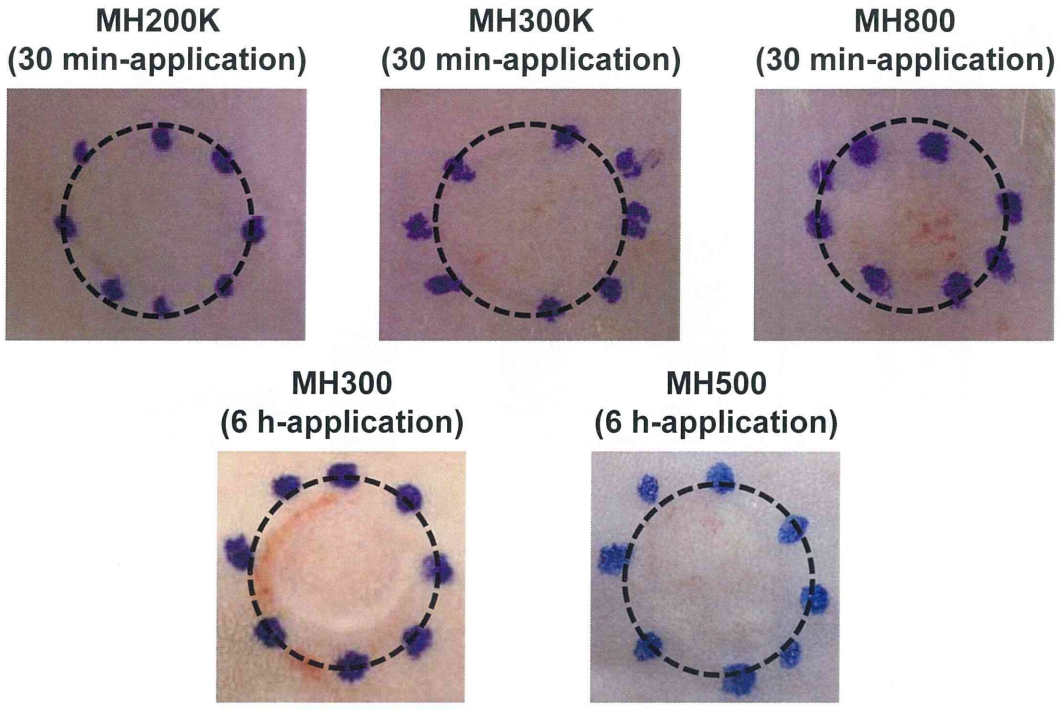
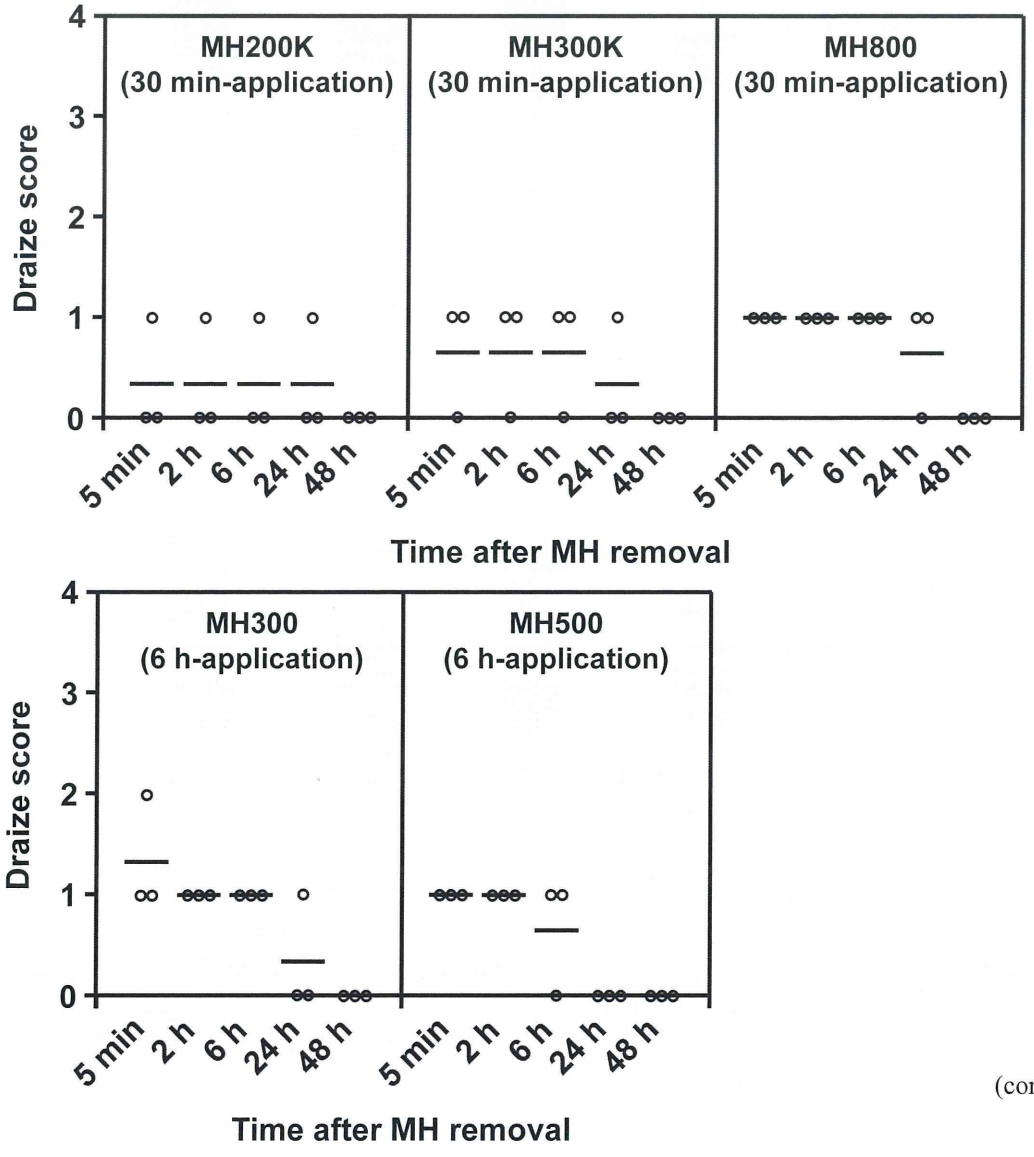


Fig. 23 HA-specific antibody titers in mice immunized subcutaneously with HA antigen solution prepared from each MH800 before and after 6-month storage at various temperatures. MH800 containing HA were stored at 4°C, 25°C, or 40°C for 6 months, and then HA antigen solution were administrated to back skin of BALB/c mice twice at 4-week interval. Two weeks after second vaccination, sera collected from these mice were assayed for the IgG titer specific for A/H1N1 (A), A/H3N2 (B), or B (C) by ELISA. Data are expressed as mean ± SE of results from 5 mice.

