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Modified Pulmonary Surfactant Is a Potent Adjuvant That Stimulates the Mucosal IgA Production in Response to the Influenza Virus Antigen[1]

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The intranasal administration of influenza hemagglutinin (HA) vaccine with Surfacten, a modified pulmonary surfactant free of antigenic c-type lectins, as a mucosal adjuvant induced the highest protective mucosal immunity in the airway. The intranasal immunization of mice with HA vaccine (0.2 μg)-Surfacten (0.2 μg) selectively induced the neutralizing anti-HA IgA, but not IgG, and conferred nearly maximal protection in the airway, without inducing a systemic response. In contrast, intranasal inoculation of vaccine with 0.2 μg of the potent mucosal adjuvant cholera toxin B* (CT-B*), prepared by adding 0.2% native CT to the B subunit of CT, induced both anti-HA IgA and IgG in the airway and in the serum. The intranasal administration of HA vaccine alone induced a limited amount of mucosal IgA against influenza virus. Although the s.c. administration of HA vaccine prominently induced serum IgG and IgA, Surfacten and CT-B* did not enhance their induction, and the concentrations of Abs leaking into the airways were insufficient to prevent viral multiplication. The intranasal administration of HA-Surfacten stimulated the expression of MHC class II, CD40, and CD86 molecules in the CD11c-positive cells isolated from the nasal mucosa, but not the expression of cells from the lungs or spleens. Lymphocytes isolated from the airway mucosa after intranasal HA-Surfacten immunization prominently induced TGF-β1 which, compared with inoculation without Surfacten, promoted an Ag-specific mucosal IgA response. Surfacten alone, however, did not induce TGF-β1. Our observations suggest that Surfacten, by mimicking the natural surfactant, is an effective mucosal adjuvant in the process of airway immunization. ***The Journal of Immunology,*** **2006, 176: 1122–1130.**

The respiratory mucosal surface is the site of viral infections as well as a major site of immune defense by the interconnecting inductive nose-associated lymphoid tissue (NALT)[3] (1). Influenza A virus (IAV) causes an annual epidemic infection by altering the antigenic properties of glycoproteins of the viral envelope. Although several currently available vaccines are administered i.m. or s.c., inducing a predominantly IgG-mediated protection in the systemic immune compartment, this systemic immunization offers insufficient protection at the mucosal surface (2). Furthermore, IgG leaking from the circulatory compartment into the mucus of airways protects only against homologous strains of virus. Mucosal IgA, in contrast, is the Ig primarily involved in the cross-protection of mucosal surfaces against variant virus infection (3–6). Consequently, the efficacy of current influenza vaccines, such as inactive or hemagglutinin (HA) vaccines, against epidemics of heterologous viruses is low (1). Intranasal vaccines have been studied to prevent infection at the site of initial invasion of the respiratory tract (3, 7, 8). However, most protein Ags are poor immunogens when delivered to the mucosa without adjuvant and, in some cases, result in immunological tolerance.

The coadministration of an adjuvant with the vaccine is essential to induce the highest mucosal protective immunity. Mucosal adjuvants are broadly divided into those acting as immunostimulatory molecules and those that facilitate the vaccine delivery (9). In the first group, the powerful oral or nasal adjuvants that have been extensively studied are toxin based, such as cholera toxin (CT) and *Escherichia coli* heat-labile toxin (HLT), which induce Ag-specific serum IgG and mucosal IgA (10, 11). These enterotoxins, however, cause severe diarrhea and are a threat to the CNS. The isolation of the B subunits of these toxins (cholera toxin B subunits (CTB) and HTL B subunits), or the creation of mutant toxins by substituting a single amino acid in the active center of ADP-ribosyltransferase in their toxin A subunits (7), has successfully removed their toxicity while increasing the induction of mucosal and systemic immunity (2, 12, 13). Recently, an inactivated influenza vaccine adjuvanted with HLT, highly effective in inducing IgA neutralizing Abs at the mucosa after its intranasal inoculation, has been tested in healthy volunteers in preclinical trials (14, 15); however, a high incidence of Bell's palsy was reported among recipients of the virosomal-influenza vaccine containing the HLT adjuvant, after its introduction in the Swiss market, in October 2000 (16). To develop safe and effective mucosal adjuvants conferring mucosal protective immunity in humans, we have searched among natural compounds, other than toxins, exhibiting strong mucosal adjuvant activity in the lungs. We observed that the pulmonary surfactant, a lamellated and/or film-like structure of lipid-protein complex, has an innate host defense activity (17, 18) and is rapidly turned over in alveolar type II cells, macrophages (19), and probably dendritic cells (DCs) in the airway.

