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Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines

Tomonori Nochi, Yoshikazu Yuki, Haruko Takahashi, Shin-ichi Sawada, Mio Mejima, Tomoko Kohda, Norihiro Harada, Il Gyu Kong, Ayuko Sato, Nobuhiro Kataoka, Daisuke Tokuhara, Shiho Kurokawa, Yuko Takahashi, Hideo Tsukada, Shunji Kozaki, Kazunari Akiyoshi and Hiroshi Kiyono

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The Airway Antigen Sampling System: Respiratory M Cells as an Alternative Gateway for Inhaled Antigens

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The Airway Antigen Sampling System: Respiratory M Cells as an Alternative Gateway for Inhaled Antigens

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In this study, we demonstrated a new airway Ag sampling site by analyzing tissue sections of the murine nasal passages. We revealed the presence of respiratory M cells, which had the ability to take up OVA and recombinant *Salmonella typhimurium* expressing GFP, in the turbinates covered with single-layer epithelium. These M cells were also capable of taking up respiratory pathogen group A *Streptococcus* after nasal challenge. Inhibitor of DNA binding/differentiation 2 (Id2)-deficient mice, which are deficient in lymphoid tissues, including nasopharynx-associated lymphoid tissue, had a similar frequency of M cell clusters in their nasal epithelia to that of their littermates, $Id2^{+/-}$ mice. The titers of Ag-specific Abs were as high in $Id2^{-/-}$ mice as in $Id2^{+/-}$ mice after nasal immunization with recombinant *Salmonella*-ToxC or group A *Streptococcus*, indicating that respiratory M cells were capable of sampling inhaled bacterial Ag to initiate an Ag-specific immune response. Taken together, these findings suggest that respiratory M cells act as a nasopharynx-associated lymphoid tissue-independent alternative gateway for Ag sampling and subsequent induction of Ag-specific immune responses in the upper respiratory tract. *The Journal of Immunology*, 2011, 186: 4253–4262.

The initiation of Ag-specific immune responses occurs at special gateways, M cells, which are located in the epithelium overlying MALT follicles such as nasopharynx-associated lymphoid tissue (NALT) and Peyer's patches (1). Peyer's patches contain all of the immunocompetent cells that are required for the generation of an immune response and are the key inductive tissues for the mucosal immune system. Peyer's patches are interconnected with effector tissues (e.g., the lamina propria of the intestine) for the induction of IgA immune responses specific to ingested Ags (2). NALT also contains all of the necessary lymphoid cells, including T cells, B cells, and APCs, for the induction and regulation of inhaled Ag-specific mucosal immune responses (1, 3). This tissue is rich in Th0-type $CD4^+$ T cells, which can become either Th1- or Th2-type cells (4). NALT is also equipped with the molecular and cellular environments for class-switch recombination of μ to α genes for the generation of IgA-committed B cells and the induction of memory B cells (5, 6). It is thus widely accepted that NALT M cells are key players in the uptake of nasally delivered Ags for the subsequent induction of Ag-specific IgA immune responses (1). As a result, NALT is considered a potent target for mucosal vaccines (1).

A recent study identified NALT-like structures of lymphocyte aggregates with follicle formation in the human nasal mucosa, especially in the middle turbinate of children <2 y old (7). Another recent study showed that, postinfection of mice with influenza via the upper respiratory tract, the levels of Ag-specific Ig observed in the serum and in nasal mucosal secretions after surgical removal of NALT were comparable to those in tissue-intact mice (8). Other studies have demonstrated that Ag-specific immune responses are induced in lymphotoxin-$\alpha^{-/-}$ and $CXCL13^{-/-}$ mice, in which the NALT exhibits structural and functional defects (9, 10). Thus, despite the central role of NALT in the generation of Ag-specific Th cells and IgA-committed B cells against inhaled Ags, these tissues do not appear essential for the induction of Ag-specific immune responses, suggesting that additional inductive sites and/or M cells are present in the upper respiratory tract.

The major goal of our study was to search for an NALT-independent M cell-operated gateway by examining and characterizing the entire nasal mucosa. We were able to identify M cells developed in the murine nasal passage epithelium as an alternative and NALT-independent gateway for the sampling of respiratory Ags and the subsequent induction of Ag-specific immune

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Abbreviations used in this article: DC, dendritic cell; dLN, draining lymph node; GAS, group A *Streptococcus*; GFP-*Salmonella*, GFP-expressing *Salmonella*; Id2, inhibitor of DNA binding/differentiation 2; NALT, nasopharynx-associated lymphoid tissue; *Salmonella*-GFP, *Salmonella typhimurium* expressing GFP; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TT, tetanus toxoid; UEA-1, *Ulex europaeus* agglutinin-1; WGA, wheat germ agglutinin.

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responses. Characterization of respiratory M cells should accelerate our understanding of the Ag sampling system at work in the upper respiratory tract.

Materials and Methods

Mice

BALB/c mice were purchased from SLC (Shizuoka, Japan). Inhibitor of DNA binding/differentiation 2 $(Id2)^{-/-}$ mice (129/Sv), generated as previously described (11), were maintained together with their littermate $Id2^{+/-}$ mice in a specific pathogen-free environment at the experimental animal facility of the Institute of Medical Science, University of Tokyo. All experiments were carried out according to the guidelines provided by the Animal Care and Use Committees of the University of Tokyo.

M cell staining

For the preparation of nasal cavity samples for confocal microscopy, we decapitated euthanized mice and then, with their heads immobilized, removed the lower jaw together with the tongue. Using the hard palate as a guide, we then used a large scalpel to remove the snout with a transverse cut behind the back molars. After removing the skin and any excess soft tissue, we flushed the external nares with PBS to wash out any blood within the nasal cavity before freezing the nasal passage tissue in Tissue-Tek OCT embedding medium (Miles, Elkhart, IN) in a Tissue-Tek Cryomold. For immunofluorescence staining, we prepared 5-μm–thick frozen sections by using a CryoJane Tape-Transfer System (Instrumedics, St. Louis, MO), allowed the sections to air dry, and then fixed them in acetone at 4°C. We then rehydrated the sections in PBS and incubated them for a further 30 min in Fc blocking solution. For M cell staining, sections were incubated overnight with rhodamine-labeled *Ulex europaeus* agglutinin-1 (UEA-1; Vector Laboratories, Burlingame, CA) at a concentration of 20 μg/ml and FITC-labeled M cell-specific mAb NKM 16-2-4 (12) at 5 μg/ml or FITC-labeled wheat germ agglutinin (WGA; Vector Laboratories, Burlingame, CA) at 10 μg/ml and counterstained with DAPI (Molecular Probes, Eugene, OR) at 0.2 μg/ml in PBS (13).

Electron microscopic analysis of respiratory M cells

For electron microscopic analysis, the nasal cavity sample was prepared and vigorously washed as described above, and then fixed on ice for 1 h in a solution containing 0.5% glutaraldehyde, 4% paraformaldehyde, and 0.1 M sodium phosphate buffer (pH 7.6). After being washed with 4% sucrose in 0.1 M phosphate buffer, the tissues were incubated in an HRP-conjugated UEA-1 solution (20 μg/ml) for 1 h at room temperature. The peroxidase reaction was developed by incubating the tissues for 10 min at room temperature with 0.02% 3,3′-diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl (pH 8) containing 0.01% H_2O_2. After being washed with the same buffer, the tissues were fixed again with 2.5% glutaraldehyde in 0.1 M phosphate buffer overnight. The nasal passage tissue was decalcified with 2.5% EDTA solution for 5 d. After being washed three times with the same buffer, samples were fixed with 2% osmium tetraoxide on ice for 1 h before being dehydrated with a series of ethanol gradients. For scanning electron microscopy (SEM), dehydrated tissues were freeze-embedded in *t*-butyl alcohol and freeze-dried, then coated with osmium and observed with a Hitachi S-4200 scanning electron microscope (Hitachi, Tokyo, Japan). For transmission electron microscopy (TEM) analysis, the samples were embedded in Epon 812 Resin mixture (TAAB Laboratories Equipment, Berks, U.K.), and ultrathin (70-nm) sections were cut with a Reichert Ultracut N Ultramicrotome (Leica Microsystems, Heidelberg, Germany). Ultrathin sections were stained with 2% uranyl acetate in 70% ethanol for 5 min at room temperature and then in Reynolds lead citrate for 5 min at room temperature. Sections were examined with a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Elucidation of M cell numbers

To examine the numbers of respiratory and NALT M cells, mononuclear cells (including M cells, epithelial cells, and lymphocytes) were isolated from the nasal passages and NALT as previously described, with some modifications (4). In brief, the palatine plate containing NALT was removed, and then NALT was dissected out. Nasal passage tissues without NALT were also extracted from the nasal cavity, and mononuclear cells from individual tissues were isolated by gentle teasing using needles through 40-μm nylon mesh. The total numbers of cells isolated from the two preparations were counted. These single-cell preparations were then labeled with PE–UEA-1 (Biogenesis, Poole, England), and the percentages of UEA-1–positive epithelial cells in the nasal passages and NALT were determined with a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ). The numbers of M cells and goblet cells in the nasal passages and NALT were counted by confocal microscopic analysis according to the patterns of staining with UEA-1 and WGA. That is, the frequencies of M cells ($UEA\text{-}1^{+}WGA^{-}$) and goblet cells ($UEA\text{-}1^{+}WGA^{+}$) were determined by the enumeration of each type in 100 $UEA\text{-}1^{+}$ cells. The formula used to estimate the number of M cells was: [(total number of mononuclear cells × percentage of $UEA\text{-}1^{+}$ epithelial cells) × M cells/$UEA\text{-}1^{+}$ epithelial cells]. The number of respiratory M cells in $Id2^{-/-}$ mice was calculated in the same manner.

Ag uptake in situ

DQ OVA was purchased from Molecular Probes. *Salmonella typhimurium* PhoPc strain transformed with the pKKGFP plasmid was kindly provided by F. Niedergang (14, 15). Group A *Streptococcus* (GAS; *Streptococcus pyogenes* ATCC BAA-1064) was obtained from the American Type Culture Collection (Manassas, VA), and immunofluorescence staining with FITC-conjugated goat anti-*Streptococcus* A Ab (Cortex Biochem, San Leandro, CA) was used to detect GAS uptake. DQ OVA (0.5 mg), GFP-expressing *Salmonella* (GFP-*Salmonella*) (5×10^8 CFU), or GAS (5×10^8 CFU) was intranasally administered and incubated in situ. Thirty minutes after the intranasal administration, the nasal passages were removed as described above and extensively washed with cold PBS with antibiotic solution to remove weakly adherent and/or extracellular DQ OVA or bacteria, as described (13).