(A)



(B)



(continued)

(C)

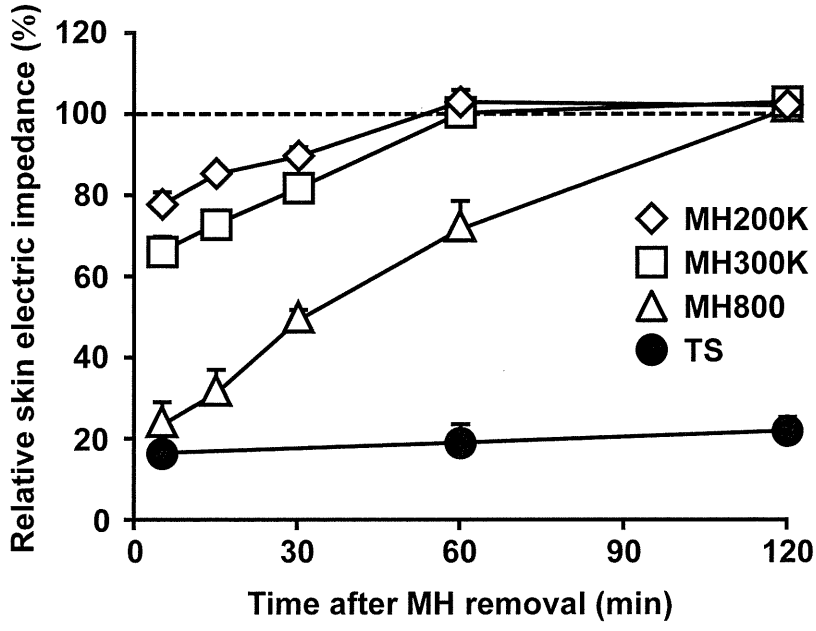


Fig. 24 Assessment of skin irritation caused by application of each MH. The MH200K, MH300K, MH300, MH500, or MH800 was applied to the back skin of Wistar ST rats for 30 min (MH200K, MH300K, and MH800) or 6 h (MH300 and MH500). (A) Each panel shows photographs of application areas 5 min after MH removal. (B) The degree of erythema on the skin of Wistar ST rats was scored using the Draize scoring system 5 min, 2 h, 6 h, 24 h, or 48 h after removing MHs. The mean score is shown as a bar. (C) Skin impedance of MH application areas and non-application areas was measured 5, 15, 30, 60, and 120 min after 30-min applications. As controls, back skin of Wistar ST rats were tape-stripped (TS). Data are expressed as mean ± SE of results from 3 rats.

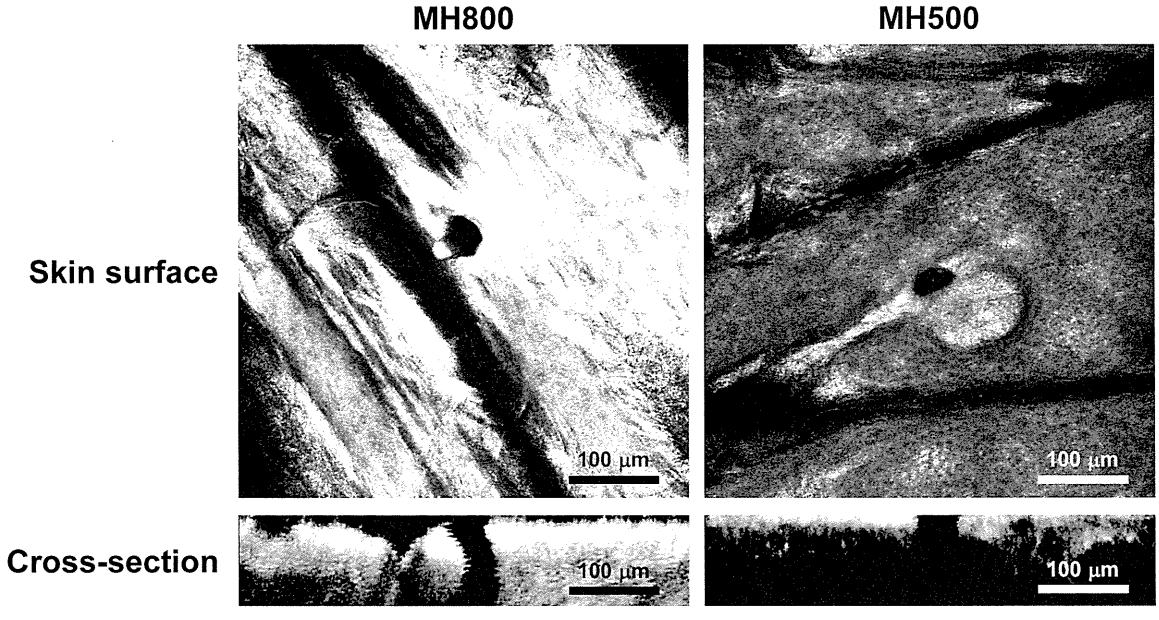


Fig. 25 Status of human skin treated with MHs. MH800 or MH500 was applied to the skin of the left lateral upper arms of 2 healthy volunteers for 5 s, and skin images were immediately photographed under in vivo confocal scanning laser microscope.