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[3] Abbreviations used in this paper: NALT, nose-associated lymphoid tissue; IAV, influenza A virus; HA, hemagglutinin; CT, cholera toxin; HLT, *Escherichia coli* heat-labile toxin; CTB, cholera toxin B subunit; DC, dendritic cell; SP, surfactant protein.

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In this study, we examined the mucosal adjuvant activity of pulmonary surfactant and of Surfacten, a modified preparation free of pulmonary surfactant protein A (SP-A) and SP-D, administered intranasally with the influenza virus HA vaccine in BALB/c mice. We studied both the mucosal and systemic IgA Ab response, as well as the protective efficacy they conferred in comparison with a s.c. inoculation. Surfacten has been used in the treatment of respiratory distress syndrome in premature babies (20). To our surprise, intranasal administration of pulmonary surfactant and Surfacten as mucosal adjuvants complexed with IAV HA vaccine both induced mucosal neutralizing IgA against IAV in the airway as effectively as the most potent mucosal adjuvant, CT, though Surfacten (up to 0.2 μg) did not induce systemic IgG or IgA. The present study reports novel biological activities of pulmonary surfactant and Surfacten as effective mucosal adjuvants.

Materials and Methods

Virus and animals

The mouse-adapted IAV A/Aichi/2/68(H3N2) used for this study was provided by Dr. M. Ohuchi from the Kawasaki Medical School (Okayama, Japan). IAV was propagated in the allantoic cavity of 10-day-old embryonated hen's eggs at 35°C for 48 h. The infectivity of the virus was measured by plaque assay as described below. All experiments were performed in 6-wk-old BALB/c female mice obtained from Japan SLC. All animals were treated according to the guidelines for animal experimentation set by Tokushima University.

HA vaccines, surfactant, and Surfacten

HA vaccine (split-product virus vaccine) was prepared from IAV A/Aichi/2/68(H3N2) by the method of Davenport et al. (21). The virus preparation from the allantoic cavity was washed with PBS and incubated on ice with β-propiolactone (WAKO) for 18 h. Then β-propiolactone was degraded by incubation at 37°C for 90 min. Subsequently, the virus preparation was mixed with 0.1% Tween 20 and an equal volume of ether and then was incubated for 2 h at 4°C with stirring. To remove ether from the solution, the mixture was centrifuged at 800 $\times$ *g* for 5 min, and the aqueous phase was collected after evaporation at room temperature for 15 min. The protein concentration of HA vaccine was measured with the bicinchononic acid protein assay reagent (Pierce) by the method of Smith et al. (22). Pulmonary surfactant was prepared from bovine bronchoalveolar lavage by the method of Hawgood et al. (23), and the commercially available modified bovine pulmonary surfactant, Surfacten (Mitsubishi Pharma), a product free from SP-A and SP-D, was prepared as reported previously (24, 25). Because ~90% of pulmonary surfactant and Surfacten consist of lipids, their weights were expressed as those of lipids. To detect bacterial LPS contamination in the products of Surfacten, pulmonary surfactant, and HA vaccine, we measured LPS by the HEK-Blue LPS detection kit (InvivoGen).

Immunization of mice

Freshly prepared 0.02- to 2.0-μg suspensions of pulmonary surfactant or Surfacten and 0.2 μg of HA vaccine in 2 μl of PBS were treated for 3 min by a model 1510 sonic oscillator (Branson Ultrasonics) and kept at room temperature for 2 h until use. The mice were anesthetized with ether, immunized intranasally on day 0, and boosted on day 28 by instilling 1 μl of the adjuvant-combined HA vaccine into each nostril. CTB* as a positive mucosal adjuvant was prepared by adding 0.2% of native CT (Sigma-Aldrich) to CTB (Sigma-Aldrich) in all experiments (26). Alternatively, the animals received primary immunization on day 0 and a booster dose on day 28 by an s.c. injection of Surfacten, CTB*, or combined HA vaccine in 50 μl of PBS. Two weeks after the second immunization, serum, nasal wash, and lung wash specimens were prepared, or the lymphocytes from the nose, lung, and spleen were isolated. All animals that received vaccine and/or Surfacten were healthy and no apparent toxicity was detected during the study period.