The airway fluorescence-labeled Ag-treated nasal passages were processed for confocal microscopy as described above or for FACSCalibur flow cytometric analysis as follows. Mononuclear cells (including M cells, epithelial cells, and lymphocytes) were physically isolated from the nasal passages and NALT as described above, fixed in 4% paraformaldehyde, and labeled with PE–UEA-1 (Biogenesis, Poole, England). The percentage of green fluorescence (BODYPI FL or GFP)/UEA-1 double-positive nasal passage epithelial cells was determined by using an FACSCalibur (BD Biosciences).

To clarify the uptake of the bacteria by M cells, $UEA\text{-}1^{+}GFP^{+}$ cells, which were sorted from the nasal passages of mice intranasally infected with GFP-*Salmonella* by using an FACSAria cell sorter (BD Biosciences) were analyzed under three-dimensional confocal microscopy (Leica Microsystems).

To demonstrate the presence of dendritic cells (DCs) in the submucosa of the nasal passages, especially underneath respiratory M cells, after intranasal instillation of GAS, we used FITC- or allophycocyanin-conjugated anti-mouse CD11c (BD Pharmingen, San Jose, CA) Abs for subsequent confocal microscopic analysis.

Immunization

The recombinant *S. typhimurium* BRD 847 strain used in this study was a double *aroA aroD* mutant that expressed the nontoxic, immunogenic 50-kDa ToxC fragment of tetanus toxin from the plasmid pTET*nir*15 under the control of the anaerobically inducible *nirB* promoter (recombinant *Salmonella*-ToxC) (16). As a control, recombinant *Salmonella* that did not express ToxC was used. The recombinant *Salmonella* organisms were resuspended in PBS to a concentration of 2.5×10^{10} CFU/ml. Bacterial suspensions were intranasally administered by pipette (10 μl/mouse) three times at weekly intervals. To eliminate any possible GALT-associated induction of Ag-specific immune responses from the swallowing of bacterial solutions after intranasal immunization, mice were given drinking water containing gentamicin from 1 wk before the immunization to the end of the experiment and were also subjected to intragastric lavage with 500 μl gentamicin solution before and after intranasal immunization. This protocol successfully eliminated the possibility of the intranasally delivered bacteria becoming deposition in the intestine (Supplemental Fig. 1). The titers of tetanus toxoid (TT)-specific serum IgG and mucosal IgA Abs were determined by end-point ELISA, as described previously (17).

To measure GAS-specific immune responses, GAS was suspended in PBS to a concentration of 2×10^{10} CFU/ml. Ten microliters bacterial suspension was intranasally administered once using a pipette. Six weeks after the administration, serum and nasal washes were prepared, and the titers of GAS-specific Ab were measured by ELISA using a previously described protocol (18).

Statistical analysis

Data are expressed as means ± SD, and the difference between groups was assessed by the Mann–Whitney *U* test. The p values <0.05 were considered to be statistically significant.

Results

Respiratory M cells in single-layer epithelium of the nasal passage

The nasal respiratory epithelium of the mouse is composed mainly of pseudostratified ciliated columnar epithelium (19). However, when H&E-stained sections of the whole nasal cavity were examined, a single-layer epithelium was found to cover some regions of the nasal cavity, especially the lateral surfaces of the nasal turbinates (Fig. 1*A–C*). Frozen sections of nasal passages from naive BALB/c mice were prepared and stained with FITC-WGA (green) and rhodamine–UEA-1 (red), and then counterstained with DAPI (blue). Clusters of UEA-1^{+}WGA^{-} cells that shared M cell characteristics were found exclusively in the single-layer epithelium of the nasal passage covered by ciliated columnar epithelial cells (Fig. 1*D*, 1*E*). Some respiratory M cells were also occasionally found on the transitional area between the single-layer and stratified epithelium. Notably, respiratory M cells also reacted with our previously developed M cell-specific mAb NKM 16-2-4 (12), demonstrating colocalization of the signals of UEA-1 and NKM 16-2-4 (Fig. 1*F*, 1*G*).

Electron microscopic analysis of respiratory M cells

SEM of the respiratory M cells revealed the characteristic features of M cells: a depressed surface with short and irregular microvilli (Fig. 2*A*, 2*B*). TEM analysis revealed that the respiratory M cell was covered by shorter and more irregular microvilli (with definite UEA-1^{+} signals; Fig. 2*C*, 2*D*) than were found in neighboring ciliated columnar respiratory epithelial cells (Fig. 2*E*). However, no pocket formation (or pocket lymphocytes) was seen in the basal membranes of respiratory M cells, unlike in NALT M cells (Fig. 2*F*, 2*G*). These findings indicated that the newly identified respiratory M cells had most of the unique morphological characteristics of classical M cells.

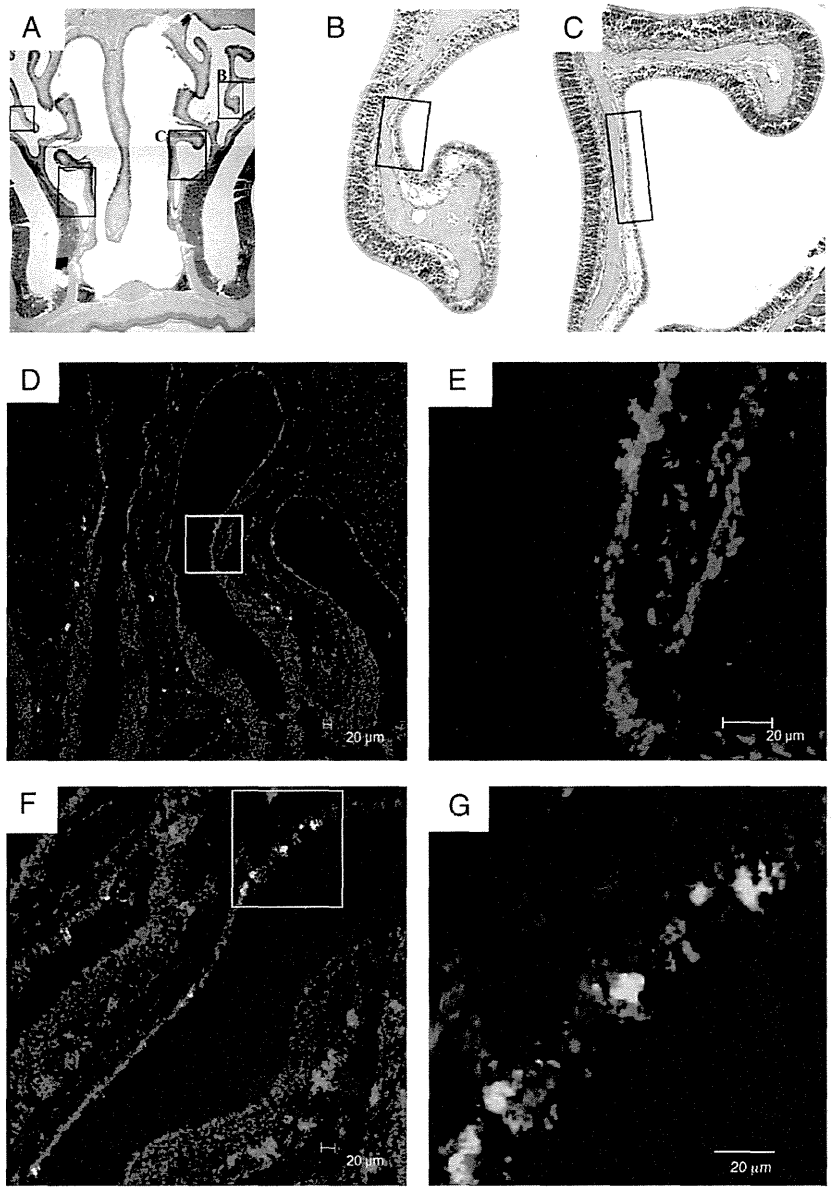

FIGURE 1. Clusters of UEA-1^{+}WGA^{-} respiratory M cells are found selectively in the single-layer epithelium of the nasal passage. *A–C*, H&E staining reveals the anatomy and general histology of the murine nasal passage (*A*, original magnification ×40). The nasal respiratory epithelium of the mouse is covered with a pseudostratified ciliated columnar epithelium. However, a single-layer epithelium was found on the lateral surfaces of the nasal turbinates (*B*, *C*). Original magnification ×100. Rectangles indicate areas covered with the single-layer epithelium. The results are representative of three independent experiments. *D–G*, Confocal views of UEA-1^{+} cells in the nasal epithelium of turbinates. Frozen sections were prepared and stained with FITC-WGA (green) and rhodamine–UEA-1 (red), and then counterstained with DAPI (blue) (*D*, *E*). Scale bars, 20 μm. The merged image is shown in *D*. An enlargement of the area in the rectangle in *D* is shown in *E*. UEA-1^{+}WGA^{-} cells are clustered on the single-layer nasal epithelium of the turbinate. *F* and *G*, UEA-1^{+} cells also reacted with our previously developed M cell-specific mAb NKM 16-2-4, demonstrating colocalization of signals of rhodamine–UEA-1 (red) and FITC-NKM 16-2-4 (green). The merged image is shown in *F*. An enlargement of an area from the rectangle in *F* is shown in *G*. The results are representative of five independent experiments.