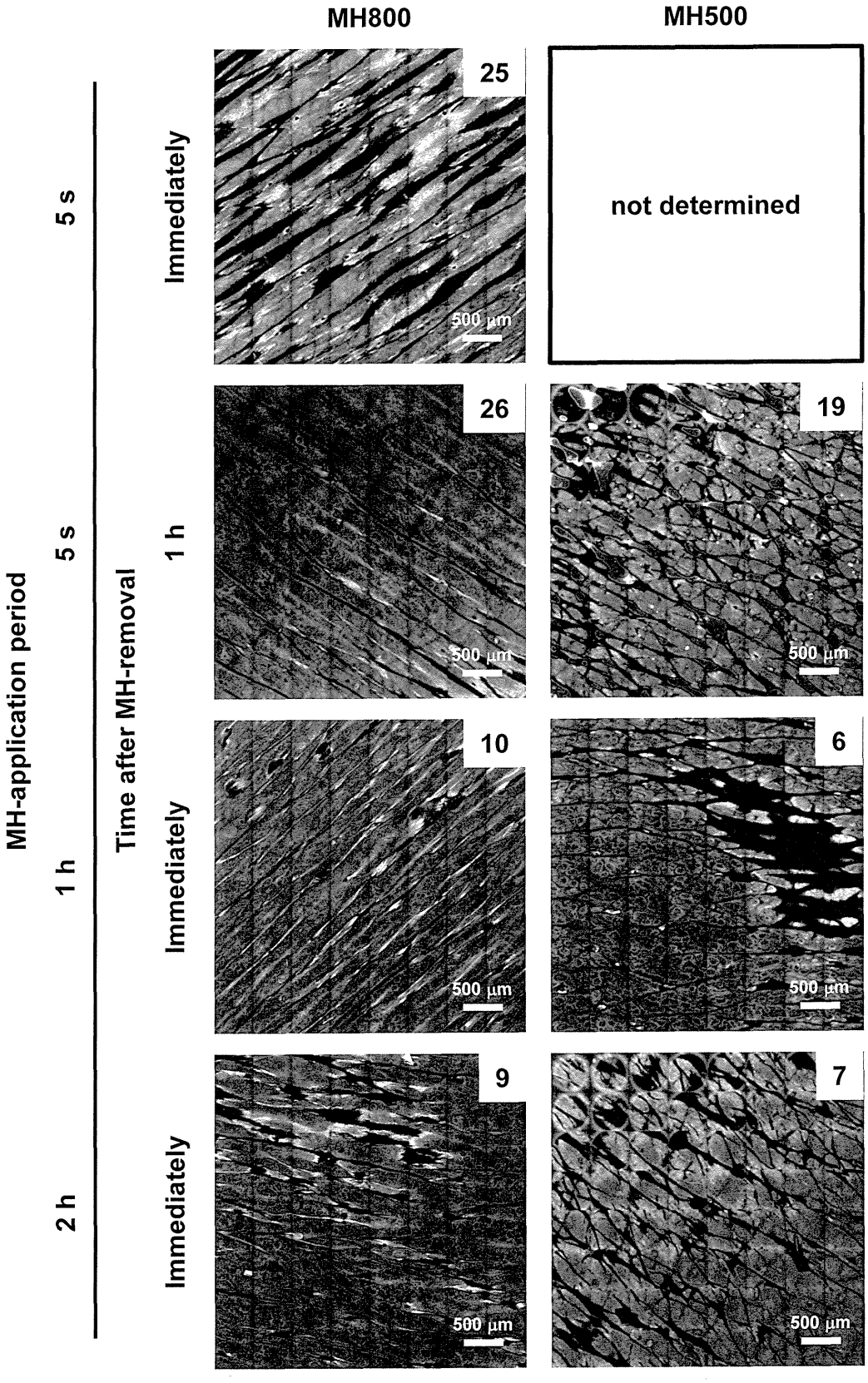


Fig. 26 Status of human skin treated with MHs. MH800 or MH500 was applied to the skin of the left lateral upper arms of 2 healthy volunteers for 5 s, 1 h, or 2 h, and skin images were immediately or 1-h later photographed under in vivo confocal scanning laser microscope. Numerical values shown at upper right in each photo indicate the number of puncture holes in the field of view.

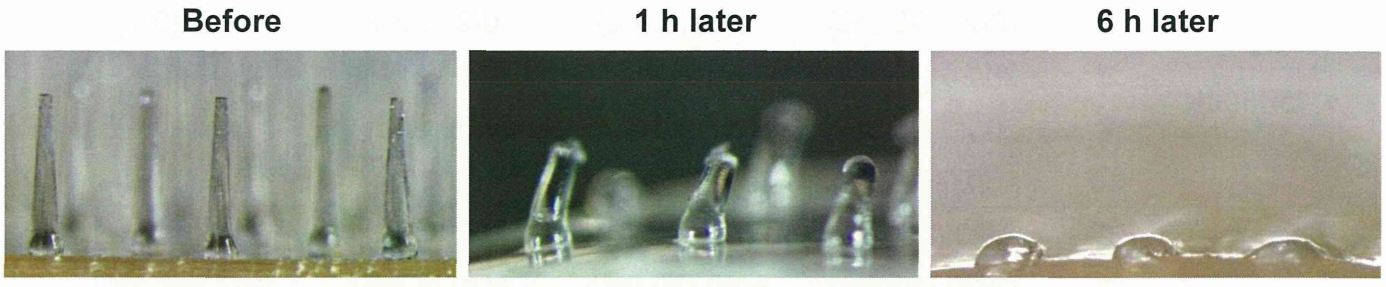


Fig. 27 Needle-dissolution of MH800 in human skin. MH800 was applied to the skin of the left lateral upper arms of 3 healthy volunteers for 1 h or 6 h, and microneedle patches were immediately observed using a stereoscopic microscope.

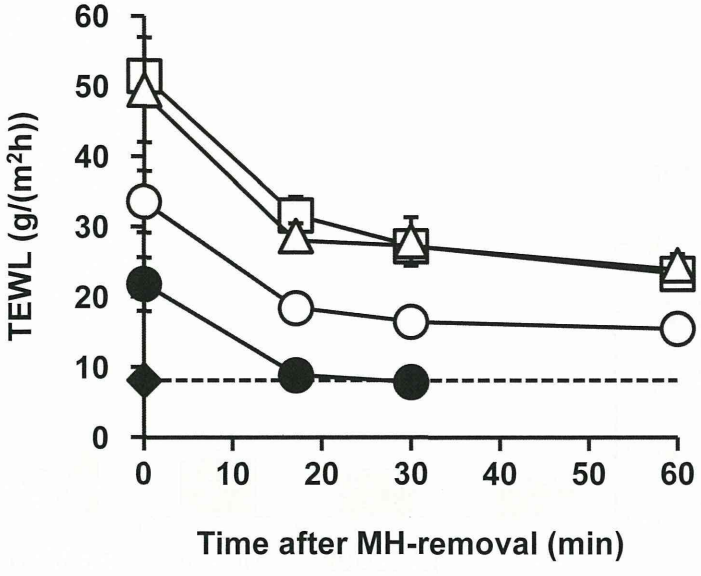


Fig. 28 Transepidermal water loss (TEWL) after MH application. MH800 were applied to the skin of the left lateral upper arms of 3 healthy volunteers for 5 s (□), 1 h (△), or 6 h (○). As a control, MH-needleless was applied for 6 h (●). At the indicated time after MH-removal, TEWL of the application sites was measured. Data are expressed as mean ± SE of results from 3 subjects. ◆; TEWL of untreated skin.

