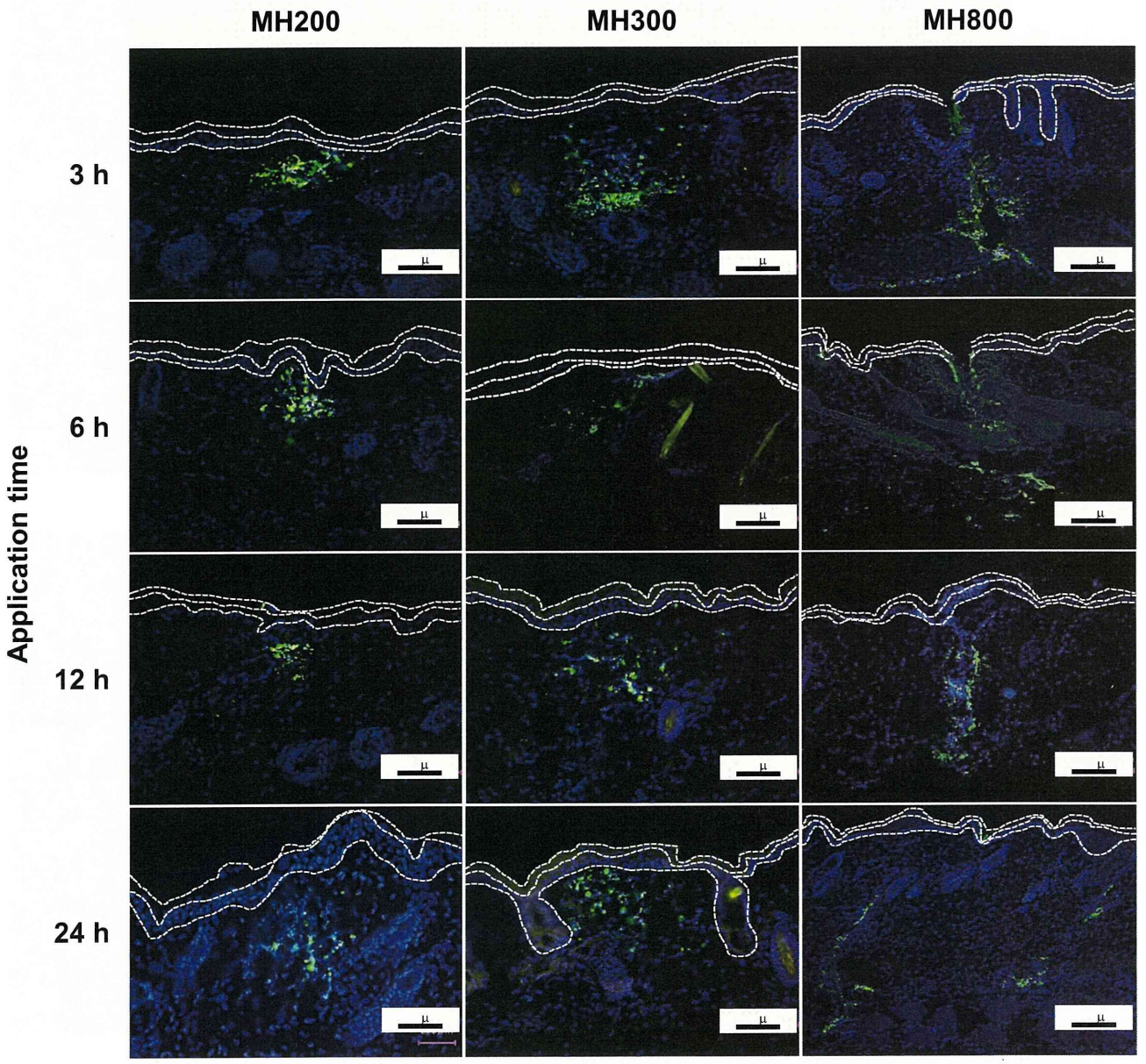

Fig. 8 Skin sections from ICR mice applied with each MH encapsulating FITC-OVA or FITC-SP for several hours. MH200, MH300, or MH800 containing FITC-OVA were placed on the back skin of ICR mice for 3, 6, 12, 24 h. The skin was harvested and frozen. Frozen section (8-μm thick) were photographed under a fluorescence microscope.

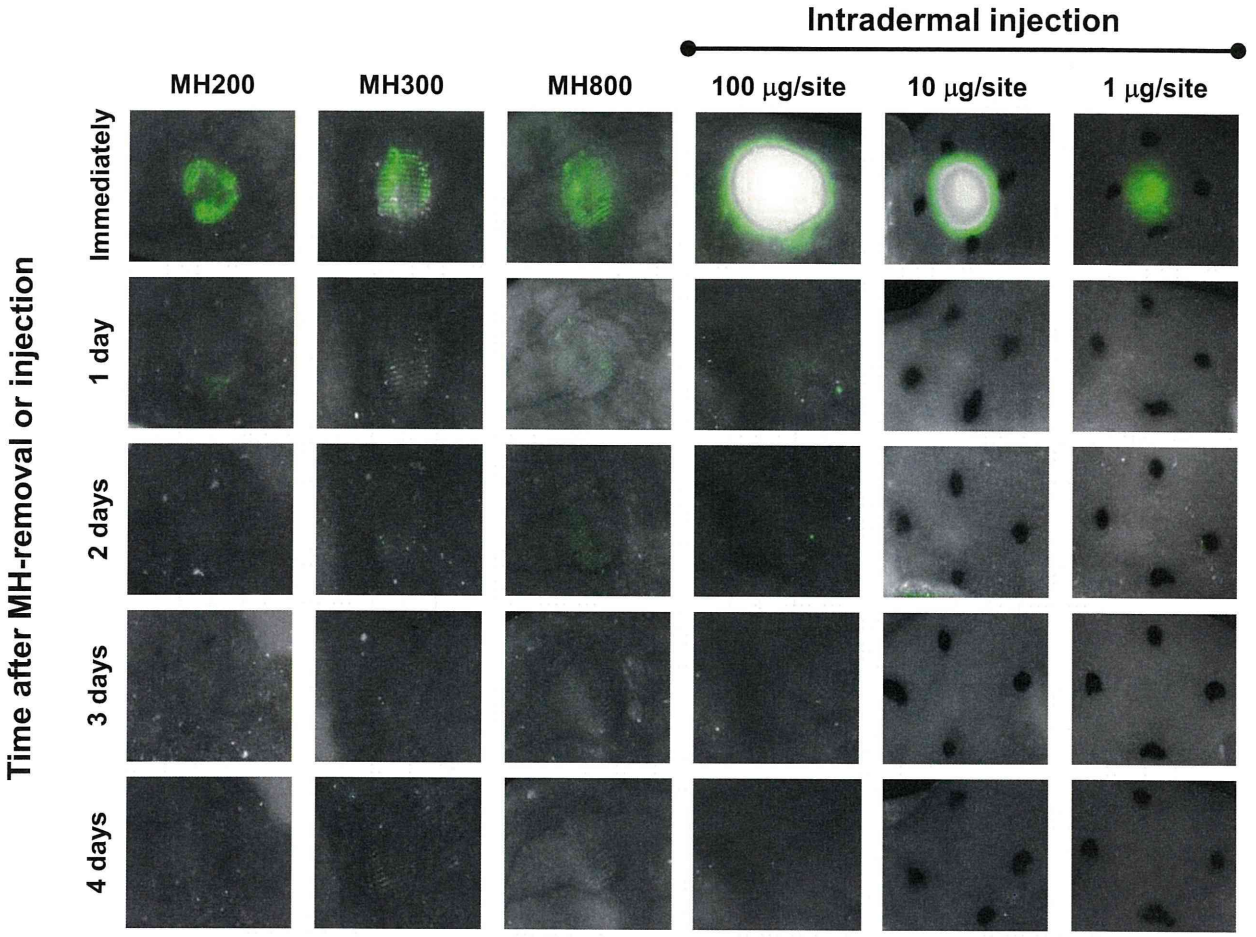

Fig. 9 Fluorescent images of mouse skin applied with MH containing FD4. ICR mice were applied with MH200, MH300, or MH800 containing FD4 on back skin for 1 h. Control groups were intradermally injected with FD4 at 1, 10, or 100 μg/site. At the indicated time, the MH-applied sites or the injected sites were photographed by using a fluorescence imager (Maestro EX) with 455-490 nm blue filter.