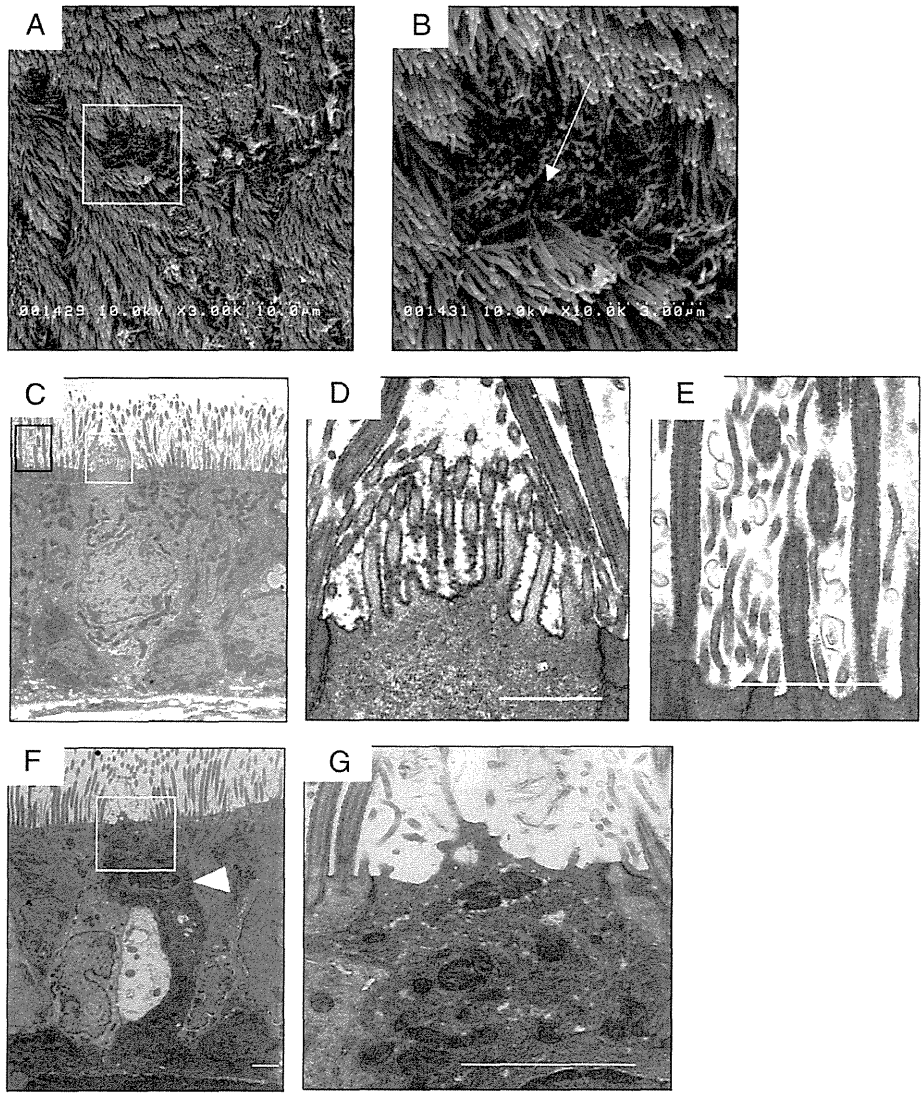

FIGURE 2. Electron microscopic analysis of respiratory M cells. *A* and *B*, SEM analysis shows that the M cells (*B*, arrow) in the nasal passage epithelium are distinguishable from adjacent respiratory epithelial cells by their relatively depressed and dark brush borders. An enlargement of the area in the rectangle in *A* is shown in *B*. As indicated in the *Materials and Methods*, the tissue specimen was incubated with HRP-conjugated UEA-1 before TEM analysis. *C–E*, TEM analysis of respiratory M cells reveals shorter and more irregular microvilli with definite UEA-1$^+$ signals (*D*), unlike the cilia of neighboring respiratory epithelial cells (*E*). *F* and *G*, TEM analysis of NALT M cells. A readily apparent intraepithelial pocket with mononuclear cells (*F*, arrowhead) and short microvilli on the apical surfaces of NALT M cells are seen. The white squares in *C* and *F* indicate UEA-1$^+$ respiratory and NALT M cells, respectively, and are magnified in *D* and *G*, respectively. The black rectangle in *C* indicates an adjacent respiratory epithelial cell and is magnified in *E*. *C–G*, Scale bars, 0.5 μm. Results are representative of four independent experiments.

Protein and bacterial Ag uptake by respiratory M cells

Because M cells were frequently found in the single layer of nasal passage epithelium (Fig. 1*D–G*), we next examined the ability of respiratory M cells to take up various forms of Ag from the lumen of the nasal cavity. DQ OVA or recombinant *Salmonella typhimurium* expressing GFP (*Salmonella*-GFP) was instilled into the nasal cavities of BALB/c mice via the nares. Thirty minutes after the intranasal instillation, immunohistological analyses revealed that the M cells located on the lateral surfaces of the nasal turbinates in the single layer of nasal epithelium had taken up DQ OVA (Fig. 3*A*, 3*B*), as had the M cells located in the NALT epithelium (Fig. 3*C*). Recombinant *Salmonella*-GFP was also observed in M cells in the single layer of nasal epithelium after intranasal administration (Fig. 4*A*, 4*B*). These findings demonstrate that, like NALT M cells (Figs. 3*C*, 4*C*), respiratory M cells were capable of taking up both soluble protein and bacterial Ags.

To further demonstrate the biological significance of respiratory M cells, the numbers of these M cells per mouse were examined and compared with those of NALT M cells (Fig. 3*D*). The number of respiratory M cells was significantly higher than that of NALT M cells. Next, we examined the efficiency of Ag uptake per respiratory M cell and NALT M cell (Figs. 3*E–J*, 4*D–I*). Nasal passage and NALT epithelial cells isolated from BALB/c mice 30 min after intranasal instillation of DQ OVA or recombinant *Salmonella*-GFP were counterstained with PE–UEA-1 for flow cytometric analysis. The UEA-1$^+$ fraction showed a significantly greater efficiency of uptake of DQ OVA Ag and recombinant *Salmonella*-GFP than did UEA-1$^-$ cells isolated from the re-

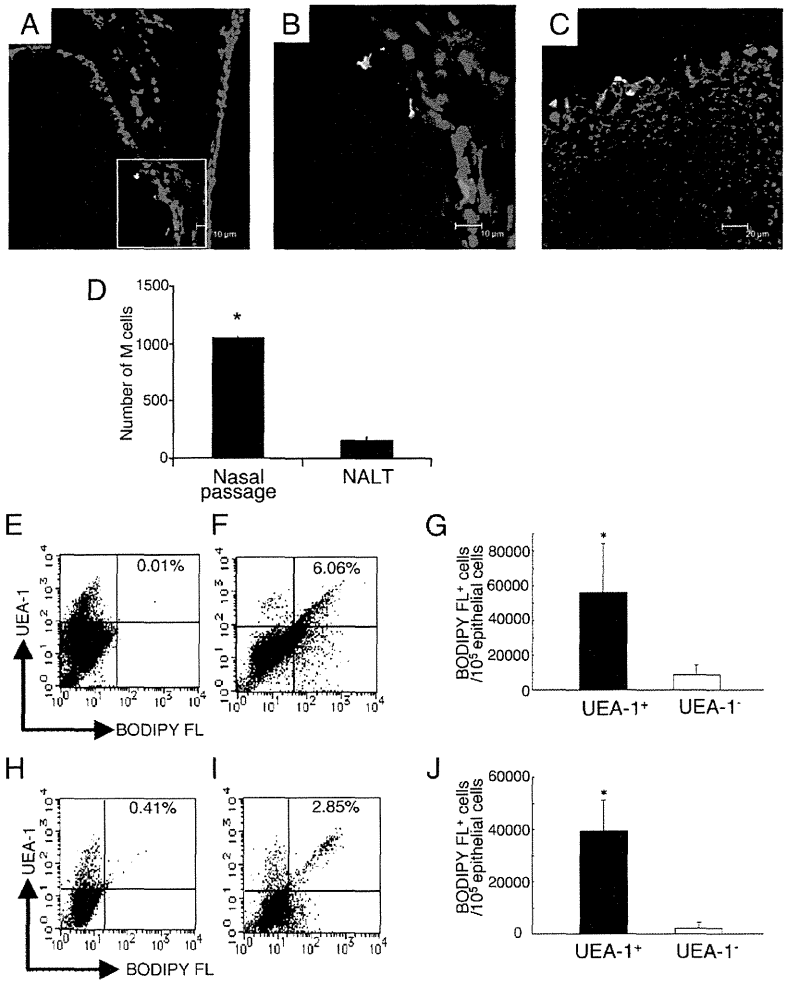

FIGURE 3. Respiratory M cells can take up DQ OVA. *A* and *B*, Immunofluorescence staining of nasal passages in BALB/c mice 30 min after DQ OVA (0.5 mg, green) instillation. Frozen sections of nasal passage were stained with rhodamine–UEA-1 (red) and DAPI (blue). Scale bars, 10 μm. The merged image is shown in *A*. An enlargement of the area in the rectangle in *A* is shown in *B*. These pictures demonstrate DQ OVA uptake by UEA-1$^+$ respiratory M cells. *C*, UEA-1$^+$ (red) NALT M cells in BALB/c mice also show an ability to take up DQ OVA (green). Scale bar, 20 μm. The results are representative of seven independent experiments. *D*, The numbers of UEA-1$^+$WGA$^-$ cells in nasal passages and NALT were quantified. The results are representative of four independent experiments. Flow cytometric analysis of DQ OVA uptake by UEA-1$^+$ respiratory (*E–G*) and NALT (*H–J*) M cells 30 min after intranasal instillation of PBS (*E*, *H*; control) or DQ OVA (*F*, *I*). *G* and *J*, UEA-1$^+$ cells showed significantly higher uptake of DQ OVA than did UEA-1$^-$ cells in the nasal passages and NALT. The results are representative of four independent experiments. $*p < 0.05$.

spiratory epithelium of the nasal passage (Figs. 3*E–G*, 4*D–F*) and NALT (Figs. 3*H–J*, 4*G–I*).

Three-dimensional confocal microscopic analysis demonstrated that UEA-1$^+$ GFP$^+$ cells, which were sorted from the nasal passages of the mice intranasally infected with GFP-*Salmonella*, had captured and taken up the bacteria (Fig. 4*J*, Supplemental Video 1).

Cluster formation by respiratory M cells and DCs in response to inhaled respiratory pathogens

Because respiratory M cells are capable of capturing bacterial Ag, we considered it important to assess these cells as potential new entry sites for respiratory pathogens such as GAS. Confocal microscopic analysis demonstrated that, after its intranasal instillation, GAS stained with FITC–anti-*Streptococcus* A Ab was taken up by UEA-1$^+$ respiratory M cells (Fig. 5*B–E*). SEM analysis also revealed the presence of GAS-like microorganisms on the membranes of respiratory M cells after nasal challenge with GAS (Supplemental Fig. 2*A*). As one might expect, GAS were found in UEA-1$^+$ NALT M cells (Supplemental Fig. 2*B*) as well, confirming a previously reported result (20). Our findings suggest that respiratory M cells act as alternative entry sites for respiratory pathogens.

When we examined the site of invasion by GAS, we noted the presence of CD11c$^+$ DCs underneath the respiratory M cells (Fig. 5). Confocal microscopic analysis of the nasal passage epithelium after intranasal instillation of GAS revealed evidence of the recruitment of DCs, some having contact with the GAS, to the area underneath the respiratory M cells (Fig. 5*B–E*). A few DCs were also observed in the nasal passages of naive mice (Fig. 5*A*); these nasal DCs might preferentially migrate to the area underneath the respiratory M cells to receive Ags from these cells for the initiation of Ag-specific immune responses.