Table 11 Local adverse event after application of MHs

MH	Day	ICDRG score			Purpura
		–	+?	+	
MH300K	2	19/20 (95.0%)	1/20 (5.0%)	0/20 (0%)	0/17 (0%)
	3	20/20 (100%)	0/20 (0%)	0/20 (0%)	0/17 (0%)
	7	20/20 (100%)	0/20 (0%)	0/20 (0%)	0/17 (0%)
	30	20/20 (100%)	0/20 (0%)	0/20 (0%)	0/17 (0%)
MH500	2	8/20 (40.0%)	12/20 (60.0%)	0/20 (0%)	6/17 (35.3%)
	3	13/20 (65.0%)	6/20 (30.0%)	1/20 (5.0%)	8/17 (47.1%)
	7	19/20 (95.0%)	0/20 (0%)	1/20 (5.0%)	6/17 (35.3%)
	30	20/20 (100%)	0/20 (0%)	0/20 (0%)	0/17 (0%)
MH800	2	7/20 (35.0%)	13/20 (65.0%)	0/20 (0%)	6/17 (35.3%)
	3	13/20 (65.0%)	7/20 (35.0%)	0/20 (0%)	10/17 (58.8%)
	7	19/20 (95.0%)	1/20 (5.0%)	0/20 (0%)	5/17 (29.4%)
	30	20/20 (100%)	0/20 (0%)	0/20 (0%)	0/17 (0%)

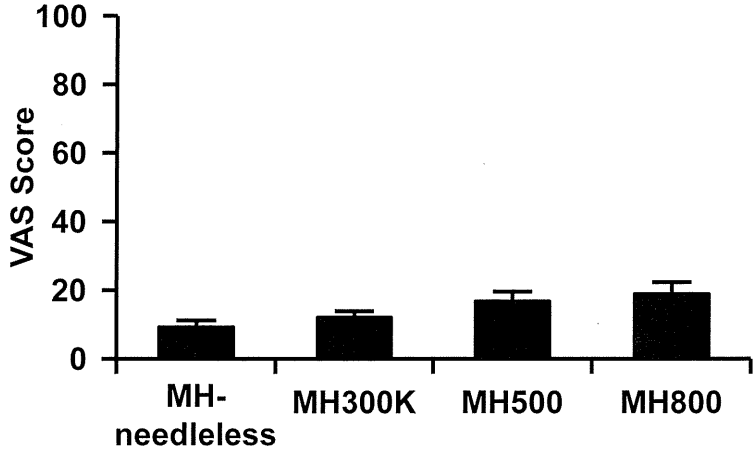


Fig. 29 Pain scale assessment on MH application. MH-needleless, MH300, MH500, and MH800 were applied to the skin of the left lateral upper arms of 17 healthy volunteers. Subjects were asked to grade the pain experienced using a VAS from 0 (no pain) to 100 (unbearable pain). Data are expressed as mean ± SE of results from 17 subjects.

Table 12 Pain scale in MN application

Subject ID	VAS score
2	2
3	16
4	7
5	0
6	13
7	0
8	36
9	25
10	10
11	0
Average	10.9

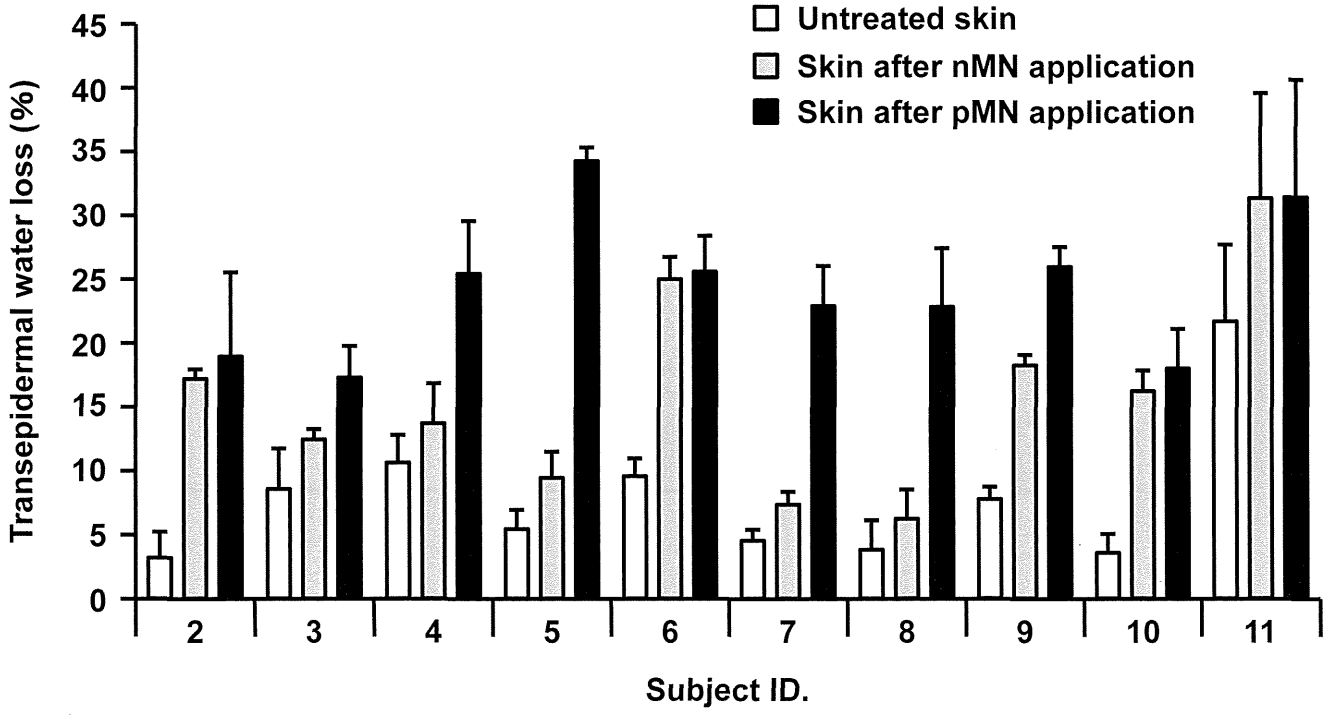


Fig. 30 TEWL after MN application. MNs were applied to the skin of the left lateral upper arm of 10 healthy volunteers for 30 min. After MN-removal, TEWL of the application sites was measured. Data are expressed as mean ± SD of results from 3 measurements.