Infection and virus titer

The mice were anesthetized with ether and infected intranasally with 3.5 $\times$ 10^4 PFU of mouse-adapted IAV/Aichi/2/68(H3N2) in 3 μl of saline instilled into each nostril. On day 3 after infection, virus titers in the nasal and lung wash, prepared as described later, were measured by a plaque assay on Madin-Darby canine kidney cells as described previously (27).

Preparation of nasal and lung washes and serum specimens

Bronchoalveolar wash (lung wash) specimens were obtained by three consecutive tracheal and lung infusions and aspirations of 1 ml of saline. Nasal wash specimens were obtained by a single 1-ml infusion of saline in the laryngeal region and then collection from the nostrils. The nasal and lung wash specimens were centrifuged at 2000 $\times$ *g* to remove cellular debris and were stored at −80°C until use. The serum specimens were prepared by drawing whole blood from the mouse's heart, and they were centrifuged at 2000 $\times$ *g* after coagulation.

Preparation of anti-IAV Abs

To separate the IgA and IgG fractions in the lung wash and serum, IgG was adsorbed on a protein G-Sepharose column (Amersham Biosciences), eluted with 0.1 M Glycine-HCl buffer (pH 2.8), and then neutralized with 0.5 M Tris-HCl buffer (pH 9.0) immediately after the elution. The eluate was dialyzed against PBS and concentrated by ultrafiltration. From the flow through fraction on a protein G-Sepharose column, IgA was purified on an affinity column constructed by coupling goat anti-mouse IgA (α-chain specific) Ab (Sigma-Aldrich) to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences). IgA was eluted with 0.1 M Glycine-HCl buffer at pH 2.8 from the column and neutralized with 0.5 M Tris-HCl buffer (pH 9.0). To purify anti-IAV Abs, purified IgA and IgG fractions were applied on an affinity column prepared by coupling the HA vaccine to the cyanogen bromide-activated Sepharose 4B. The anti-IAV Abs were eluted from the column as described earlier, lyophilized, and dissolved with PBS containing 0.01% BSA. The concentrations of Ag-specific Abs were measured by ELISA using a mouse IgA or IgG ELISA quantitation kit (Bethyl Laboratories) according to instructions provided by the manufacturer. The IgG contamination in the purified IgA fraction was under the detection level and vice versa.

Anti-HA vaccine-specific IgA and IgG levels

Total amounts of IgA and IgG in the nasal and lung washes and in the serum were measured by ELISA, using mouse IgA and IgG ELISA quantitation kits, respectively. The detection of HA vaccine-specific IgA and IgG is briefly described as follows. A 96-well plate (Nunc) was coated with HA vaccine and BSA (0.1 μg/well each) in PBS overnight at 4°C, then blocked with 1% BSA in 50 mM Tris-HCl (pH 8.0) containing 0.14 M NaCl and 0.05% Tween 20 (TTS) for 1 h at room temperature. The serum AND nasal and lung wash specimens diluted with TTS containing 1% BSA were added to each well and incubated for 2 h at room temperature. The plate was washed six times with TTS, incubated with goat anti-mouse IgA or anti-mouse IgG Ab conjugated with HRP for 1 h at room temperature, and incubated with a TMB Microwell Peroxidase Substrate System (KPL) according to the manufacturer's instructions. The chromogen produced was measured at an absorbance of 450 nm using a SPECTRA max PLUS 384 autoreader (Molecular Devices). We used 50 ng/ml purified mouse anti-HA vaccine IgA and IgG as standards.

Neutralizing Abs

The nasal wash of mice administered intranasally with HA or HA-Surfacten was collected and diluted with saline at 10- and 500-ng concentrations of anti-HA IgA per 3 ml. For neutralization, 5 $\times$ 10^4 PFU of IAV was incubated with 3 ml of diluted nasal wash on ice for 1 h. To determine neutralization, the virus titers in the neutralization mixtures serially diluted 100-fold were measured by plaque assay as described earlier. The amount of IgG was <1 ng in these diluted samples, confirming that blood contamination in the nasal wash was nearly negligible. The nasal wash of nonimmunized mice was also diluted to the same serial concentrations and then analyzed as a negative control.