Presence of respiratory M cells in NALT-deficient mice

When we examined the numbers of respiratory M cells in the lymphoid structure-deficient Id2$^{-/-}$ mice (including NALT, NALT-null), the frequency of occurrence of respiratory M cells was comparable to that found in their littermate Id2$^{+/-}$ mice (Fig. 6*A*). This finding suggested that development of respiratory M cells occurred normally under NALT-null or Id2-deficient conditions. Frozen tissue samples were next prepared from NALT-null mice that had received fluorescence-labeled bacteria by intranasal instillation. Immunohistological analysis of these samples revealed the presence of recombinant *Salmonella*-GFP in UEA-1$^+$ cells from the nasal epithelium of Id2$^{-/-}$ mice. GFP-positive bacteria were also located in the subepithelial region of the nasal passages, suggesting that, in the NALT-null mice, some of the nasally deposited bacteria were taken up by respiratory M cells (Fig. 6*B*, 6*C*). Flow cytometric analysis confirmed the uptake of recombinant *Salmonella*-GFP by UEA-1$^+$ M cells, with UEA-1$^+$ cells in the nasal passages of Id2$^{-/-}$ mice showing a significantly higher uptake than UEA-1$^-$ cells (Fig. 6*D–F*).

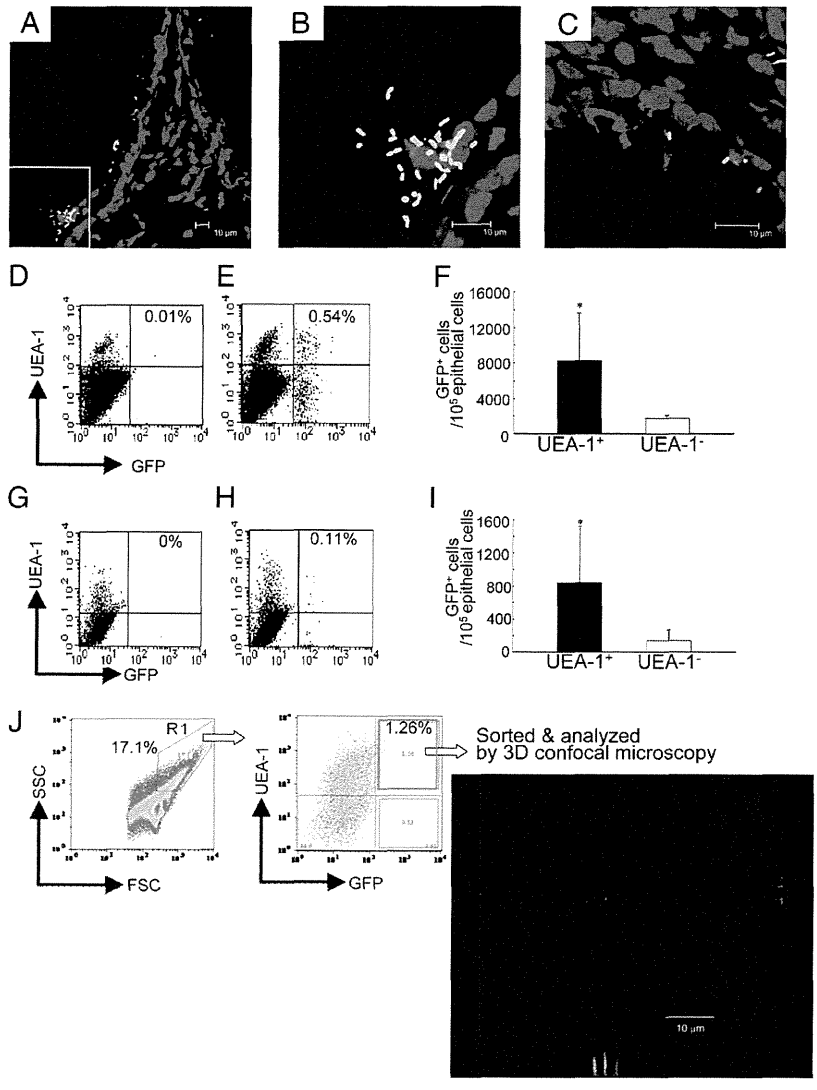

FIGURE 4. Respiratory M cells show an ability to take up recombinant *Salmonella*-GFP. *A* and *B*, Immunofluorescence staining of the nasal passages of BALB/c mice 30 min after GFP-*Salmonella* (5×10^8 CFU, green) instillation. Frozen sections of nasal passage were stained with rhodamine–UEA-1 (red) and DAPI (blue). The merged image is shown in *A*. An enlargement of the area in the rectangle in *A* is shown in *B*. These pictures demonstrate the ability of UEA-1^+ respiratory M cells, like UEA-1^+ NALT M cells (*C*), to take up GFP-*Salmonella*. The results are representative of six separate experiments. *A–C*, Scale bars, 10 μm. Flow cytometric analysis of GFP-*Salmonella* uptake by UEA-1^+ respiratory (*D–F*) and NALT (*G–I*) M cells 30 min after intranasal instillation of PBS (*D*, *G*; control) or GFP-*Salmonella* (*E*, *H*). *F* and *I*, Efficiency of uptake of GFP-*Salmonella* by UEA-1^+ cells in both nasal passages and NALT. The data showed UEA-1^+ M cells to be significantly more efficient than UEA-1^- epithelial cells at taking up GFP-*Salmonella*. The results are representative of five independent experiments. *J*, Three-dimensional confocal microscopic analysis demonstrated that UEA-1^+ GFP^+ cells, which were sorted from the nasal passages of mice intranasally infected with GFP-*Salmonella* (green), took up bacteria. Scale bar, 10 μm. The results are representative of three separate experiments. $^*p < 0.05$.

Induction of Ag-specific immune responses in NALT-deficient mice

NALT-null ($Id2^{-/-}$) mice and their littermate $Id2^{+/-}$ mice were intranasally immunized with recombinant *S. typhimurium* BRD 847 expressing a 50-kDa ToxC fragment of tetanus toxin (recombinant *Salmonella*-ToxC) to examine whether Ag sampling via respiratory M cells could induce Ag-specific immune responses in NALT-deficient mice. To eliminate any possible GALT-associated induction of Ag-specific immune responses from the swallowing of bacterial solutions after intranasal immunization, mice were given drinking water containing gentamicin from 1 wk before the immunization to the end of the experiment and were also subjected to intragastric lavage with 500 μl gentamicin solution before and after intranasal immunization. This protocol successfully eliminated the possibility of the intranasally delivered bacteria becoming deposition in the intestine (Supplemental Fig. 1). The titer of TT-specific serum IgG Ab was as high in $Id2^{-/-}$ mice as in $Id2^{+/-}$ mice (Fig. 6*G*). TT-specific IgA Abs were also detected in the nasal secretions and vaginal washes of intranasally immunized NALT-deficient mice (Fig. 6*H*, 6*I*). As expected, TT-specific Abs were not detected in either $Id2^{-/-}$ or $Id2^{+/-}$ mice intranasally immunized with a control recombinant *Salmonella* that did not express the ToxC gene (Fig. 6*G–I*). In addition to the responses to *Salmonella*, GAS-specific immune responses were induced in the absence of NALT in the experiment with $Id2^{-/-}$ mice (Fig. 6*J–L*). These data indicate that the respiratory M cell is an important Ag-sampling site for the induction of Ag-specific local IgA and serum IgG immune responses.

Discussion

In this study, we show the existence of a novel Ag sampling site for inhaled Ags in the upper respiratory epithelium. The murine nasal membrane has been reported to contain four types of epithelium: respiratory, olfactory, transitional, and squamous (21). Most of the respiratory epithelium is located in the lateral and ventral regions of the nasal cavity and is covered with pseudostratified ciliated columnar cells (21). In this study, we were also able to observe a single-layer epithelium on the lateral surfaces of the turbinates, which was comprised exclusively of UEA-1^+WGA^- M cells (Fig. 1). These respiratory M cells showed specific reactivity to our previously developed M cell-specific mAb NKM 16-2-4 (12). Because NALT is characterized by follicle-associated epithelium, we first thought that this single-layer epithelium could represent the follicle-associated epithelium of the nasal passage. However,

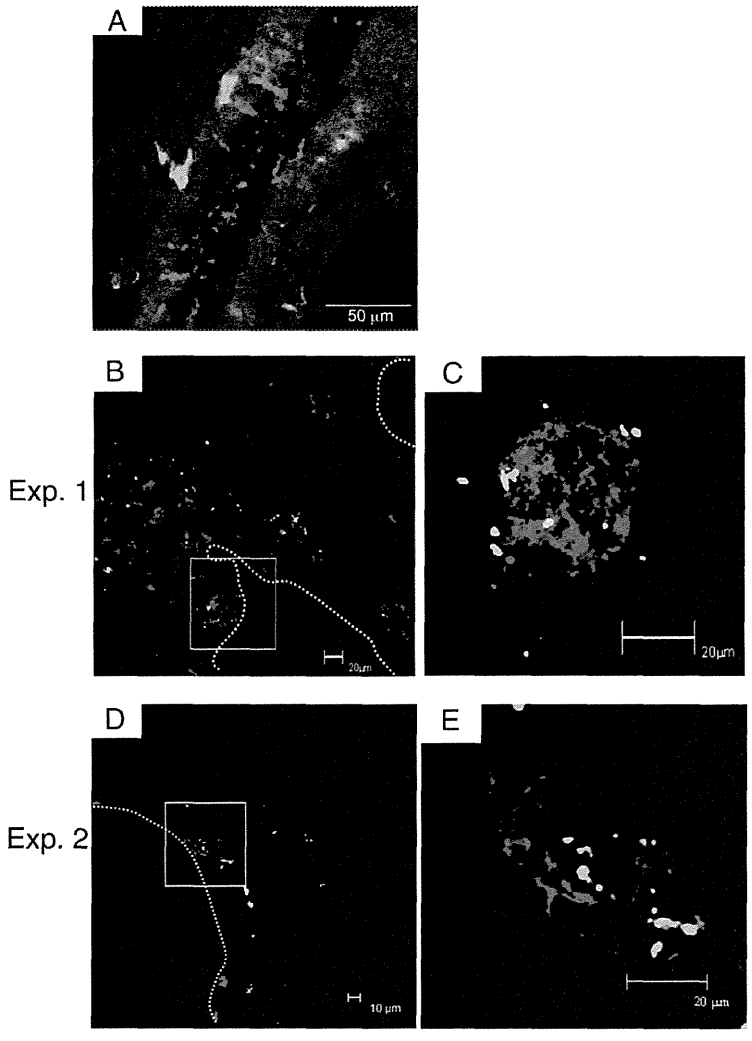

FIGURE 5. Respiratory M cells form clusters with DCs after GAS infection. *A*, Before nasal challenge with GAS, only a few DCs (FITC-CD11c$^+$, green) were associated with UEA-1$^+$ M cells (red) in the nasal passage. Scale bar, 50 μm. *B–E*, Two sets of confocal views of the nasal passage 5 d after intranasal instillation of GAS (Exp. 1 and Exp. 2, respectively). Frozen sections of the nasal passage were stained with FITC–anti-*Streptococcus* A Ab (green), rhodamine–UEA-1 (red), and allophycocyanin-CD11c (blue). These images reveal large numbers of DCs congregated underneath the UEA-1$^+$ respiratory M cells; some of the DCs were closely associated with GAS infiltrated through the UEA-1$^+$ respiratory M cells. *C* and *E* are enlargements of the areas in the squares shown in *B* and *D*, respectively. The results are representative of five independent experiments. *B*, *C*, and *E*, Scale bars, 20 μm; *D*, scale bar, 10 μm.

we ruled out this possibility when we could not find any organized lymphoid structures beneath the single-layer epithelium. The respiratory M cells had most of the classical features of M cells, including a depressed surface covered with short and irregular microvilli. However, TEM analysis revealed that, unlike NALT M cells, they lacked an intraepithelial pocket (Fig. 2). Examination of the numbers of respiratory and NALT M cells per nasal cavity revealed that there were more respiratory M cells than NALT M cells (in general six or seven times more; Fig. 3*D*), suggesting that the respiratory M cell plays a critical role as a gateway for the upper airway.