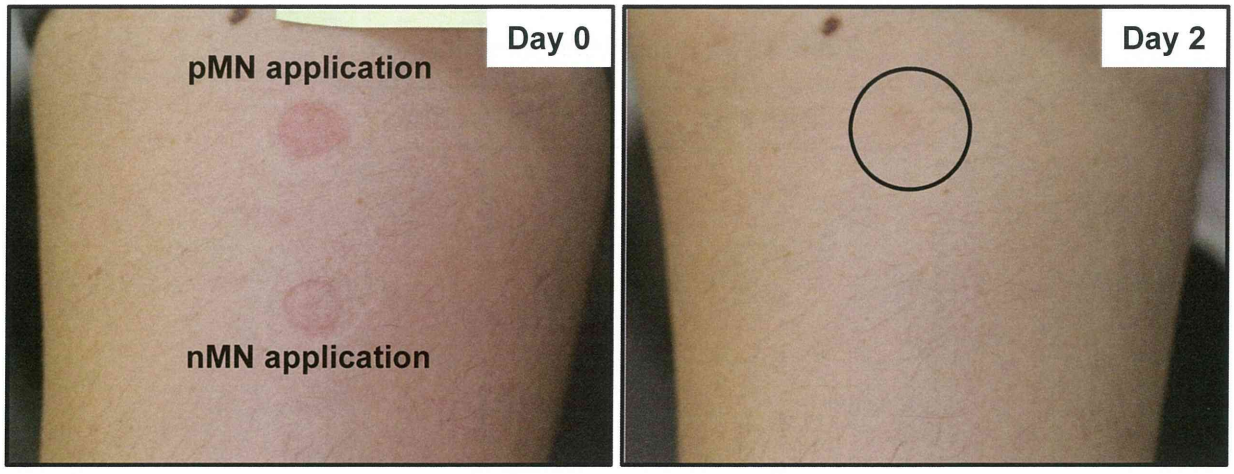


Fig. 31 Photographs of human skin (ID 5) after MN application.

Table 13 ICDRG score after application of MN

Subject ID	MN	Day 2	Day 7
2	nMN	-	-
	pMN	-	-
3	nMN	-	-
	pMN	-	-
4	nMN	-	-
	pMN	-	-
5	nMN	-	-
	pMN	+?	-
6	nMN	-	-
	pMN	-	-
7	nMN	-	-
	pMN	-	-
8	nMN	-	-
	pMN	-	-
9	nMN	-	-
	pMN	-	-
10	nMN	-	-
	pMN	-	-
11	nMN	+?	-
	pMN	+?	-