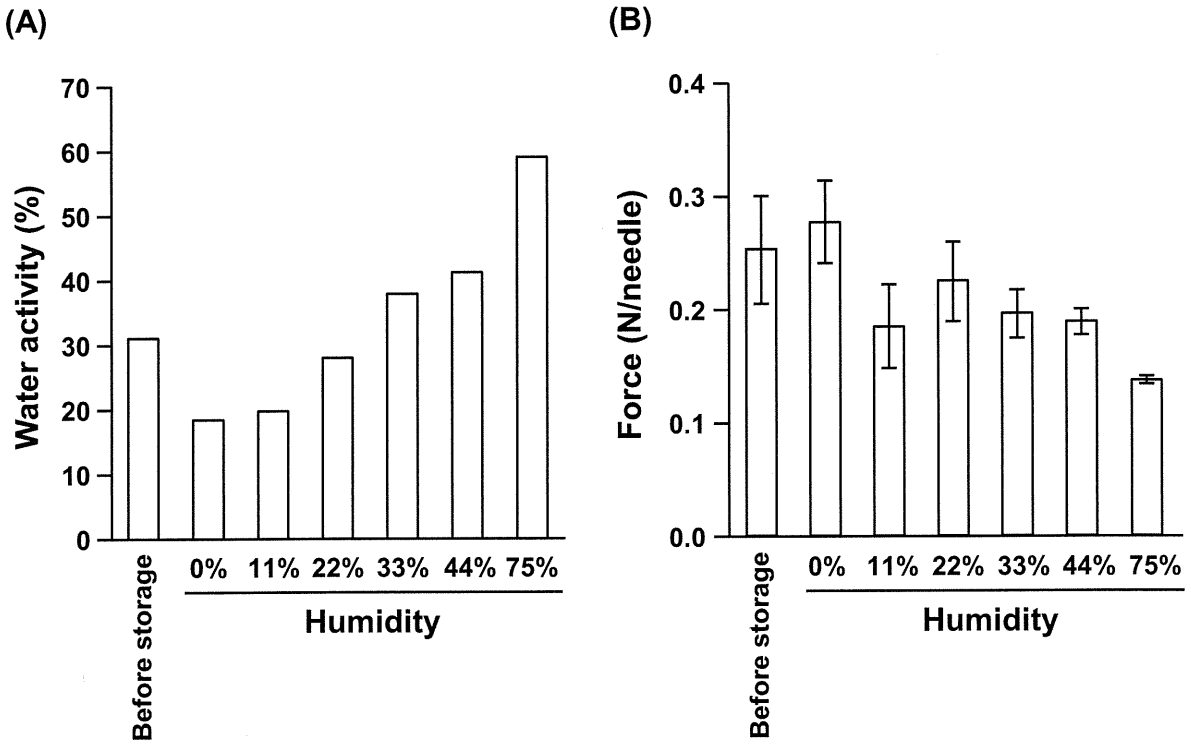

Fig. 10 Water activity and mechanical strength of MH800 after storage under various humidity conditions. MH800 were placed under 0, 11, 22, 33, 44, or 75% humidity conditions for 1 week. The water activity of them were measured by using a water activity measuring system, and the force required to fracture 40 microneedles of them were measured by using a texture analyzer.

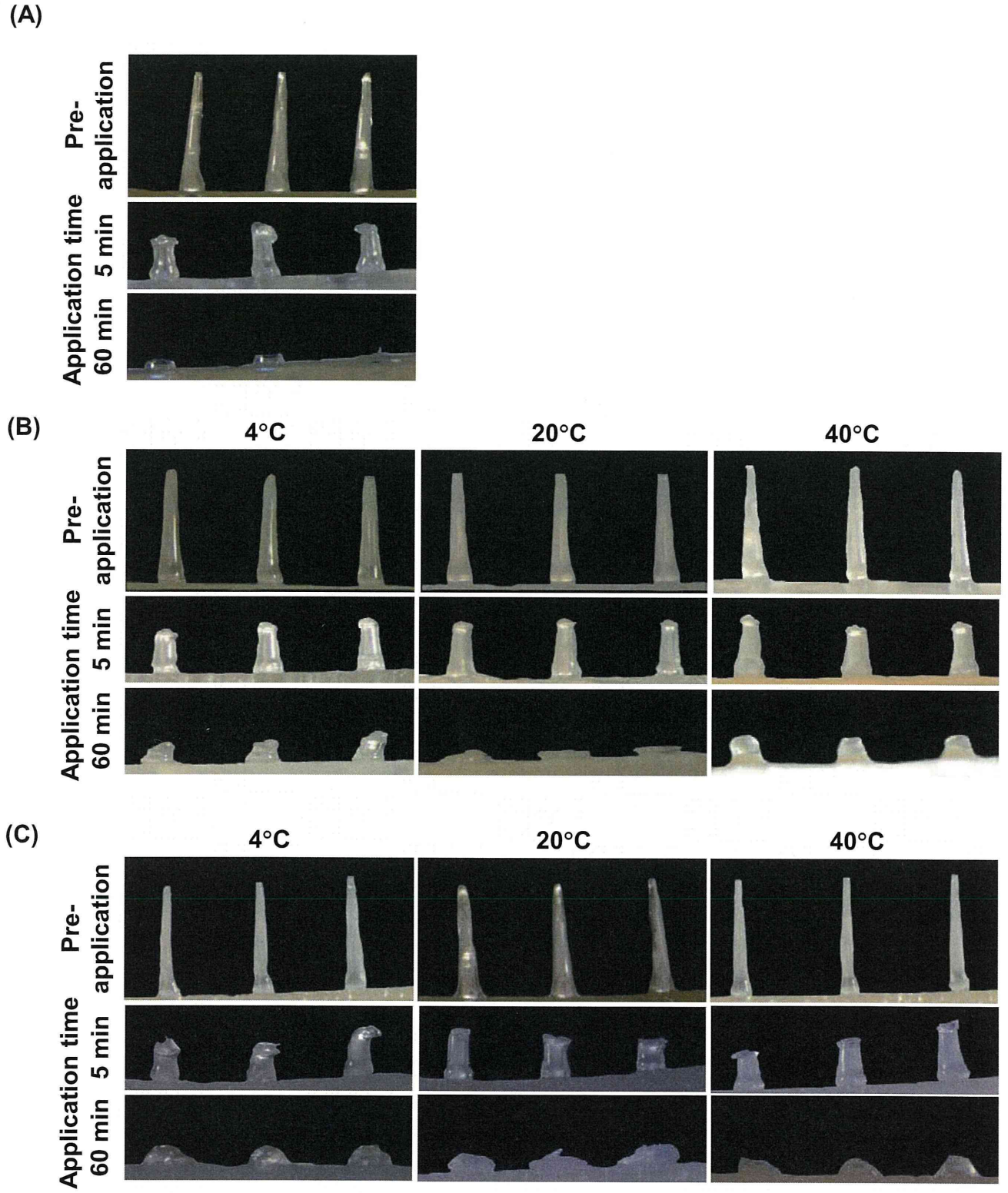

Fig. 11 Dissolution kinetics of MH800 before (A) or after long-term storage in various temperatures for 6 (B) or 12 months (C). MH800 containing TT and DT were stored at 4, 25, or 40°C for 6 or 12 months. Then, these MH800 were applied on the back skin of Wistar ST rats for the indicated time. After removal of the MH800, the microneedles remaining on each MH800 were photographed under a stereoscopic microscope.