The anatomical and histological characteristics of the nasal cavity differ markedly between humans and mice. Reflecting this fact, the occurrence of single-layer epithelium also differs between the two species. Murine respiratory epithelium consists of a typical single-layer epithelium with traditional columnar epithelial cells in the turbinate portion of the nasal cavity, whereas pseudostratified columnar epithelium covers the olfactory epithelium (21, 22). In contrast, the traditional single-layer epithelium is not observed in the human nasal cavity, and both the upper respiratory surfaces and the olfactory surfaces are covered by pseudostratified columnar epithelium (23, 24). These differences suggest that the presence of respiratory M cells in the nasal cavity might be a feature unique to the mouse. The presence or absence of respiratory M cells in the human nasal cavity still needs to be carefully examined, and, if these cells are present, their contribution to the uptake of inhaled Ags needs to be investigated in future studies.

Previously, M cells in the lower respiratory tract were found to provide a portal of entry for bacterial pathogens into the lung (25). Our study suggests that the newly identified NALT-independent M cells in the upper respiratory tract provide an alternative portal of entry for nasally inhaled pathogens. The respiratory epithelium comprises three distinct Ag-sampling and/or pathogen-invasion sites: respiratory M cells and NALT M cells in the upper respiratory tract and M cells in the lower respiratory tract. It is interesting to speculate that the nature of the respiratory pathogen may dictate its preferred entry site, with GAS preferentially invading the host via the upper respiratory tract M cells and *Mycobacterium tuberculosis* preferentially invading via the lower respiratory tract M cells. This attractive possibility requires careful examination, and such a line of investigation has been initiated in our laboratory.

Salmonella, a known gastrointestinal pathogen, may have no relevance to the immunological and physiological aspects of Ag uptake by respiratory M cells. However, when used as a live vector for the intranasal delivery of vaccine Ags, attenuated *Salmonella* effectively elicits Ag-specific immune responses (26–29). Pasetti et al. (28) compared intranasal and orogastric immunizations in

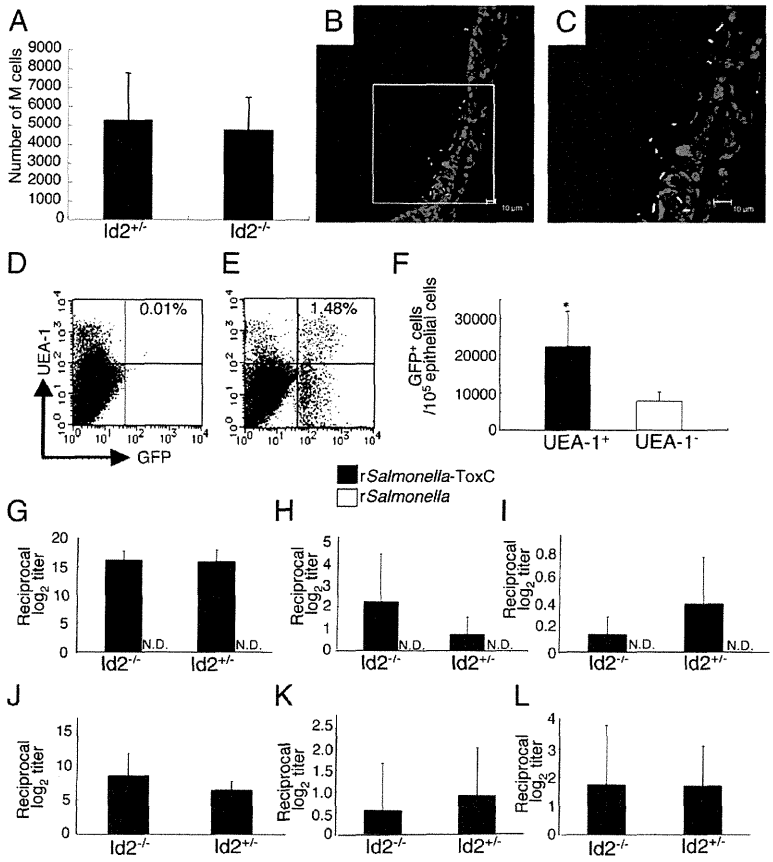

FIGURE 6. $Id2^{-/-}$ mice, which lack NALT, can take up GFP-*Salmonella*, which induce Ag-specific immune responses in UEA-1^+ respiratory M cells. *A*, The numbers of UEA-1^+WGA$^-$ cells in nasal passages of $Id2^{-/-}$ and $Id2^{+/-}$ mice were measured. The results are representative of four independent experiments. *B* and *C*, Immunofluorescence staining of nasal passages of $Id2^{-/-}$ mice in which GFP-expressing *Salmonella* (green) had been instilled. Frozen sections of nasal passages were stained with rhodamine–UEA-1 (red) and DAPI (blue). Scale bars, 10 μm. *C* is an enlargement of the area in the square shown in *B*. The results are representative of three independent experiments. *D–F*, Flow cytometric analysis of GFP-*Salmonella* uptake by UEA-1^+ M cells 30 min after intranasal instillation of PBS (*D*; control) or GFP-*Salmonella* (*E*) in the nasal passages of $Id2^{-/-}$ mice. *F*, Efficiency of uptake by UEA-1^+ cells in the nasal passages of $Id2^{-/-}$ mice was significantly greater than that by UEA-1^- cells. The results are representative of three independent experiments. *G–I*, NALT-deficient ($Id2^{-/-}$) mice and $Id2^{+/-}$ mice were intranasally immunized with recombinant *Salmonella*-ToxC (2.5×10^8 CFU) or recombinant *Salmonella* (2.5×10^8) alone three times at weekly intervals. They were given gentamicin-containing drinking water and also subjected to intragastric lavage with gentamicin solution to eliminate GALT-mediated Ag-specific immune responses. Samples were obtained 7 d after the last intranasal immunization to measure TT-specific Igs by ELISA. Serum IgG (*G*), nasal wash IgA (*H*), vaginal wash IgA (*I*). The results are representative of three independent experiments. *J–L*, As was the case with *Salmonella*, GAS-specific immune responses were induced in the absence of NALT (i.e., in $Id2^{-/-}$ mice), this time by a single intranasal injection of GAS (2×10^8 CFU). Serum IgG (*J*), nasal wash IgA (*K*), vaginal wash IgA (*L*). There were no statistical differences between $Id2^{-/-}$ and $Id2^{+/-}$ mice, as analyzed by the unpaired Mann–Whitney *U* test. The results are representative of five independent experiments. $^*p < 0.05$. N.D., not detected.

terms of both Ag-specific immune responses and in vivo distribution of vaccine organisms; they demonstrated that intranasal immunization resulted in greater humoral and cell-mediated immune responses and in the delivery of larger numbers of vaccine organisms to the nasal tissues, lungs, and Peyer's patches. Furthermore, intranasal immunization effectively induces Ag-specific IgA Abs in the reproductive secretions of mice and primates (30, 31). Notably, the levels of Ag-specific IgA Abs in the nasal secretions of NALT-deficient $Id2^{-/-}$ mice were not significantly higher than, or comparable to, those of control tissue-intact mice following intranasal immunization with recombinant *Salmonella* expressing ToxC (Fig. 6*H*) or GAS (Fig. 6*K*), respectively. In contrast, in intranasally immunized NALT-deficient mice, the levels of Ag-specific IgA Abs in remote secretions such as the vaginal wash were not significantly lower than, or comparable to, those in similarly treated tissue-intact mice (Fig. 6*I*, 6*L*). Inasmuch as these results revealed no significant differences between the two groups of intranasally immunized mice, our results at least suggest that respiratory M cells contribute to the induction of Ag-specific immune responses at both local and distant effector sites. However, we still need to carefully examine and compare the contributions of respiratory M cells and NALT M cells in the initiation of Ag-specific IgA Ab responses at local (e.g., airway) and distant (e.g., reproductive tract) effector sites.

In regard to the functional aspects of respiratory M cells, our data demonstrated that the numbers of respiratory M cells that took up OVA were comparable to those of NALT M cells (Fig. 3*G*, 3*J*). In contrast, 10 times more respiratory M cells than NALT M cells took up *Salmonella*; this result suggested that respiratory M cells are more efficient at taking up bacterial (or particulate) Ags than are NALT M cells (Fig. 4*F*, 4*I*). Although we do not have any data regarding the mechanism(s) behind these findings, these results

suggest that there may be functional differences in, for example, Ag uptake capability, between respiratory M cells and NALT M cells due to possible differences in the expression of bacterial Ag receptors, even though the morphologies and phenotypes of these two subsets of M cells are similar. In support of this possibility, it has been shown that the expression of a GP-2–specific receptor for FimH bacteria is restricted to Peyer's patches and not villous M cells; this situation may be analogous to that of NALT and respiratory M cells (32). Although the molecular mechanisms for the induction of Ag-specific immune responses by intranasal immunization and the efficacy of intranasal inoculation await elucidation, we demonstrated in this paper that respiratory M cells, like NALT M cells, are capable of sampling *Salmonella*, thereby opening a new avenue for the uptake of *Salmonella*-delivered vaccine.