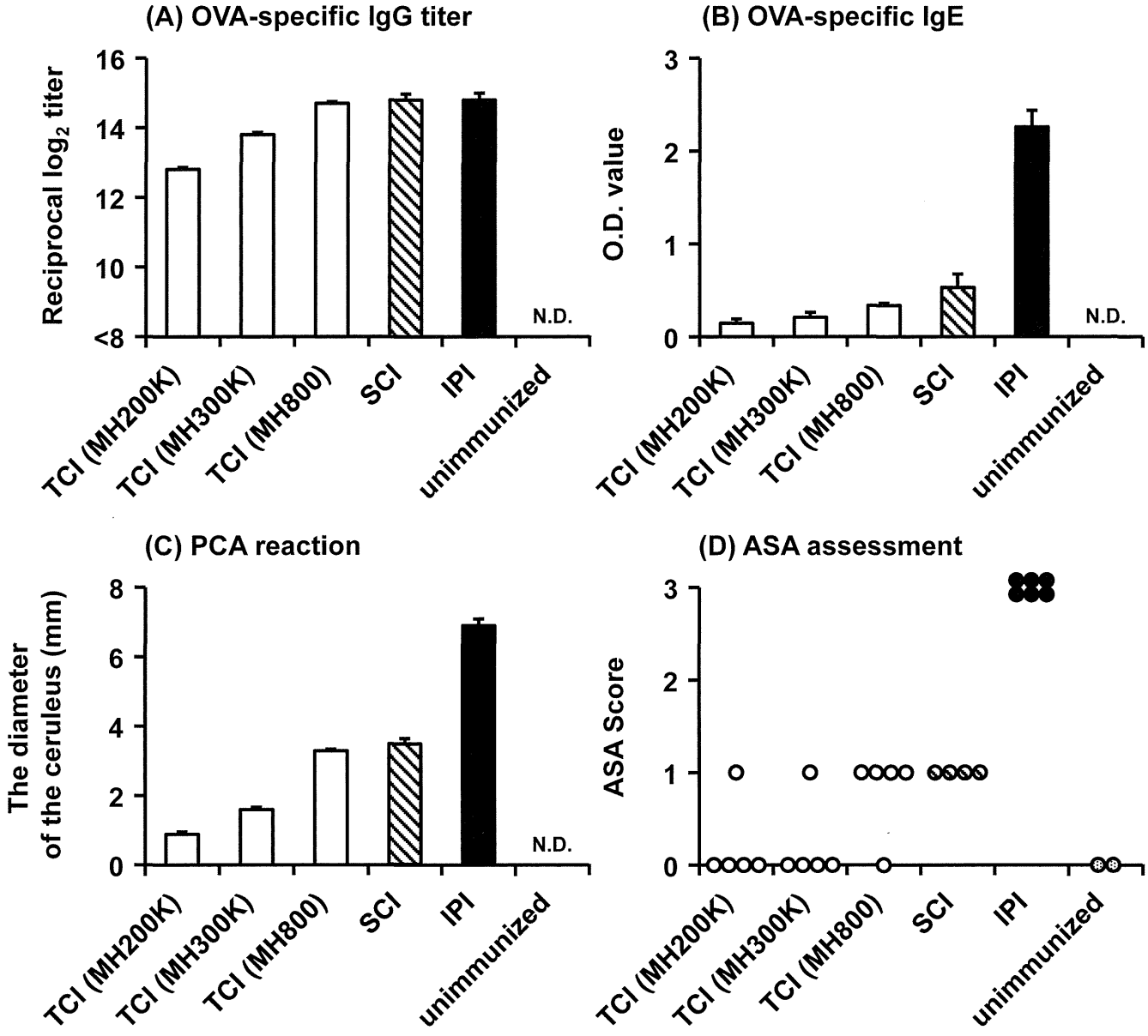
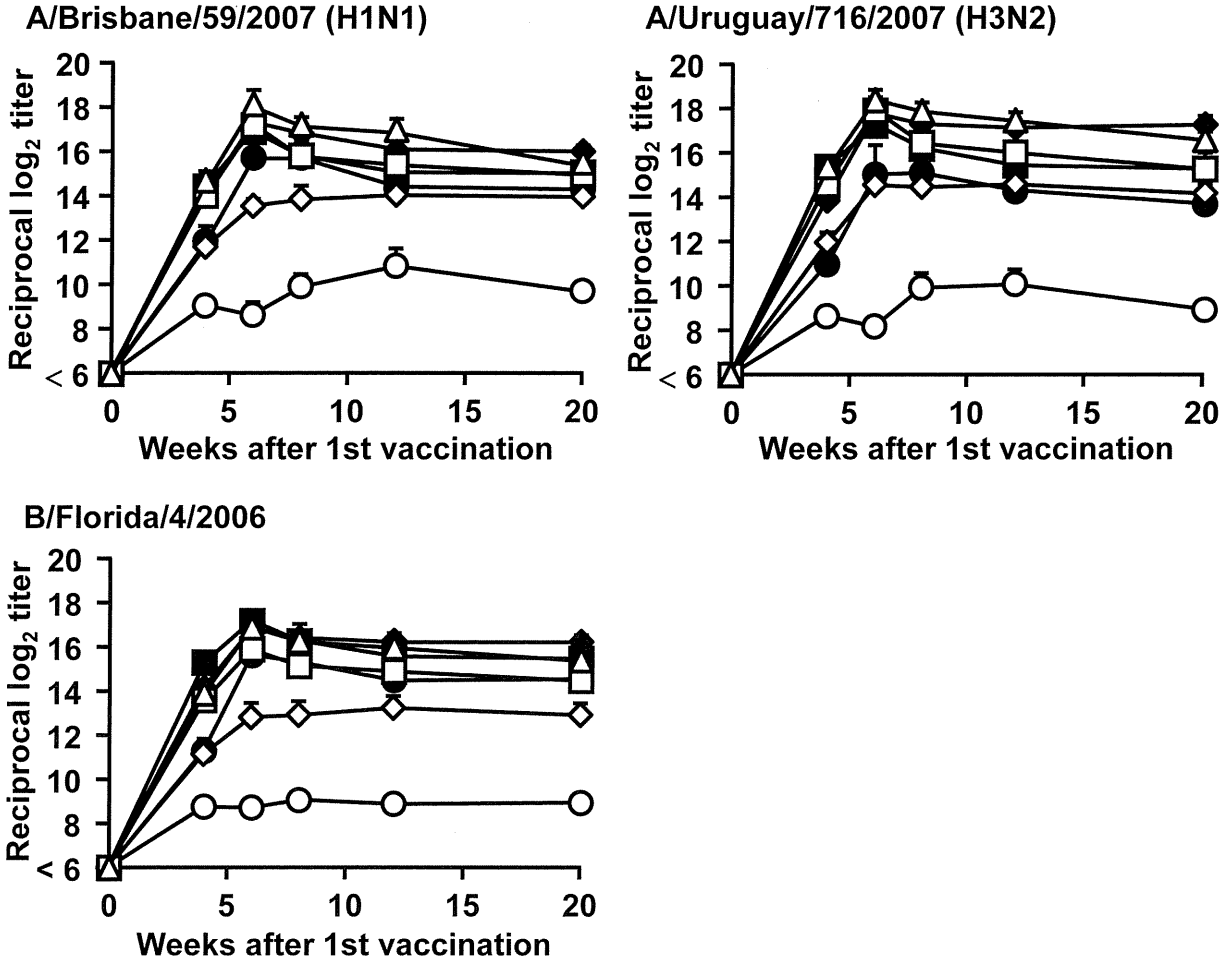
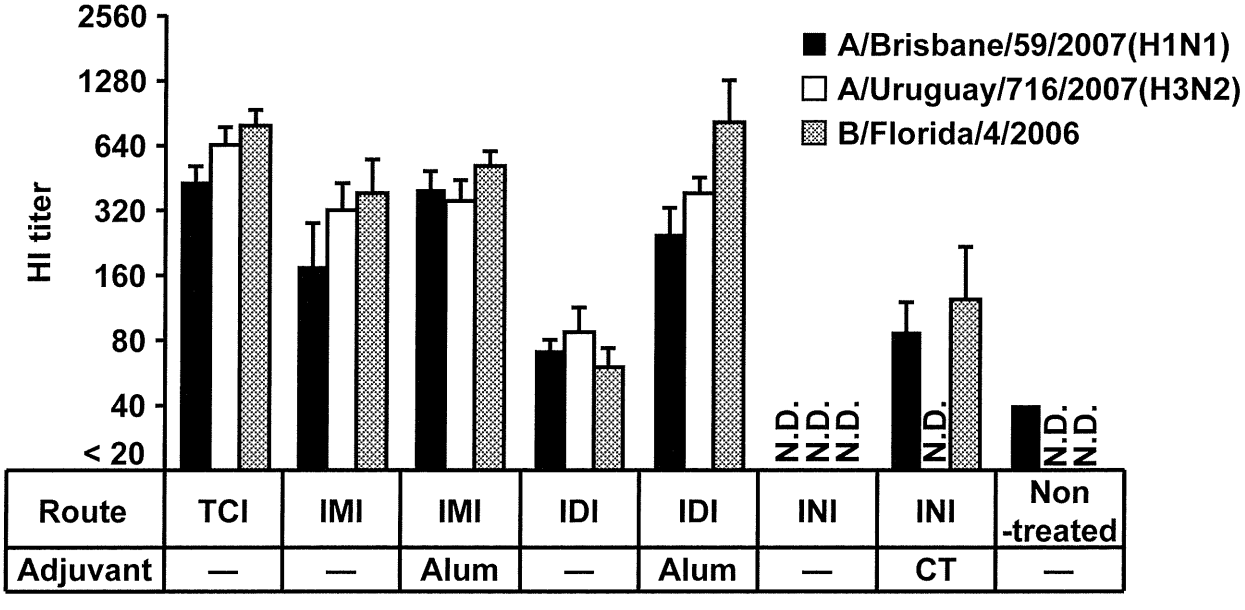


Fig. 32 Allergic immune responses induced by antigen loaded MH formulation. Hartley guinea pigs were treated with MH200K, MH300K, or MH800 containing 1 μg OVA/needles for 6 h four times at 2-week interval, or were subcutaneously immunized with 1 μg OVA (SCI) five times at 2-week interval. As positive controls, Hartley guinea pigs were immunized by intraperitoneal injection of 1-μg OVA and 5-mg Alum (IPI) twice at 2-week interval. Two weeks after the final vaccination, sera were collected from these animals and were assayed for OVA-specific IgG titers (A), and O.D. value of IgE (16-fold dilution) (B) by ELISA. (C) For the PCA reaction, non-sensitized Wistar ST rats were injected with sera from immunized-guinea pigs. Twenty-four hours later, these rats were intravenously injected with Evans blue and OVA, and a leak blue spot at the injection site was measured 30 min later. Data are expressed as mean ± SE of results from 4-6 guinea pigs. (D) In ASA assessments, guinea pigs were intravenously injected with OVA a month after the final vaccination, and the performance status of guinea pigs was scored using the ASA scoring system. N.D.; not detected.