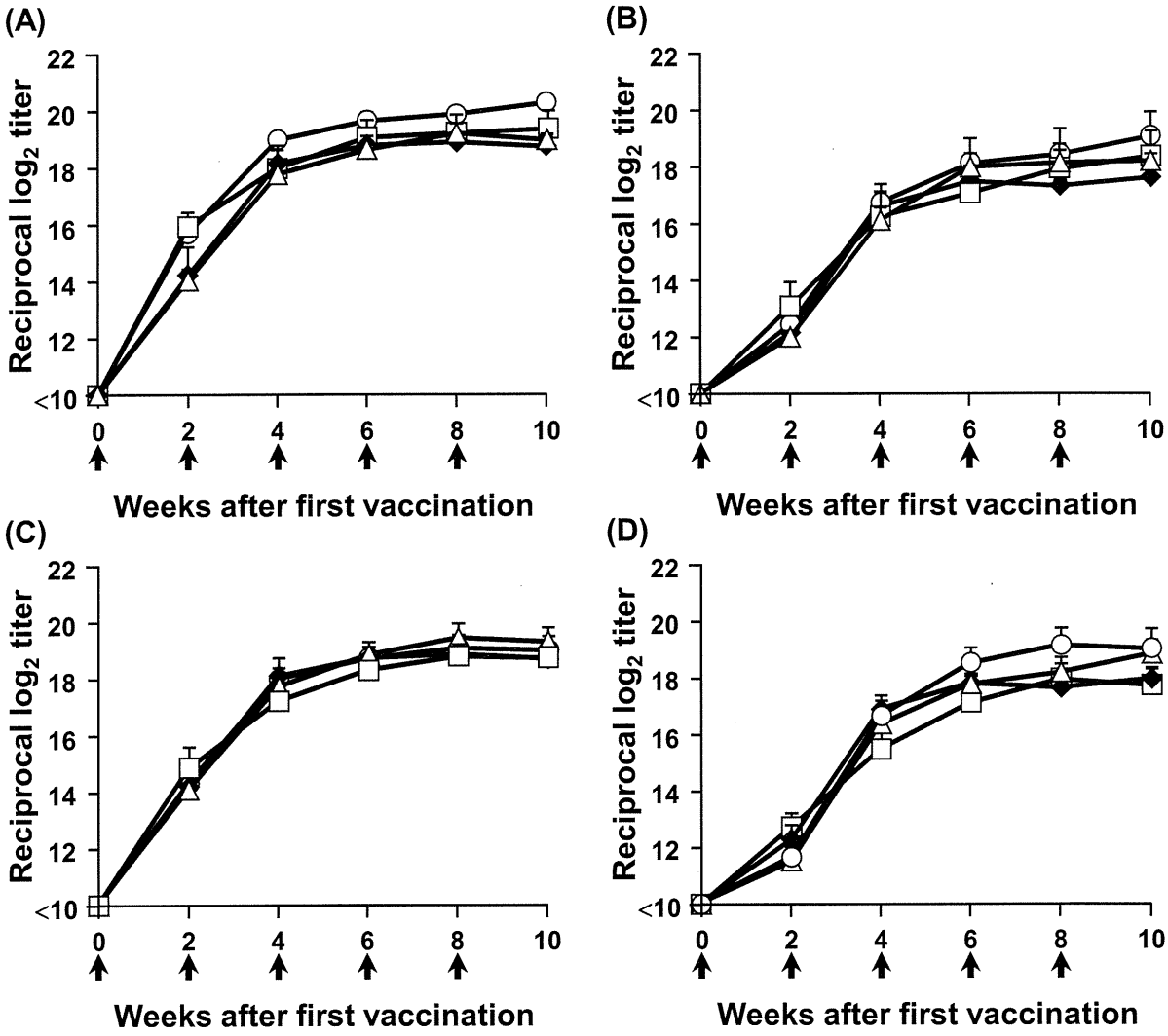

Fig. 12 OVA-specific antibody titers in rats immunized transcutaneously with MH800 which were stored for a long-term under various conditions. MH800 containing TT (20 μg) and DT (10 μg) were stored at 4°C (○), 25°C (□), or 40°C (△) for 6 (A, B) or 12 months (C, D), and then were applied to back skin of Wistar ST rats five times at 2-week intervals. As a control, TT/DT-containing MH800 just after production (◆) were applied at the same manner. At the indicated points, serum collected from these rats were assayed for the IgG titer specific for TT (A, C) or DT (B, D) by ELISA. Data are expressed as mean ± SE of results from 5 rats. Arrows indicate the vaccination point.

Table 7 Tetanus toxin challenge

Storage condition of TT/DT-containing MH800	No. of survival mice/No. of tested mice
4°C, 6 months	5/5
25°C, 6 months	5/5
40°C, 6 months	5/5
4°C, 12 months	5/5
25°C, 12 months	5/5
40°C, 12 months	5/5
Unvaccinated	0/5

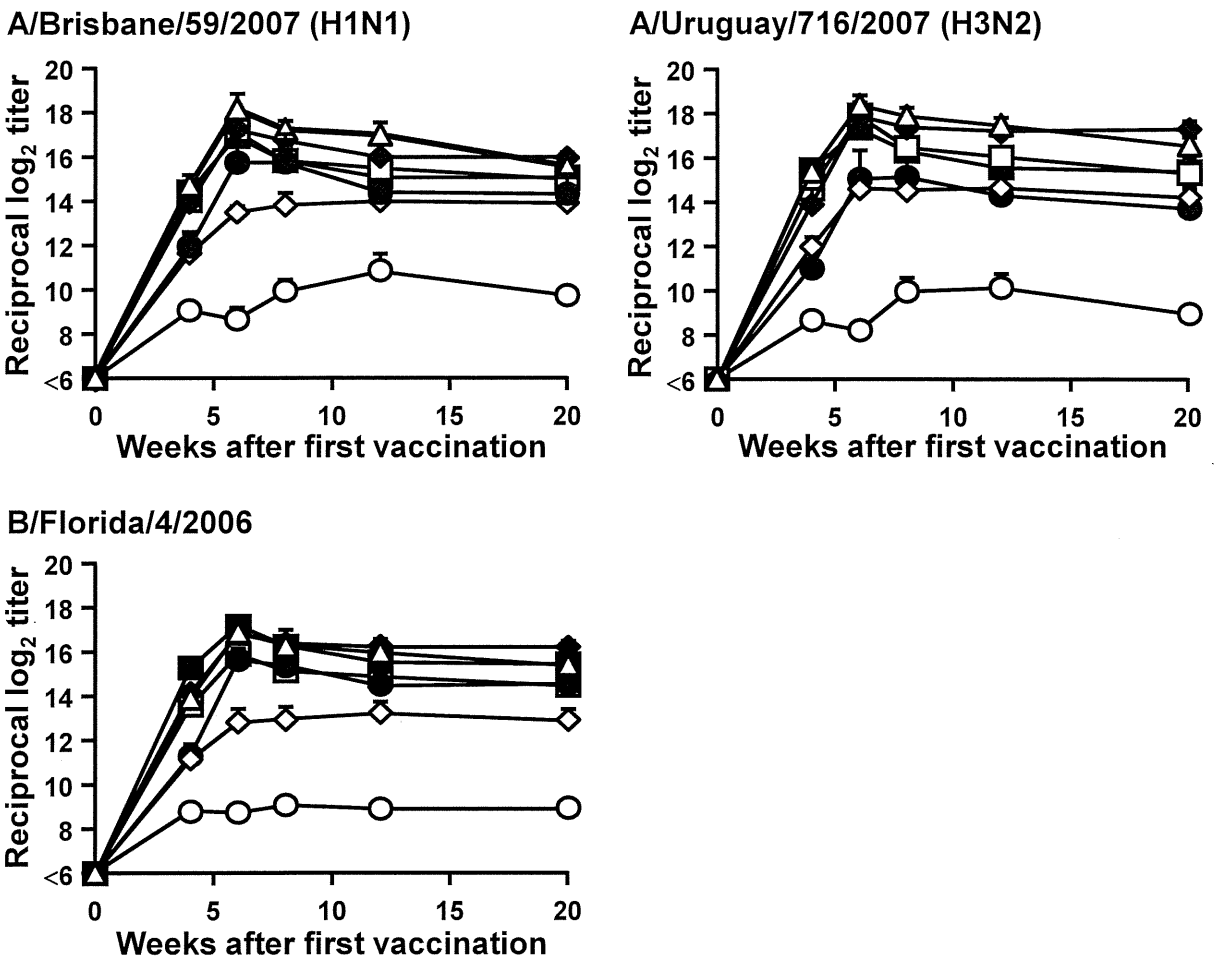

Fig. 13 Anti-HA IgG responses in BALB/c mice after transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with trivalent seasonal influenza HA antigens [A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), and B/Florida/4/2006] (each 0.2 μg) for 6 h twice at 4-week intervals (△). Control groups were intramuscularly with HA antigens (each 0.2 μg) (□), intradermally with HA antigens (each 0.2 μg) (◇) or intranasally immunized with HA antigens (each 0.2 μg) (○) twice at 4-week intervals. Another control group was intramuscularly with HA antigens (each 0.2 μg) with Alum (100 μg) (■), intradermally with HA antigens (each 0.2 μg) combined with Alum (100 μg) (◆) or intranasally immunized with HA antigens (each 0.2 μg) with CT (10 μg) (●) twice at 4-week intervals. At several points, sera collected from these mice were assayed to determined HA-specific IgG titer by ELISA. Data are expressed as mean ± SE of results from 5-7 mice.

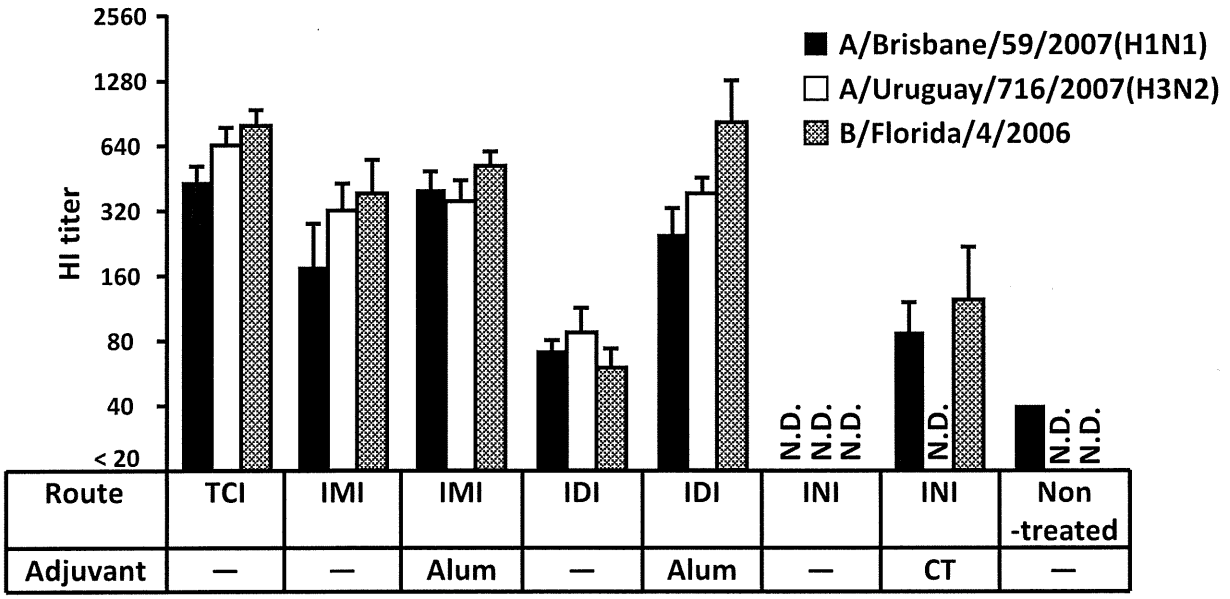

Fig. 14 HI activity of sera from BALB/c mice after transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with trivalent seasonal influenza HA antigens [A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), and B/Florida/4/2006] (each 0.2 μg) for 6 h twice at 4-week intervals. Control groups were intramuscularly with HA antigens (each 0.2 μg) , intradermally with HA antigens (each 0.2 μg) or intranasally immunized with HA antigens (each 0.2 μg) twice at 4-week intervals. Other control groups were intramuscularly with HA antigens (each 0.2 μg) combined with Alum (100 μg), intradermally with HA antigens (each 0.2 μg) combined with Alum (100 μg) or intranasally immunized with HA antigens (each 0.2 μg) with CT (10 μg) twice at 4-week intervals. Two weeks after final vaccination, sera collected from these mice were assayed. HI activity expressed as the highest dilution that resulted in complete inhibition of hemagglutination. Data are expressed as mean ± SE of results from 5-7 mice. TCI; transcutaneous immunization, IMI; intramuscular immunization, IDI; intradermal immunization, INI; intranasal immunization, N.D.; not detectable.