CD18-expressing phagocytes (33) and mucosal DCs (34) are involved in the uptake of pathogens from the lumen of the intestine, but their role in the upper respiratory tract has never been clarified. Moreover, we found no evidence that mucosal DCs take up pathogens from the lumen of the nasal passage by expanding their dendrites into the lumen after nasal challenge with GAS. It was recently shown that intranasal immunization of mice with OVA plus adenovirus vector expressing Flt3 ligand as a mucosal adjuvant selectively increases $CD11b^+$ DC numbers in the nasal passages more effectively than those in NALT and subsequently induces Ag-specific Ab and CTL responses (35). Therefore, we speculated that the induction of immune responses in the murine model of intranasal administration of bacteria (e.g., *Salmonella* and GAS) might depend on the presence of appropriate initial Ag sampling sites associated with M cells, which can internalize the vaccine organisms. In this study, DCs were rarely detected in the subepithelial layer or the epithelial layer of the nasal passage in naive mice (Fig. 5*A*). It is important to note that DCs migrated to the area underneath the respiratory M cells and accumulated there to form cell clusters after exposure to respiratory pathogens (Fig. 5*B–D*). Following mucosal exposure to pathogens, submucosal DCs accumulate underneath infected mucosal epithelium that is not associated with organized lymphoid follicles (36, 37). Furthermore, these Ag-capturing DCs are capable of migrating into the draining lymph nodes (dLNs), where they encounter naive T cells for initial Ag-priming (36, 37). The question of whether DCs resident in the nasal passages migrate to the submucosal area to receive inhaled pathogens taken up via respiratory M cells and then travel to the dLNs (e.g., the cervical lymph nodes) to initiate an Ag-specific immune response remains to be addressed. It is interesting to postulate that respiratory M cells could be alternative airway Ag sampling sites for subsequent processing or presentation by nasal passage DCs, thereby initiating Ag-specific immune responses in the dLNs. In support of this hypothesis, it has been shown that Ag-specific Th cells are generated and found in the NALT and dLNs of mice given GAS intranasally (38). Our current study offers proof in support of this hypothesis by showing that *Salmonella* were effectively taken up by upper respiratory tract M cells in NALT and respiratory M cells and that a live vector-containing vaccine Ag induced Ag-specific immune responses via the nasal route.

We showed that TT-specific serum IgG and nasal wash IgA immune responses after intranasal immunization with recombinant *Salmonella*-ToxC were as high in $Id2^{-/-}$ mice as in $Id2^{+/-}$ mice (Fig. 6*G*, 6*H*) and that the frequency of occurrence of respiratory M cells in $Id2^{-/-}$ mice was comparable to that in their littermate $Id2^{+/-}$ mice (Fig. 6*A*). Generally, as discussed above, submucosal and dermal DCs have been shown to migrate to (or to be located in) the area just beneath infected epithelium and to then migrate into the dLNs after they have captured Ags. The DCs then present the peptides derived from these Ags to naive T cells, which subsequently undergo differentiation to Ag-specific effector T cells (36, 37). It has further been suggested that, rather than the DCs harboring Ag-derived peptides migrating to the systemic compartments, such as spleen and other secondary lymphoid tissues, the effector T cells generated in the dLNs after mucosal or vaginal Ag application migrate to these compartments and initiate Ag-specific immune responses (36).

If the cross-talk system between the airway mucosal and systemic immune compartments is similar to that between the reproductive mucosal and systemic immune compartments, it is unlikely that, in $Id2^{-/-}$ mice, the initiation of Ag-specific immune responses, including the presentation of Ags to naive T cells, occurs through migration of nasal DCs into the spleen after the capture of GAS-Ags by respiratory M cells and DCs. However, we cannot rule out this possibility, because it is possible that the nasal immune system, including the system by which Ags are taken up by respiratory M cells, offers distinct Ag-capture, -processing, and -presentation mechanisms via nasal DCs for the generation and migration of Ag-specific effector T cell and B cells. We have also found B-1 cell populations in the nasal passages (N. Tanaka, S. Fukuyama, T. Nagatake, K. Okada, M. Murata, K. Goda, D-Y. Kim, T. Nochi, S. Sato, J. Kunisawa, T. Kaisho, Y. Kurono, and H. Kiyono, manuscript in preparation), and it is possible that these cells may contribute to the induction of Ag-specific Ig responses without any help from $CD4^+$ T cells. At this stage, this is mere speculation, and the precise mechanism needs to be addressed in the future.

Taken together, these findings led us to conclude that respiratory M cells are effective alternative sampling sites for nasally inhaled bacterial Ags and thus play a key role in the induction of systemic and local mucosal immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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Okamatsu et al. Virology Journal 2013, 10:47
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Potency of a vaccine prepared from A/swine/Hokkaido/2/1981 (H1N1) against A/Narita/1/2009 (H1N1) pandemic influenza virus strain

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Abstract

Background: The pandemic 2009 (H1N1) influenza virus has spread throughout the world and is now causing seasonal influenza. To prepare for the emergence of pandemic influenza, we have established a library of virus strains isolated from birds, pigs, and humans in global surveillance studies.

Methods: Inactivated whole virus particle (WV) and ether-split (ES) vaccines were prepared from an influenza virus strain, A/swine/Hokkaido/2/1981 (H1N1), from the library and from A/Narita/1/2009 (H1N1) pandemic strain. Each of the vaccines was injected subcutaneously into mice and their potencies were evaluated by challenge with A/Narita/1/2009 (H1N1) virus strain in mice.

Results: A/swine/Hokkaido/2/81 (H1N1), which was isolated from the lung of a diseased piglet, was selected on the basis of their antigenicity and growth capacity in embryonated chicken eggs. Two injections of the WV vaccine induced an immune response in mice, decreasing the impact of disease caused by the challenge with A/Narita/1/2009 (H1N1), as did the vaccine prepared from the homologous strain.

Conclusion: The WV vaccine prepared from an influenza virus in the library is useful as an emergency vaccine in the early phase of pandemic influenza.

Keywords: Influenza A (H1N1)pdm, Vaccine, Swine influenza virus

Background

A pandemic influenza caused by swine-origin H1N1 virus appeared in Mexico in 2009 and spread throughout the world [1-3]. The pandemic virus isolates were antigenically similar to classical swine influenza viruses and distinct from H1N1 virus strains circulating in humans since 1977 [2,4]. A pandemic 2009 (H1N1) vaccine was produced and evaluated in clinical trials [5]. The production of a large amount of egg-produced pandemic 2009 (H1N1) vaccine was, however, limited due to its poor yield in chicken embryos [6], leading to a delay in the efficient control of the pandemic.

It was revealed that the H3 HA gene of A/Hong Kong/68 (H3N2) strain originated from that of isolates from migratory ducks and that pigs served as a mixing vessel for the generation of reassortants with the precedent human H2N2 influenza virus [7-10]. To prepare for pandemic influenza, we have conducted a global surveillance of influenza in birds and mammals since 1977, and have established a vaccine strain library of influenza A viruses [11-15]. Their pathogenicity, antigenicity, genetic information, and yield in chicken embryos have been analyzed and the data are available at http://virusdb.czc.hokudai.ac.jp/.

In the present study, a vaccine strain against pandemic 2009 (H1N1) influenza was selected from 42 H1N1 influenza viruses in the virus library. The potency of inactivated whole virus particle (WV) and ether-split (ES) vaccines prepared from a virus strain in the library was evaluated.

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Results

Antigenic analysis of H1N1 influenza viruses

Eighteen H1N1 influenza virus strains were selected from 42 strains in the library, showing good growth in embryonated chicken eggs (data not shown). The 18 virus strains were antigenically analyzed by hemagglutination-inhibition (HI) test with chicken antisera to H1N1 viruses isolated from birds, pigs and humans (Table 1). The pandemic strain, A/Narita/1/2009 (H1N1) (Narita/09), which was the first isolate in Japan in 2009, reacted with the antiserum to Sw/Hok/81 at a titer of 1:640, 8-fold lower than that to homologous virus. The antiserum to Narita/09 reacted with swine influenza viruses, especially the isolates in 1930–1981 at a titer of 1:1,280-2,560, which was 2- to 4-fold lower than that to homologous virus. These results indicate that the antigenicity of Narita/09 was to some extent related to those of H1N1 classical swine flu virus strains.

Genetic analyses of H1N1 viruses

Nucleotide sequences of the HA genes of the 18 H1N1 viruses were phylogenetically analyzed by the neighbor-joining method with those of other H1N1 strains, including H1N1 viruses isolated from humans. Based on the results of phylogenetic analysis, H1 HA genes were grouped into human, swine, or avian origin clusters (Figure 1). Swine influenza viruses isolated in Japan during 1977–1981 were clustered with pandemic 2009 (H1N1) viruses. Identity of amino acid of HA between Sw/Hok/81 and Narita/09 was 89.9% and glycosylation sites of HA were not different.

Growth of H1N1 viruses in embryonated chicken eggs

The growth of 18 H1N1 viruses in embryonated chicken eggs was assessed. All the viruses replicated efficiently and had reached a plateau by 48 hours post-infection (p.i.). No significant difference in peak titers of vaccine candidates was detected (data not shown). Sw/Hok/81 showed the highest titer at $10^{8.3}$ plaque-forming units (PFU)/ml 48 hours p.i., which was 10 times higher than that of Narita/09 ($10^{7.3}$ PFU/ml).

Potency test of the vaccine against H1N1 pandemic virus in mice

Four, 20, and 100 μg protein of WV or ES vaccines of Narita/09 and Sw/Hok/81, respectively, were subcutaneously

Table 1 The cross-reactivity of H1N1 viruses isolated from pigs, humans, and birds

	HI titer of chicken antisera against representative H1 viruses					
Virusesa	**Narita/09**	**Sw/Iowa/15/30**	**Sw/Hok/81**	**PR/8/34**	**Hok/4/96**	**Dk/Mong/540/01**
A/Narita/1/2009	**5,120b**	80	640	40	40	80
Swine isolates						
A/swine/Iowa/15/1930	1,280	**1,280**	2,560	20	80	640
A/swine/Niigata/1/1977	1,280	1,280	2,560	40	160	640
A/swine/Shimane/1/1978	2,560	1,280	5,120	40	160	640
A/swine/Shizuoka/1/1978	2,560	1,280	5,120	40	160	640
A/swine/Toyama/1/1978	2,560	1,280	5,120	40	160	640
A/swine/Kanagawa/1/1978	1,280	1,280	640	40	320	640
A/swine/Hokkaido/2/1981	1,280	1,280	**5,120**	80	80	640
A/swine/Miyagi/5/2003 (H1N2)	640	320	2,560	160	80	80
Human isolates						
A/PR/8/1934	20	40	40	**2,560**	160	20
A/Hokkaido/2/1996	320	80	80	160	**5,120**	320
A/Hokkaido/11/2002	160	80	80	320	5,120	80
Avian isolates						
A/duck/Miyagi/66/1977	160	80	80	40	40	640
A/swan/Hokkaido/55/1996	320	80	40	80	80	1,280
A/duck/Hokkaido/1130/2001	160	80	40	<20	<20	1,280
A/duck/Hokkaido/1203/2001	160	80	80	<20	<20	640
A/duck/Mongolia/540/2001	80	160	40	<20	20	**1,280**
A/duck/Hokkaido/83/2004	160	80	40	<20	<20	640
A/duck/Hokkaido/W73/2007	80	80	80	<20	<20	640

a: Subtype of viruses was H1N1 exept for A/swine/Miyagi/5/2003 (H1N2).
b: Homologous titer was shown in bold.