(A)



(B)

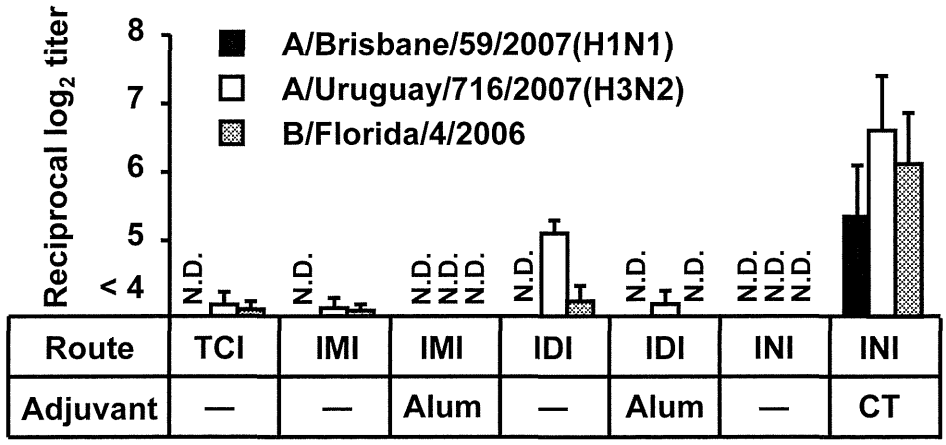


Route	TCI	IMI	IMI	IDI	IDI	INI	INI	Non -treated
Adjuvant	—	—	Alum	—	Alum	—	CT	—

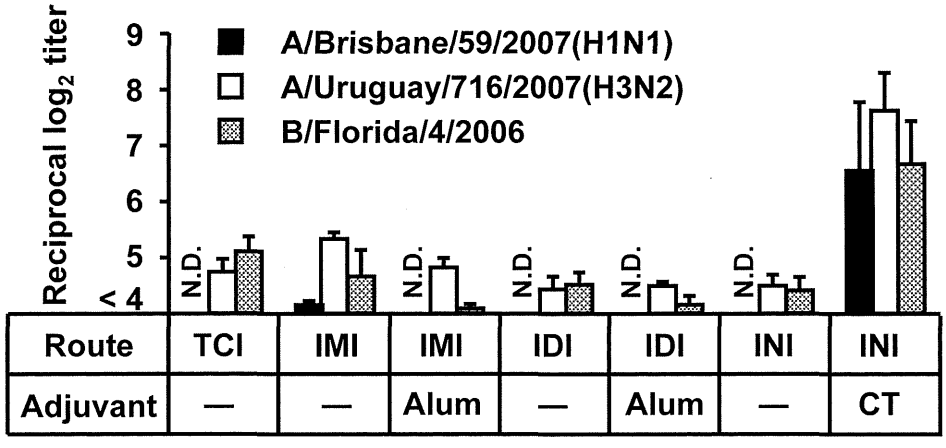
(continued)

(C)