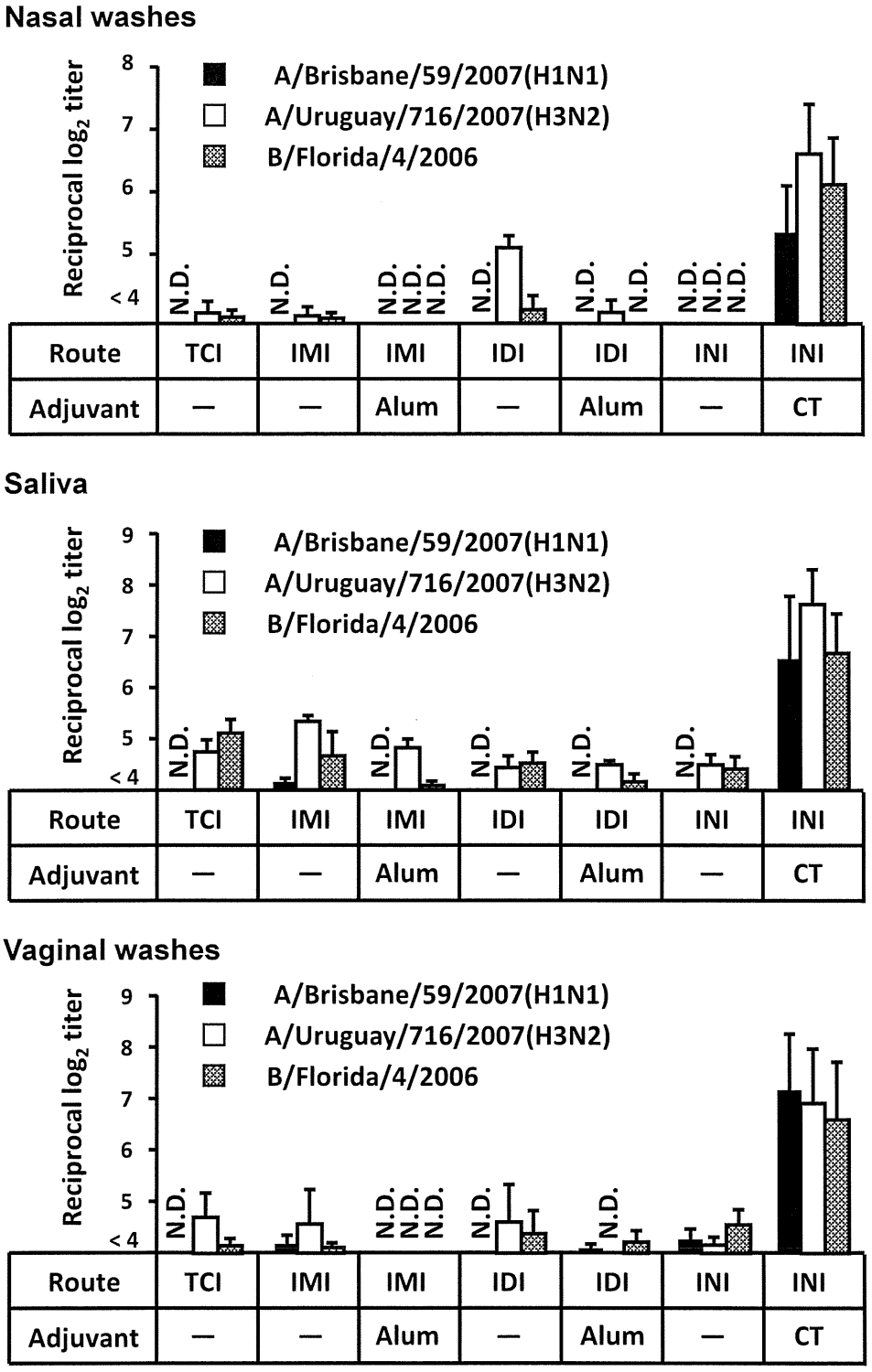

Fig. 15 Anti-HA IgA responses in BALB/c mice 16 weeks after final transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with trivalent seasonal influenza HA antigens [A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), and B/Florida/4/2006) (each 0.2 μg) for 6 h twice at 4-week intervals. Control groups were intramuscularly with HA antigens (each 0.2 μg) , intradermally with HA antigens (each 0.2 μg) or intranasally immunized with HA antigens (each 0.2 μg) twice at 4-week intervals. Other control groups were intramuscularly with HA antigens (each 0.2 μg) combined with Alum (100 μg) , intradermally with HA antigens (each 0.2 μg) combined with Alum (100 μg) or intranasally immunized with HA antigens (each 0.2 μg) with CT (10 μg) twice at 4-week intervals. Sixteen weeks after final vaccination, nasal washes, saliva and vaginal washes were collected from these mice were assayed to determined HA-specific IgA titer by ELISA. Data are expressed as mean ± SE of results from 5-7 mice.

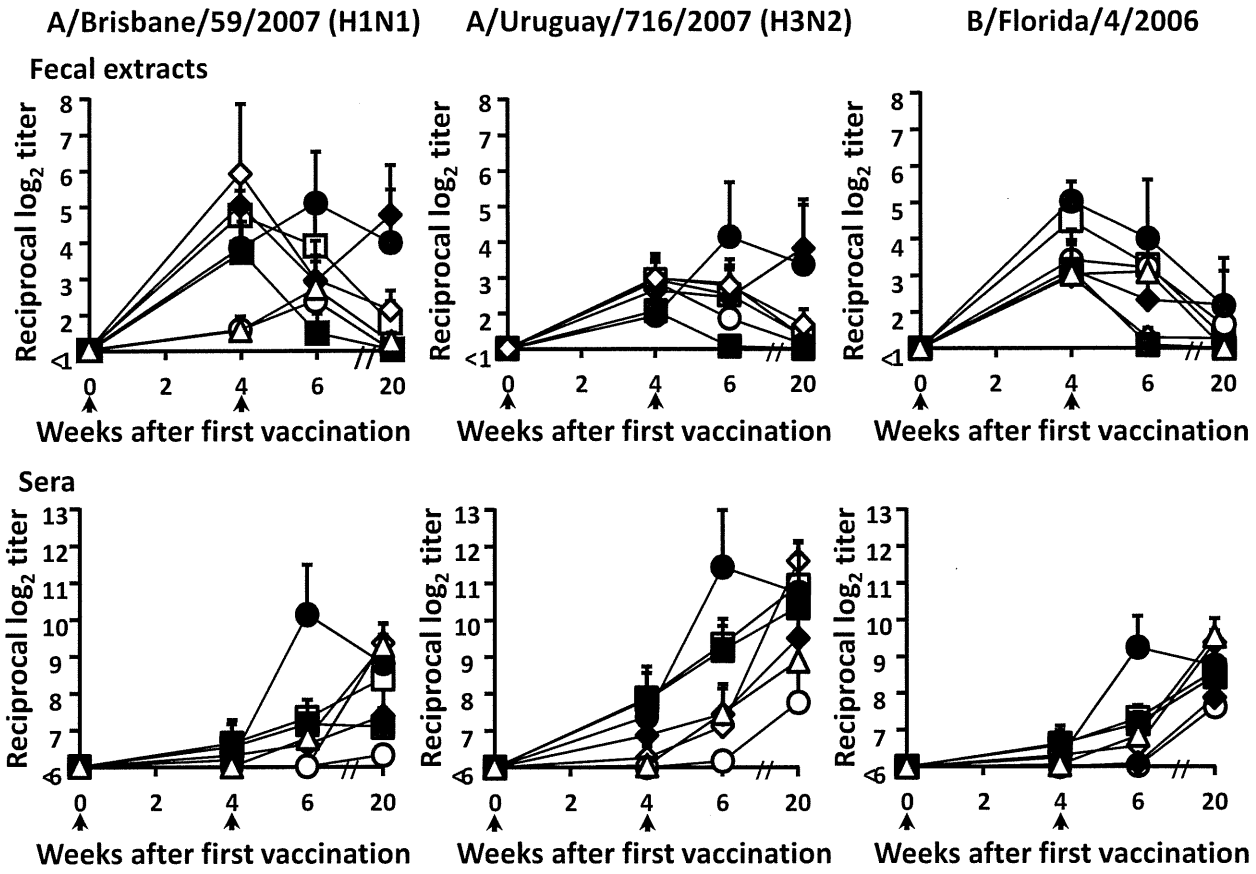

Fig. 16 Anti-HA IgA responses in BALB/c mice at several points after transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with trivalent seasonal influenza HA antigens [A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), and B/Florida/4/2006] (each 0.2 μg) (△) for 6 h twice at 4-week intervals. Control groups were intramuscularly with HA antigens (each 0.2 μg) (□) , intradermally with HA antigens (each 0.2 μg) (◇) or intranasally immunized with HA antigens (each 0.2 μg) (○) twice at 4-week intervals. Other control groups were intramuscularly with HA antigens (each 0.2 μg) combined with Alum (100 μg) (■), intradermally with HA antigens (each 0.2 μg) combined with Alum (100 μg) (◆) or intranasally immunized with HA antigens (each 0.2 μg) with CT (10 μg) (●) twice at 4-week intervals. At several points, fecal extracts and sera collected from these mice were assayed to determined HA-specific IgA titer by ELISA. Data are expressed as mean ± SE of results from 5-7 mice.