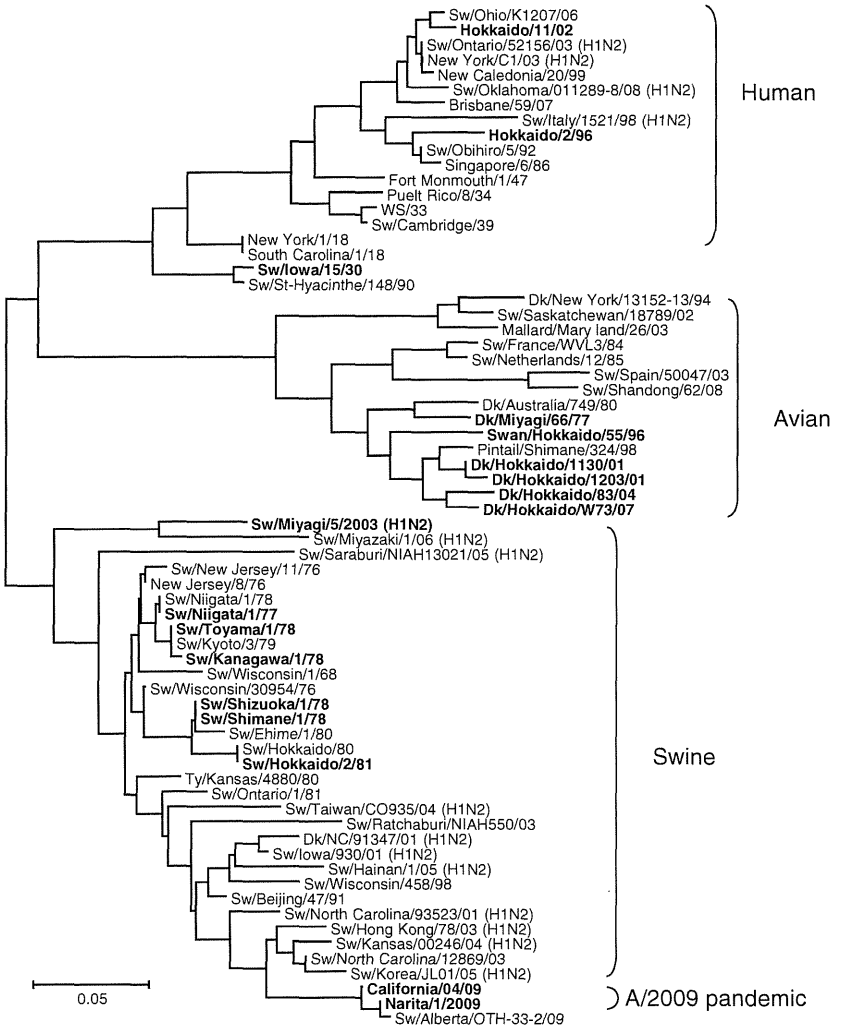

Figure 1 Phylogenetic tree of H1 HA gene of influenza viruses. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at the nodes indicate confidence levels in a boot strap analysis with 1,000 replications. Viruses shown in bold were used in this study and classified into 3 groups according to the animal from which they were isolated (human, avian and swine). Subtypes of viruses were H1N1 except for those in parentheses. Sw: swine, Dk: duck.

injected once into 5 mice. The serum antibody titers of mice against the vaccine and challenge strains were examined (Table 2). The neutralization (NT) antibodies were induced by each vaccine in a dose-dependent manner. Serum NT antibodies induced by injection of WV or ES vaccine of Sw/Hok/81 were not detected with Narita/09.

To assess the potency of the vaccine against the challenge with pandemic 2009 (H1N1) virus, $10^{6.0}$ PFU of Narita/09 were intranasally inoculated into mice which were injected subcutaneously once with each of the test vaccines. The rate of weight loss of the mice after virus challenge is shown in Figure 2. The mice injected with Narita/09 or Sw/Hok/81 vaccines survived for 14 days, although they showed some weight loss, while the non-vaccinated control mice showed significant weight loss and had died by day 14 after the challenge. In the mice injected with Narita/09 vaccine, no significant difference in weight loss was observed in the mice vaccinated with WV or ES vaccine. The mice injected with ES vaccine of Sw/Hok/81, however, showed significant weight loss compared with mice injected with WV vaccine. The rate of weight loss of mice injected with ES vaccine of Sw/Hok/81 correlated in a dose-dependent manner. The potency of vaccines was also evaluated by measuring the virus titer in the lower respiratory tract of mice (Table 2). The virus titers in the lungs were $10^{4.3}$–$10^{4.7}$ PFU/g in mice injected with

Table 2 Neutralizing antibody titers of mice injected once with the vaccine and virus titers in the lungs after challenge

Vaccine			NT titer to		Virus titer in lungs, mean log PFU/g ±Se[a]
Strain	Protein, μg	Formulation	Narita/09	Sw/Hok/81	
PBS	-	-	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	5.0±0.17
Narita/09	4	ES	<10, <10, <10, <10, <10	ND	4.6±0.10
	20	ES	<10, 20, 20, 20, 40	ND	4.6±0.12
	100	ES	20, 40, 80, 160, 160	ND	4.1±0.35
Narita/09	4	WV	40, 40, 40, 80, 160	ND	4.2±0.17
	20	WV	40, 80, 160, 160, 320	ND	3.9±0.25*
	100	WV	320, 320, 640, 1280, 1280	ND	2.4±0.50**
Sw/Hok/81	4	ES	<10, <10, <10, <10, <10	<10, <10, <10, 10, 10	4.6±0.04
	20	ES	<10, <10, <10, <10, <10	<10, 10, 10, 20, 80	4.4±0.02
	100	ES	<10, <10, <10, <10, <10	10, 20, 20, 40, 40	4.7±0.02
Sw/Hok/81	4	WV	<10, <10, <10, <10, <10	20, 40, 40, 40, 80	4.5±0.06
	20	WV	<10, <10, <10, <10, <10	20, 40, 80, 80, 80	4.4±0.04
	100	WV	<10, <10, <10, <10, <10	160, 160, 160, 160, 320	4.3±0.09

Mice were injected with each vaccine subcutaneously. Serum samples were collected 3 weeks after injection.
The animals were challenged by intranasal administration of 106.0 PFU of A/Narita/09.
At 3 days after challenge, lungs samples were collected and virus titers were measured. ES: ether split vaccine, WV: whole inactivated vaccine
a: Data are for 5 mice.
*: P<0.05, vs. virus titers in PBS group.
**: P<0.01, vs. virus titers in PBS group.

100, 20, and 4 μg protein of each vaccine of Sw/Hok/81, and $10^{5.0}$ PFU/g in the non-vaccinated mice.

To improve the efficacy of the Sw/Hok/81 vaccine, WV or ES vaccine of Sw/Hok/81 was injected twice into mice. At 2 weeks p.i., the serum NT antibody titers of the mice injected with the vaccine were higher than that of mice injected once (Table 3). Although the challenge appeared to be less severe compared to first experiment (Figure 3), the virus titers of the lungs of the mice were similar to those of mice injected once with Narita/09 vaccine (Table 3). These results indicate that even if an antigenic difference was observed between vaccine and challenge strains, the WV vaccine induced immunity in mice, decreasing the impact of disease caused by the challenge strain.

Discussion

Vaccination is a measure to reduce the impact of influenza; however, it takes 6 months to prepare a vaccine [16]. Virus isolates from humans usually do not grow well in embryonated chicken eggs, which poses significant limitations for influenza vaccine production. Attempts to increase the yield of candidate vaccine strains have been made by multiple passages in eggs over time or genetic reassortment with a high growth laboratory strain [17,18]. To prepare for pandemic influenza, a virus library of non-pathogenic influenza A viruses with 144 combinations of 16 HA and 9 NA subtypes has been established [15]. In the present study, we selected vaccine strains from 18 H1N1 virus isolates from birds, pigs, and humans on the basis of their growth in embryonated chicken eggs and their antigenicity. Among these viruses, the yield of Sw/Hok/81 in embryonated chicken eggs showed $10^{8.3}$ PFU/ml, which is higher than that of Narita/09 ($10^{7.3}$ PFU/ml), indicating that a virus strain selected from the influenza virus library could be used for the vaccine strain.

The 1957 and 1968 pandemic influenza virus strains were reassortants of avian and human strains [19]. Kida *et al.* showed that viruses in pigs are in antigenically stasis, as are those in ducks, compared with influenza viruses in humans [9,10]. The present results of antigenic analysis of H1N1 viruses indicate that pandemic 2009 (H1N1) virus was antigenically similar to that of classical swine influenza viruses, not to that of human influenza viruses, as previously described by Garten *et al.* [2]. Although we cannot predict the subtype of the pandemic strain, the antigenicity of the virus is conserved in pigs or ducks. Thus, antigenically related strains isolated from natural hosts could be used for human pandemic influenza vaccines. In order to update the influenza virus library as a seed of vaccine strains, continuous surveillance of avian and swine influenza and the study of pathogenicity, antigenicity, genetic information, and yield in chicken embryo of virus strains are needed.