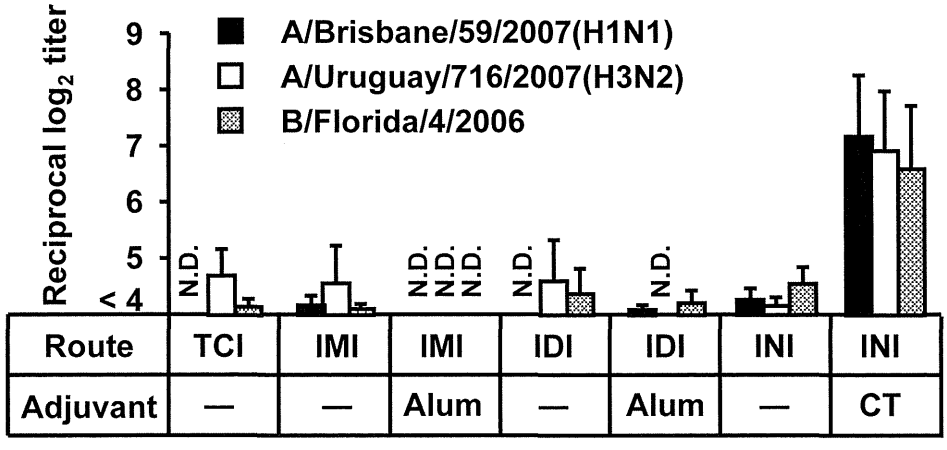
Nasal washes



Saliva



Vaginal washes



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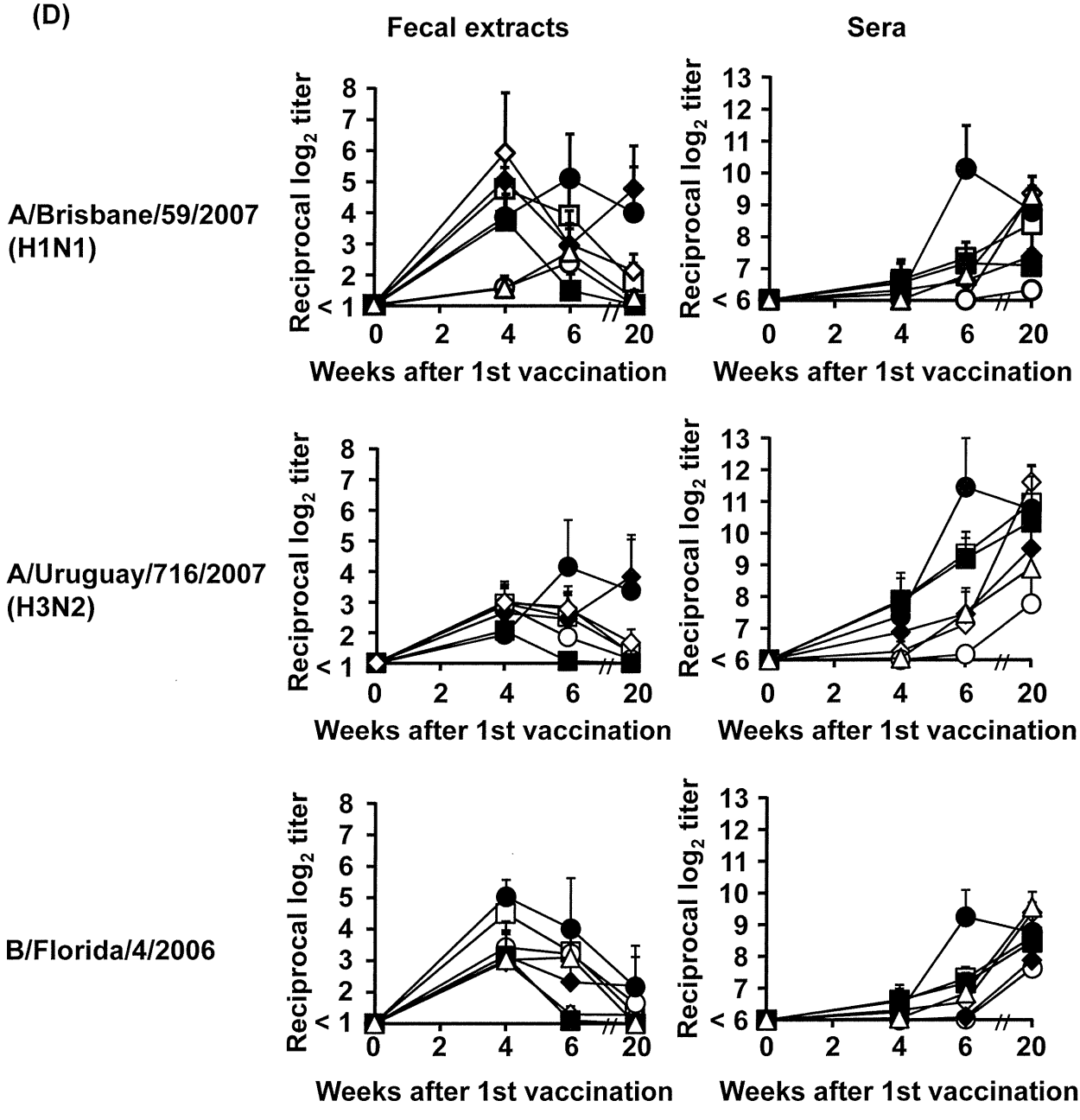
(D)


Fecal extracts
Sera
A/Brisbane/59/2007 (H1N1)
A/Uruguay/716/2007 (H3N2)
B/Florida/4/2006
Reciprocal log2 titer
< 1
< 6
0 2 4 6 20
Weeks after 1st vaccination

(continued)

(E)

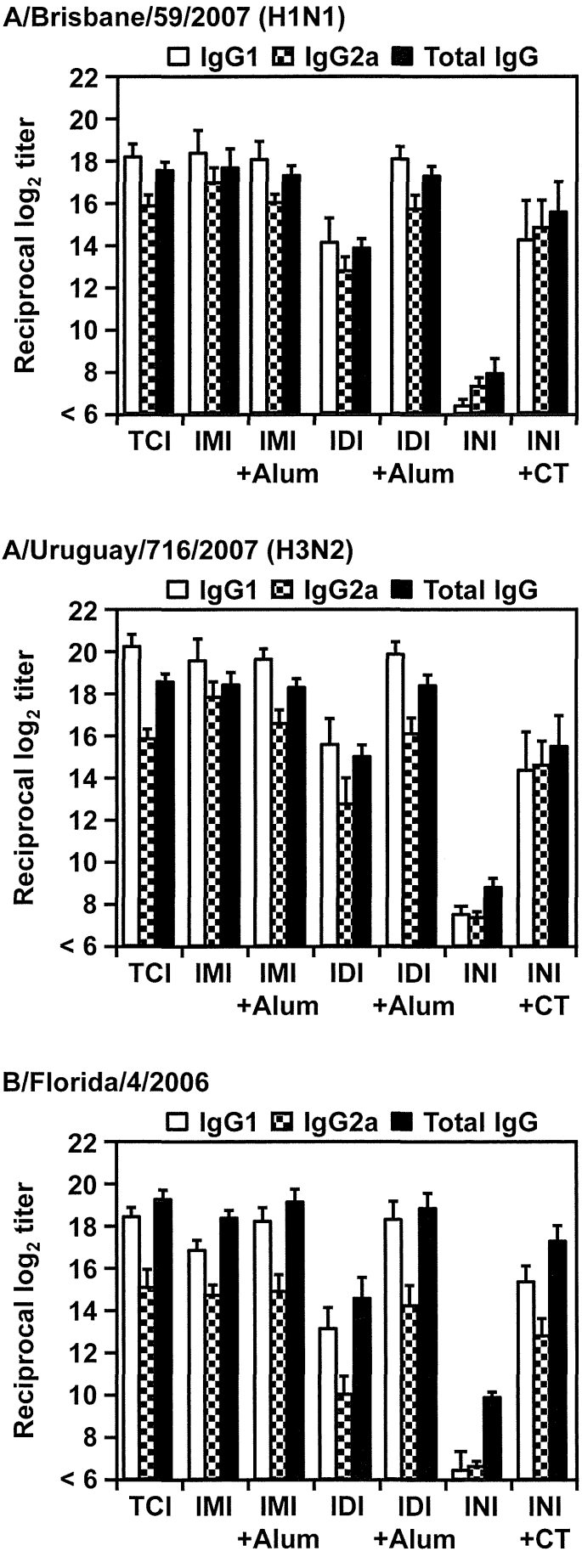


Fig. 33 Anti-HA immune responses in BALB/c mice after TCI. BALB/c mice were transcutaneously vaccinated with trivalent seasonal influenza HA antigens from [A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), and B/Florida/4/2006] (0.2 μg each) for 6 h twice at 4-week interval (△). Control groups were treated with intramuscular (□), intradermal (◇), or intranasal (○) application of HA antigens (0.2 μg each) twice at 4-week interval. Another control group was treated with intramuscular injection of HA antigens (0.2 μg each) 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 interval. (A) At the indicated points, sera collected from these mice were assayed to determine the HA-specific IgG titer by ELISA. (B) 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. (C) Sixteen weeks after the final treatment, nasal washes, saliva, and vaginal washes collected from these mice were assayed to determine the HA-specific IgA titer by ELISA. (D) At the indicated points, fecal extracts and sera collected from these mice were assayed to determine the HA-specific IgA titer by ELISA. (E) Sera collected 2 weeks after the last treatment were assayed for HA-specific IgG subclass (IgG1 and IgG2a) by ELISA. Data are expressed as mean ± SE of results from 5-7 mice. TCI; transcutaneous immunization, IMI; intramuscular immunization, IDI; intradermal immunization, INI; intranasal immunization, N.D.; not detectable.