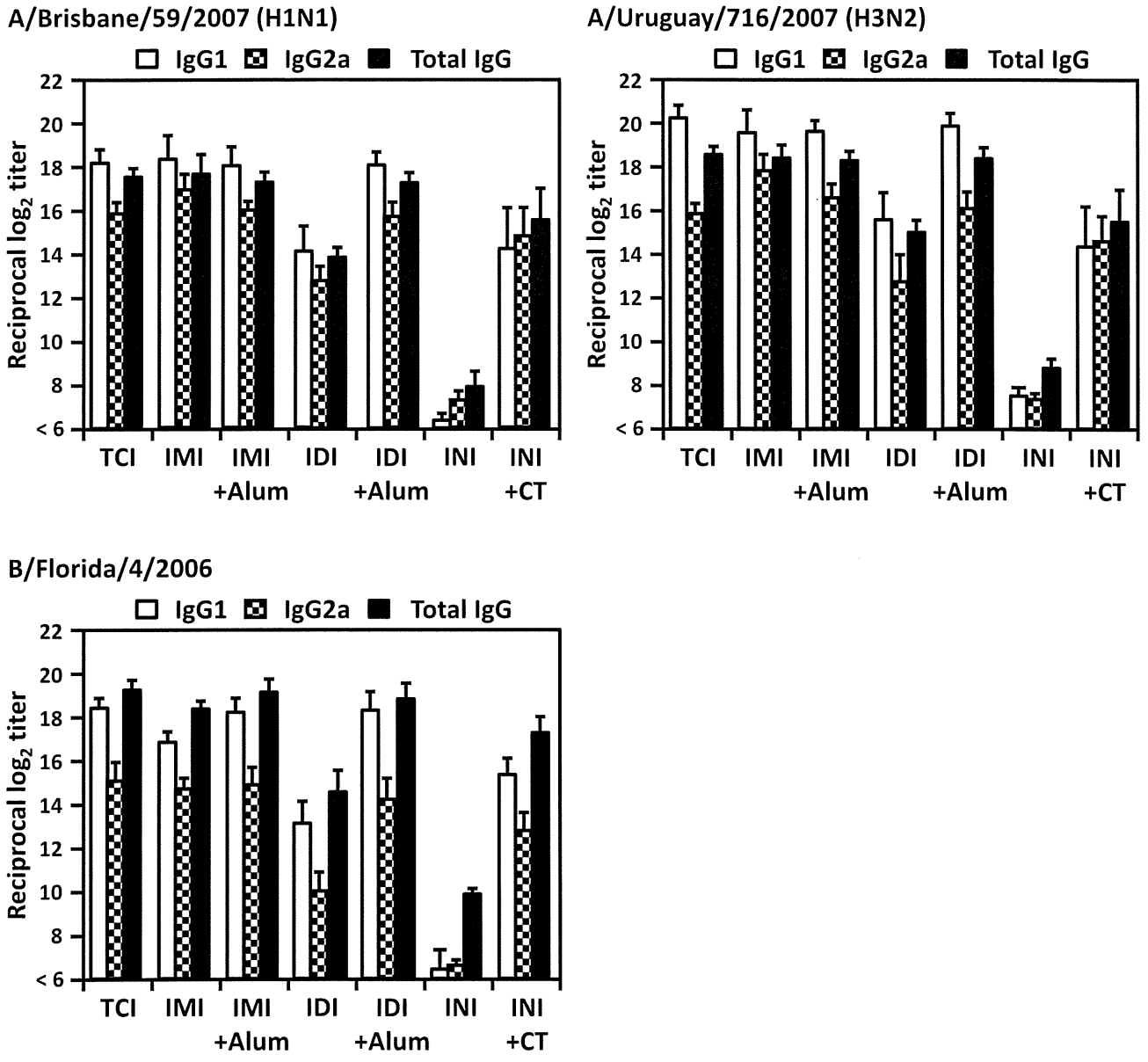

Fig. 17 Analysis of HA-specific IgG subclass. BALB/c mice were transcutaneously vaccinated with trivalent seasonal influenza HA antigens [(A) A/Brisbane/59/2007 (H1N1), (B) A/Uruguay/716/2007 (H3N2), and (C) B/Florida/4/2006] (each 0.2 μg) for 6 h twice at 4-week intervals. Control groups were intramuscularly with HA antigens (each 0.2 μg) , intradermally with HA antigens (each 0.2 μg) or intranasally immunized with HA antigens (each 0.2 μg) twice at 4-week intervals. Other control groups were intramuscularly with HA antigens (each 0.2 μg) combined with Alum (100 μg), intradermally with HA antigens (each 0.2 μg) combined with Alum (100 μg) or intranasally immunized with HA antigens (each 0.2 μg) with CT (10 μg) twice at 4-week intervals. Two weeks after the final vaccination, sera collected from these mice were assayed for HA-specific IgG subclass (IgG1, IgG2a) titer by ELISA. Data are expressed as mean ± SE of results from 5-7 mice.

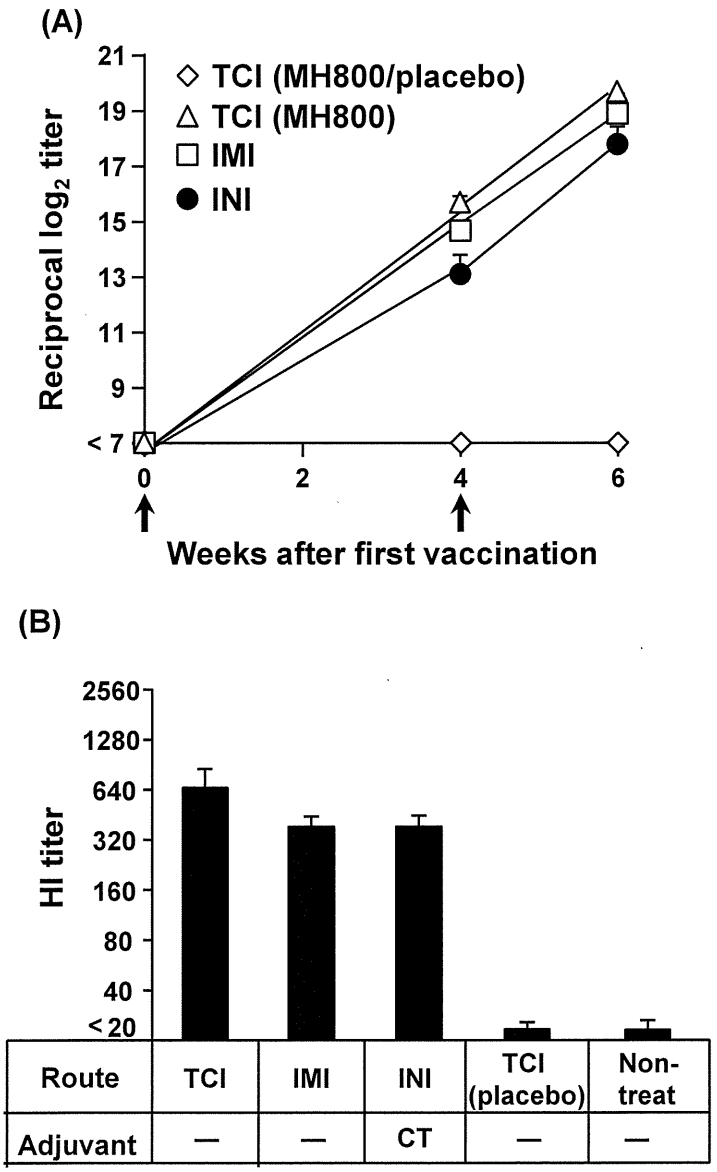

Fig. 18 Anti-HA IgG titer and HI activity of sera from BALB/c mice after transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 μg) for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 μg), or intranasal application of HA (0.4 μg) combined with CT (10 mg) twice at 4-week intervals. At the several points, sera collected from these mice were assayed for the HA-specific IgG titer by ELISA. Two weeks after final vaccination, sera collected from these mice were assayed for the HI titer. HI activity expressed as the highest dilution that resulted in complete inhibition of hemagglutination. Data are expressed as mean ± SE of results from 13 mice.