In the present study, to prepare for future pandemics, we evaluated the potency of a vaccine prepared from Sw/Hok/81 against the pandemic 2009 (H1N1) virus. It was revealed that mice injected with WV or ES vaccine prepared from Sw/Hok/81 induced immunity to suppress the disease manifestation after challenge with Narita/09, although an

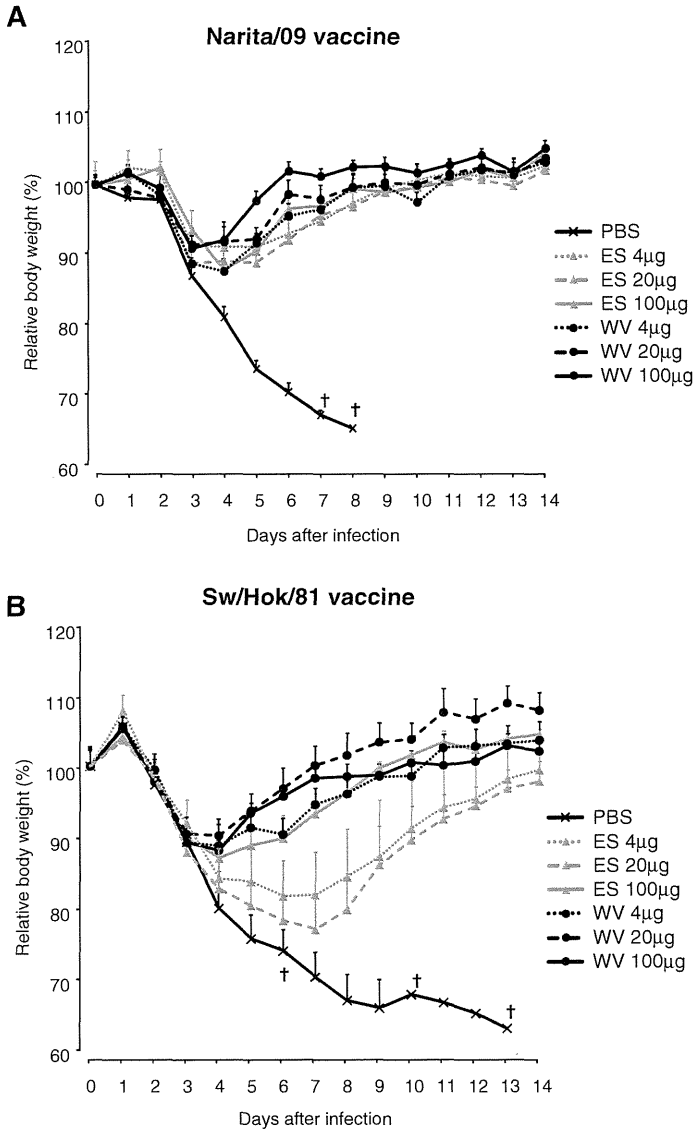

Figure 2 Changes in body weight of mice injected subcutaneously once with Narita/09 (A) or Sw/Hok/81 (B) vaccine after the challenge with Narita/09. Data are shown as mean body weight ± standard error. ES: ether split, WV: whole inactivated. †: Mice died.

antigenic difference was observed in these viruses. WV vaccine induces higher immune responses after intramuscular immunization and is superior to ES and subunit vaccine in human populations [20,21]. The reason for these immune responses to WV vaccine is the stimulation of innate [22] and cell-mediated immune responses to internal viral proteins. Indeed, identity of NP protein between Sw/Hok/81 and Narita/09 were 96.9%. In the previous studies, WV vaccine prepared from a virus strain selected from the library also showed protective efficacy against H5 and H7 virus infection in chicken, mice and cynomolgus macaques [23-28]. These results suggest that WV vaccine should work best in immunologically naive people in the early phase of a pandemic and two injections of the vaccine will be more effective even if the antigenicity of the pandemic strain is partially different from the vaccine strain.

Conclusion

The potency of the vaccine prepared from Sw/Hok/81 for the pandemic 2009 (H1N1) virus was evaluated. Mice injected once with WV vaccine prepared from Sw/Hok/81 induced immunity to suppress weight loss and virus growth

Table 3 Neutralizing antibody titers of mice injected twice with the vaccine and virus titers in the lungs after challenge

Vaccine			NT titer to		Virus titer in lungs, mean log PFU/g ±Se[a]
Strain	Protein, μg	Formulation	Narita/09	Sw/Hok/81	
PBS	-	-	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	4.4±0.08
Sw/Hok/81	4.0	ES	<10, <10, <10, <10, <10	20, 40, 80, 160, 160	4.4±0.07
	20	ES	<10, <10, <10, <10, <10	80, 80, 160, 160, 160	4.2±0.19
	100	ES	<10, <10, <10, <10, <10	80, 80, 160, 320, 310	3.9±0.14*
Sw/Hok/81	4.0	WV	<10, <10, <10, <10, <10	160, 320, 320, 640, 640	4.2±0.11
	20	WV	<10, <10, <10, <10, <10	160, 320, 640, 640, 640	3.9±0.28
	100	WV	<10, 10, 40, 40, 160	160, 320, 640, 640, 640	2.9±0.30**

Mice were injected twice with each vaccine subcutaneously with a 2-week interval. Serum samples were collected 2 weeks after the final immunization.
The animals were challenged by intranasal administration of 106.0 PFU of A/Narita/09.
At 3 days after challenge, lungs samples were collected and virus titers were measured. ES: ether split vaccine, WV: whole inactivated vaccine
a: Data are for 5 mice.
*: P<0.05, vs. virus titers in PBS group.
**: P<0.01, vs. virus titers in PBS group.

in the lungs after challenge with Narita/09. The suppression of virus recovery from lungs of mice injected twice with WV vaccine was similar to that in mice injected once with Narita/09 vaccine. These results suggest that WV vaccine should work best in immunologically naive people in the early phase of a pandemic, and two injections of the vaccine will be more effective if the antigenicity of the pandemic strain is partially different from the vaccine strain.

Materials and methods

Viruses and cells

Eighteen H1N1 influenza viruses isolated from humans, pigs and wild birds were used in the present study as representative of 42 H1N1 virus strains in our virus library (http://virusdb.czc.hokudai.ac.jp/). Narita/09 was provided by the National Institute of Infectious Diseases (Tokyo, Japan). Viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 hours.

Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (Nissui, Japan) supplemented with calf serum and used for titration of viral infectivity.

Sequencing and phylogenetic analysis

Viral RNA was extracted with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) from the allantoic fluid of chicken

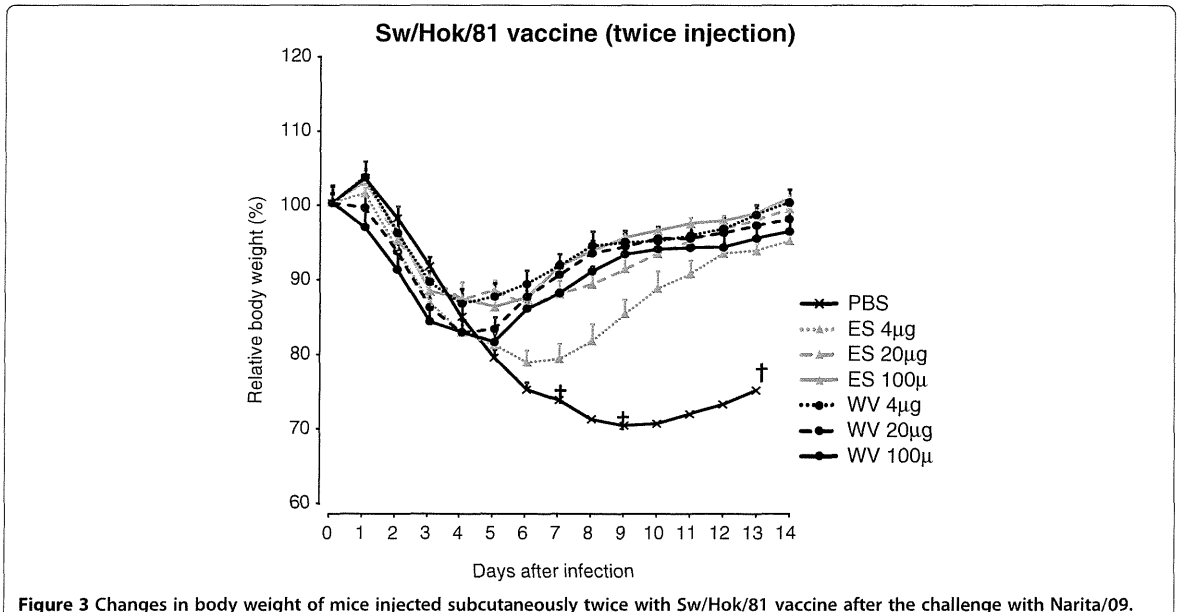

Figure 3 Changes in body weight of mice injected subcutaneously twice with Sw/Hok/81 vaccine after the challenge with Narita/09. Data are shown as mean body weight ± standard error. ES: ether split, WV: whole inactivated. †: Mice died.

embryos infected with the virus. Nucleotide sequences of all eight gene segments were determined after RT-PCR, as described previously [29]. The sequence data were analyzed using GENETYX ver. 9.1 (GENETYX Corporation, Tokyo, Japan). Phylogenetic analysis of the HA gene was performed by BioEdit ver. 7.0 and MEGA 5 by the neighbor-joining method with 1,000 bootstraps.

Serological tests

HI tests were performed by the microtiter method [30]. The HI titer was expressed as the reciprocal of the highest serum dilution showing complete inhibition of the hemagglutination of 4 HA units of the virus. In NT tests, titers were determined as the reciprocals of serum dilution of the complete inhibition of the cytopathic effect of 100 PFU of viruses using MDCK cells.

Viral growth in embryonated chicken eggs

Viruses of 100 50% egg infectious dose (EID_{50}) were inoculated into 10-day-old embryonated chicken eggs and incubated at 35°C for 48 hours. Allantoic fluid was harvested to determine viral titers at different time points (0, 12, 24, 48, and 72 hr). The PFU of each virus in the allantoic fluid was determined.

Vaccine preparation

To assess the potency of vaccines, inactivated WV vaccines of Sw/Hok/81 and Narita/09 were prepared as described previously [31]. ES vaccine of each strain was also prepared according to Kida *et al.* [32]. Briefly, purified viruses were disrupted with 0.1% Tween 80 and an equal volume of diethyl-ether for 30 min at room temperature. After centrifugation for 30 min at 6,000 g, the water phase was collected and ether dissolved in water was blown out with a stream of nitrogen.

Potency test of vaccine against Narita/09 in mice

WV or ES vaccines of each strain with 4, 20 and 100 μg protein were injected subcutaneously into ten 4-week-old female BALB/c mice (CLEA Japan Inc., Tokyo, Japan), respectively. PBS was injected into control mice. Three weeks after immunization, serum samples were collected and 30 μl of $10^{6.0}$ PFU of Narita/09 was intranasally inoculated into the mice under anesthesia. Three days after the challenge, five mice in each group were sacrificed and the lungs were collected. The virus titers in the lung homogenates were quantified by plaque assay of MDCK cells. Five other mice were observed for clinical signs and weight loss for 14 days. WV and ES vaccines of Sw/Hok/81 were also injected into mice twice with a 2-week interval. Two weeks after the final injection, the serum samples were collected and Narita/09 was inoculated into mice. Animal experiments were authorized by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approved numbers: 9148 and 1052) and all experiments were performed according to the guidelines of this committee.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MO drafted the manuscript and prepared the vaccines used in the present study. MO, TH, NM carried out animal experiment. YS, and HK participated in the coordination of the study. All authors read and approved the final manuscript.

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