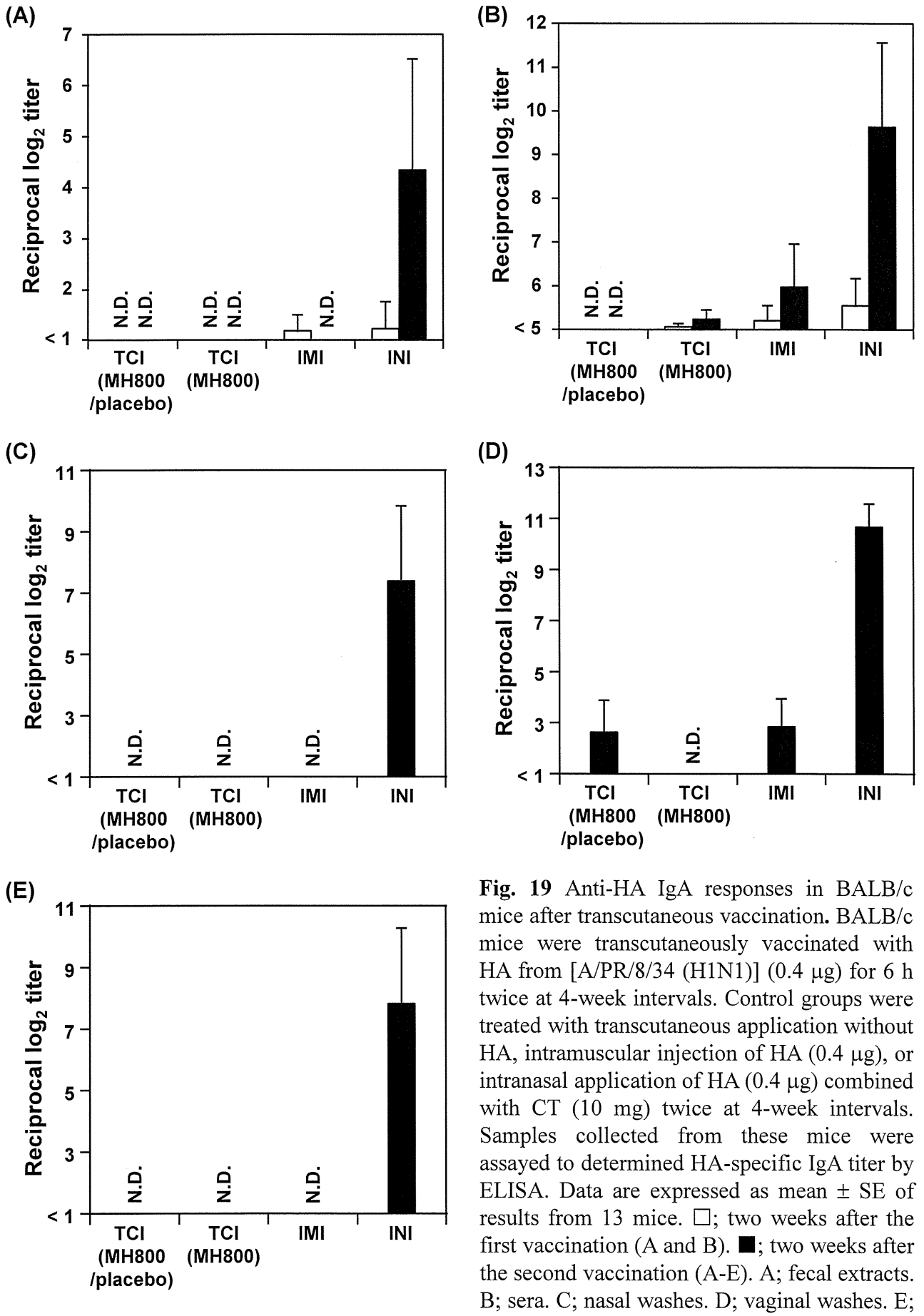

Fig. 19 Anti-HA IgA responses in BALB/c mice after transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 μg) for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 μg), or intranasal application of HA (0.4 μg) combined with CT (10 mg) twice at 4-week intervals. Samples collected from these mice were assayed to determined HA-specific IgA titer by ELISA. Data are expressed as mean ± SE of results from 13 mice. □; two weeks after the first vaccination (A and B). ■; two weeks after the second vaccination (A-E). A; fecal extracts. B; sera. C; nasal washes. D; vaginal washes. E; saliva. N.D.; not detectable.

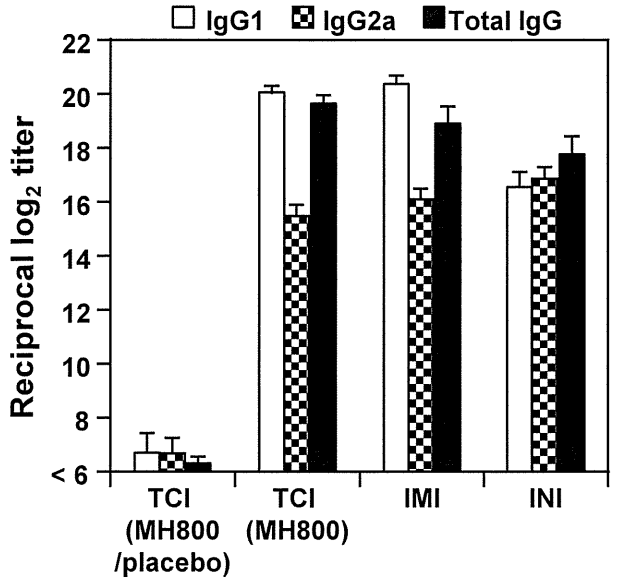

Fig. 20 Analysis of HA-specific IgG subclass. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 μg) for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 μg), or intranasal application of HA (0.4 μg) combined with CT (10 mg) twice at 4-week intervals. Two weeks after the final vaccination, sera collected from these mice were assayed for HA-specific IgG subclass (IgG1, IgG2a) titer by ELISA. Data are expressed as mean ± SE of results from 13 mice.

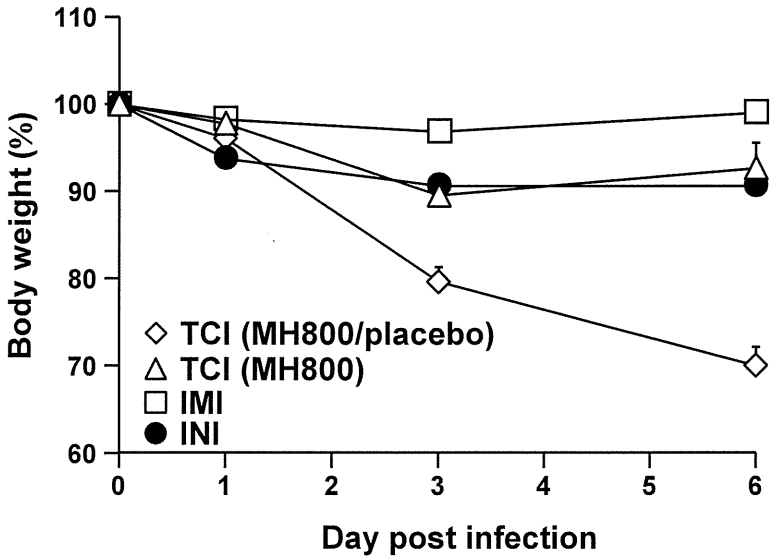

Fig. 21 Weight loss after influenza infection. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 μg) for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 μg), or intranasal application of HA (0.4 μg) combined with CT (10 mg) twice at 4-week intervals. Then, These mice were each infected intranasally with 6×10^5 PFU of the A/PR/8/34(H1N1) virus. Body weight was measured at each day and presented as percent of original weight before infection (day 0). Data are expressed as mean ± SE of results from 10 mice.

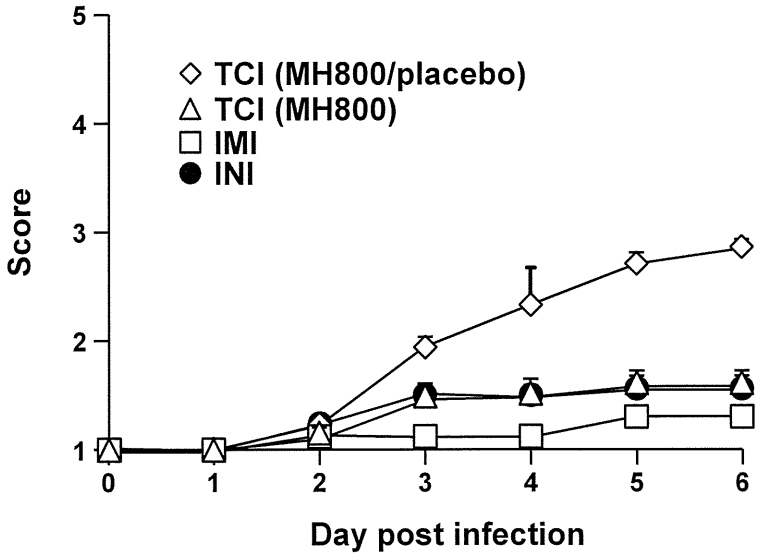

Fig. 22 Assessment of performance status after influenza infection. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 μg) for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 μg), or intranasal application of HA (0.4 μg) combined with CT (10 mg) twice at 4-week intervals. These mice were each infected intranasally with 6×10^5 PFU of the A/PR/8/34(H1N1) virus. The degree of performance status of mice was scored at each day. Data are expressed as mean ± SE of results from 10 mice.

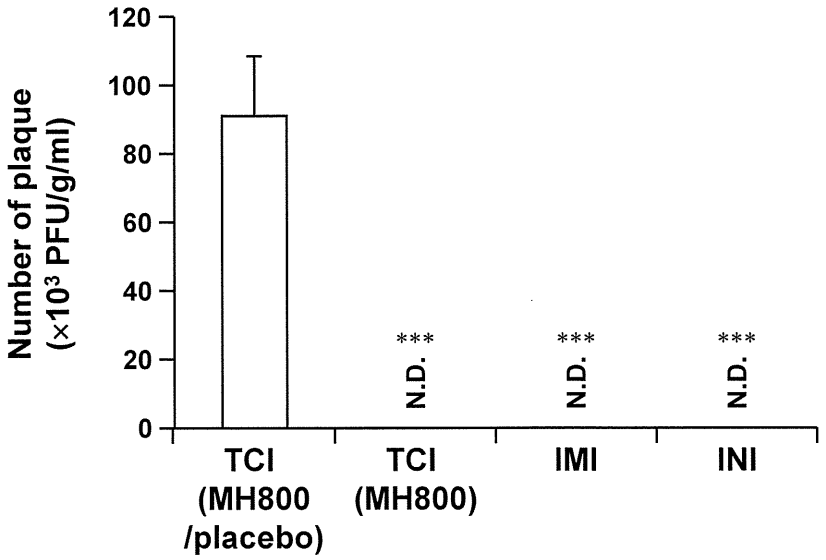

Fig. 23 Number of plaque in the lung. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 μg) for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 μg), or intranasal application of HA (0.4 μg) combined with CT (10 mg) twice at 4-week intervals. These mice were each infected intranasally with 6×10^5 PFU of the A/PR/8/34(H1N1) virus. Six days after infection, lung was collected from these mice and number of virus in lung homogenate was determined using a plaque assay system. Data are expressed as mean ± SE of results from 10 mice. N.D.; not detectable. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for nonparametric multiple comparison. :* * *; $p<0.001$ versus TCI (MH800/placebo) group.

Table 8 Lung weight

Vaccination	Lung weight (g; Mean ± SE)	
	Left lobe	Right lobe
TCI (placebo)	0.154 ± 0.007	0.243 ± 0.022
TCI	0.068 ± 0.007	0.142 ± 0.013
IMI	0.059 ± 0.003	0.123 ± 0.005
INI	0.057 ± 0.002	0.117 ± 0.003

Table 9 The degree of consolidation of lung

Vaccination	Consolidation (/10 tested mice)				Score (Mean ± SE) [a]	
	Left lobe	Right lobe	Right and left lobe	No	Left lobe	Right lobe
TCI (placebo)	1	0	9	0	4.2 ± 0.2	3.4 ± 0.5
TCI	0	1	0	9	1.0 ± 0.0	1.1 ± 0.1
IMI	0	0	0	10	1.0 ± 0.0	1.0 ± 0.0
INI	0	0	0	10	1.0 ± 0.0	1.0 ± 0.0

[a] Degree of consolidation: 1; no consolidation, 2; ≤1/3 lobe, 3; >1/3 to ≤1/2 lobe, 4; >1/2 to ≤ 2/3 lobe, 5; >2/3 lobe.

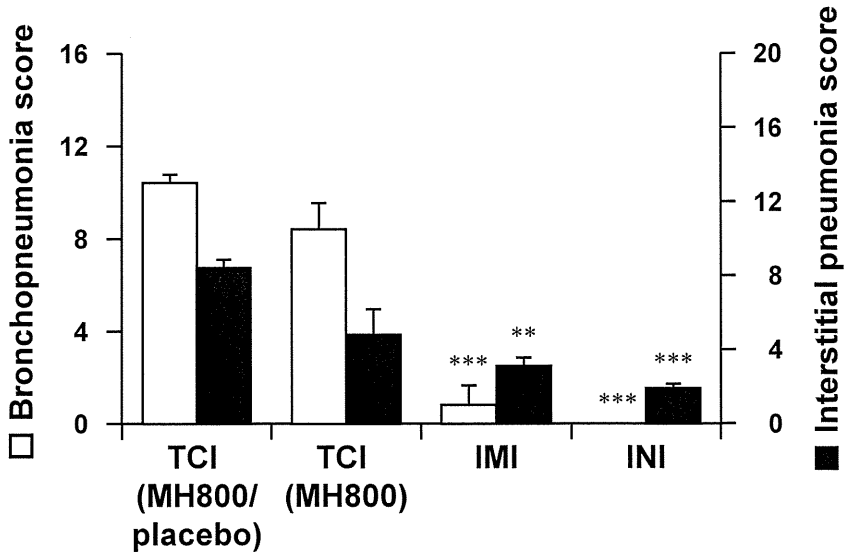

Fig. 24 Assessment of inflammation of lung after influenza infection. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 μg) for 6 h twice at 4-week intervals. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 μg), or intranasal application of HA (0.4 μg) combined with CT (10 mg) twice at 4-week intervals. These mice were each infected intranasally with 6×10^5 PFU of the A/PR/8/34(H1N1) virus. The degree of inflammation of lung was scored. 0; no inflammation, 1; very slight, 2; well-defined, 3; moderate, and 5; severe. Pathological findings were classified into bronchopneumonia and interstitial pneumonia. Bronchopneumonia; hypertrophy of bronchus mucous epithelium, hyperplasia of bronchus mucous epithelium, denaturation or necrosis of bronchus mucous epithelium and infiltration with mononuclear cells and polymorphonuclear neutrophil leukocyte of bronchial submucosa. Interstitial pneumonia; focal atelectatic lung or alveolus of the lung, focal hydrops or alveolus of the lung, focal bleed or alveolus of the lung, infiltration with mononuclear cells and polymorphonuclear neutrophil leukocyte of alveolar septum and infiltration with mononuclear cells and polymorphonuclear neutrophil leukocyte of alveolus of the lung. Data are expressed as mean ± SE of results from 10 mice. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's test for nonparametric multiple comparison. :**; $p<0.01$, ***; $p<0.001$ versus TCI (MH800/placebo) group.

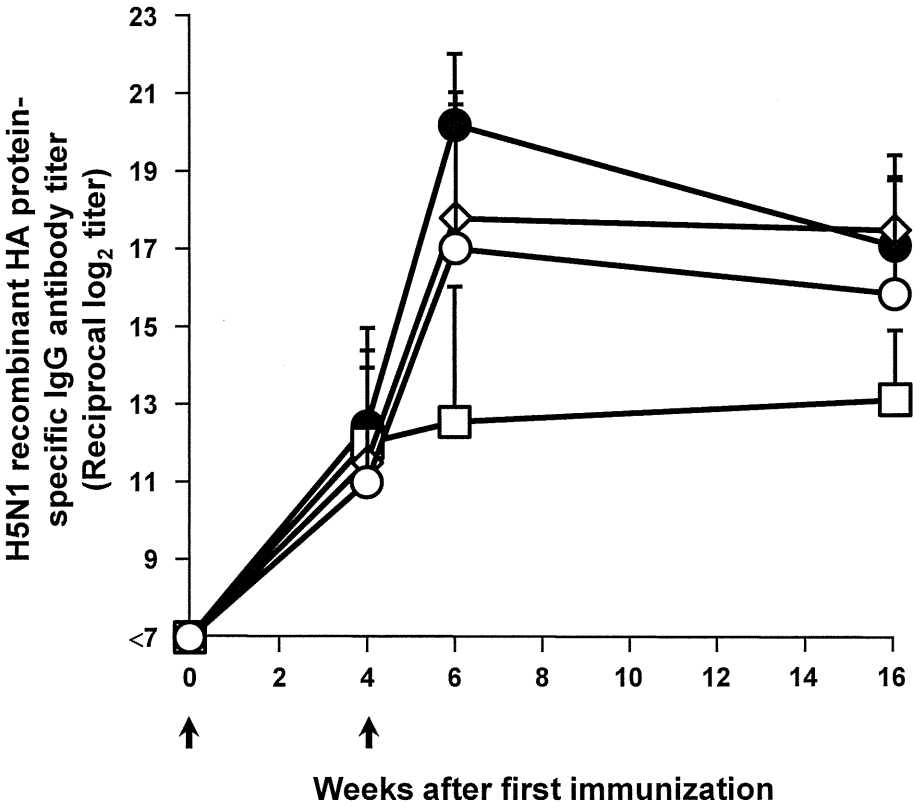

Fig. 25 Anti-HA IgG responses in BALB/c mice after transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with 0.05 μg H5N1 recombinant HA protein using by MH800 (◇) or MH300 (□) for 6 h twice at 4-week intervals. Control groups were intramuscularly vaccinated with 0.05 μg H5N1 recombinant HA protein without (○) or with (●) 10 μg Alum twice at 4-week intervals. At several points, sera collected from these mice were assayed to determine HA-specific IgG titer by ELISA. Data are expressed as mean ± SE of results from 3 mice. Arrow; immunization point.

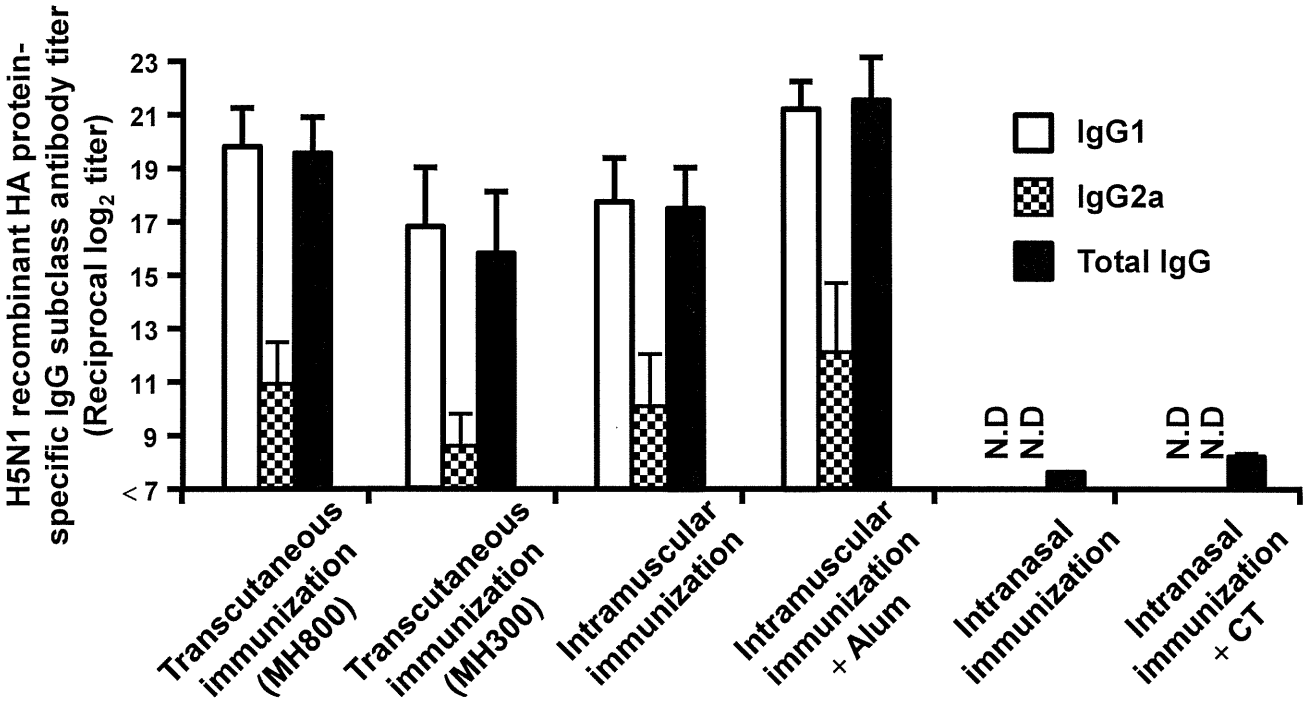

Fig. 26 Analysis of HA-specific IgG subclass. BALB/c mice were transcutaneously vaccinated with 0.05 μg H5N1 recombinant HA protein using by MH800 or MH300 for 6 h twice at 4-week intervals. Control groups were intramuscularly or intranasally vaccinated with 0.05 μg H5N1 recombinant HA protein. Other control groups were intramuscularly combined with 10 μg Alum or intranasally combined with 10 μg CT immunized with 0.05 μg H5N1 recombinant HA protein twice at 4-week intervals. Two weeks after final vaccination, sera collected from these mice were assayed for HA-specific IgG subclass (IgG1, IgG2a) titer by ELISA. Data are expressed as mean ± SE of results from 6 mice.

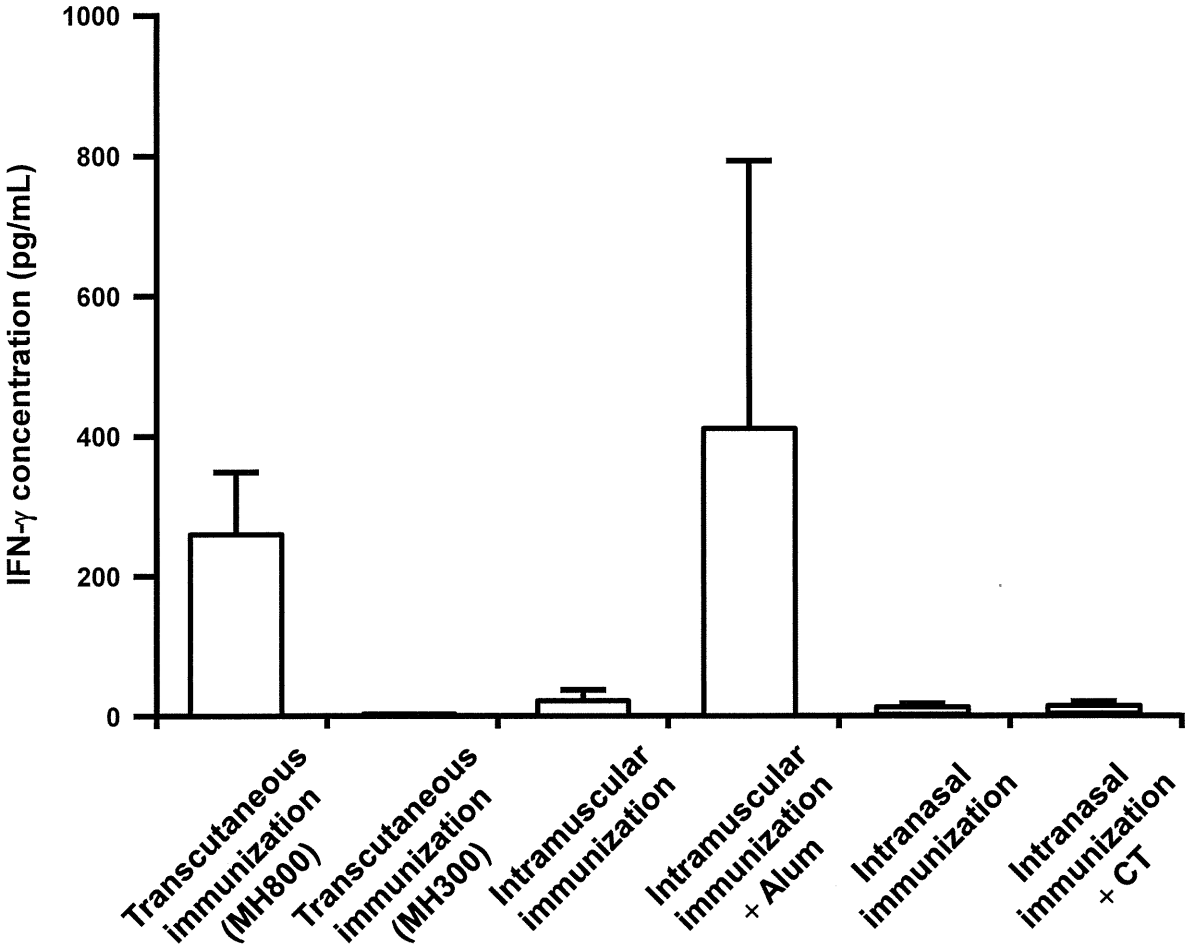

Fig. 27 Cytokine production in splenocytes of mice after transcutaneous vaccination. BALB/c mice were transcutaneously vaccinated with 0.05 μg H5N1 recombinant HA protein using by MH800 or MH300 for 6 h twice at 4-week intervals. Control groups were intramuscularly or intranasally vaccinated with 0.05 μg H5N1 recombinant HA protein. Other control groups were intramuscularly combined with 10 μg Alum or intranasally combined with 10 μg CT immunized with 0.05 μg H5N1 recombinant HA protein twice at 4-week intervals. Two weeks after final vaccination, single-cell suspensions of splenocytes were prepared and IFN-γ concentration was assayed by ELISA after stimulating the cells with 1 μg/mL H5N1 recombinant HA protein for 24 h. Data are expressed as mean ± SE of results from 3 mice.

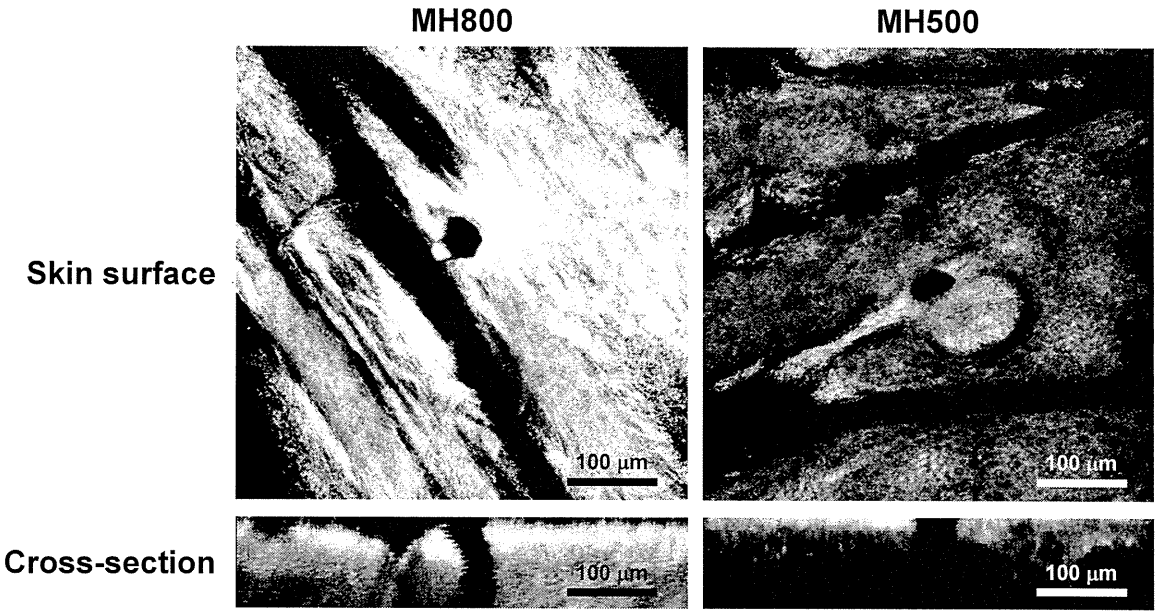

Fig. 28 In vivo confocal microscopic images after MH application on human skin. MH800 or MH500 was applied to the skin of left lateral upper arm of 2 healthy volunteers for 5 second, and then skin images were immediately photographed under in vivo confocal scanning laser microscope.