DC isolation and flow cytometry

Nasal, pulmonary, and spleen cells from groups of 15 immunized or nonimmunized mice were isolated by collagenase digestion as described elsewhere (28, 29). The selection of DCs from these tissues was performed by magnetic cell sorting with a VarioMACS separator (Miltenyi Biotech) using anti-CD11c (N-418)-conjugated magnetic beads (no. 520-01) and an LS column according to the manufacturer's instructions. Positively selected cells were then passed through a second selection MS column (Miltenyi Biotec) to increase their purity (29). After primary intranasal immunization for 48 h, the DCs were immediately used for flow cytometry analyses by a FACS. DCs from nonimmunized mice were cultured for 7 days in cRPMI consisting of RPMI 1640 medium supplemented with 1% glutamine, 0.1 mM nonessential amino acids, 50 μM 2-ME, 1% penicillin-streptomycin (Sigma-Aldrich), 10% FCS, and 20 ng of recombinant murine granulocyte-macrophage CSF/ml. DCs (2 $\times$ 10^5 cells/well) were

seeded in new flat-bottom, 24-well plates (Nunc) with 1 ml of cRPMI, with or without 1 μg/ml HA vaccine, 1 μg/ml Surfacten, or both. After incubation for 24 h, the cells were harvested, washed twice with PBS containing 1 mM EDTA, and then subjected to flow cytometry.

DCs were exposed to FITC-conjugated anti-mouse I-A/I-E Ab and PE-anti-mouse CD40 or to FITC-anti-mouse CD80 and PE-anti-mouse CD86 (BD Pharmingen) for 30 min on ice. Concentration of all Abs used was 1 μg/ml. The cells were then washed twice with EDTA/PBS and fixed with 1% paraformaldehyde in PBS on ice for 15 min. Flow cytometry analysis was performed using a FACSCalibur cytometer (BD Biosciences), and the data were analyzed using CellQuest software (BD Biosciences).

Measurements of cytokines

Lymphocytes were prepared by Percoll density gradient centrifugation from the nose, lung, and spleen of mice immunized intranasally with HA-Surfacten (28) and suspended in RPMI 1640 medium supplemented with 10% FCS. The consistently >98% viability was verified by staining the cells with trypan blue, and the viable cells were cultured at a density of 1×10^6 cells/1 ml of medium/well in a 24-well plate, with or without 1 μg/ml HA vaccine, for 3 days at 37°C. Concentrations of IL-4, IL-5, IL-6, IL-13, and TGF-β1 in culture medium were measured by a competitive ELISA kit (BioSource International) according to the manufacturer's instructions. The concentrations of HA vaccine-induced cytokines were calculated by subtracting the concentrations in the conditioned medium of cultured lymphocytes treated without from those of lymphocytes treated with HA vaccine.

Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the unpaired Student t test. A value of $p < 0.01$ denoted the presence of a statistically significant difference.

Results

Effects of intranasal administration of pulmonary surfactant or Surfacten together with HA vaccine on anti-HA IgA production

To examine the adjuvant activity of pulmonary surfactant and Surfacten, we analyzed anti-HA IgA concentrations in the nasal and lung washes and in the serum 2 wk after booster intranasal immunization of mice with 0.2 μg of HA vaccine, with or without 0.2 μg of bovine pulmonary surfactant or Surfacten. In comparison with the intranasal inoculation of HA vaccine alone, immunization with both HA-pulmonary surfactant and HA-Surfacten increased the concentrations of HA-specific IgA by ~2- to 3.6-fold in the nasal and lung washes (Fig. 1, *A* and *B*), but not significantly in serum (Fig. 1*C*). Under the same experimental conditions, however, pulmonary surfactant and Surfacten did not increase the HA-specific IgG concentrations in those washes. Because the natural pulmonary surfactant contains antigenic C-type lectins, such as 26- to 38-kDa glycoprotein SP-A and 43-kDa SP-D, and because its lipid composition is variable among specimens, we used Surfacten (25), a reconstituted lung surfactant free from SP-A and SP-D and with a constant lipid composition, instead of the natural pulmonary surfactant in the following experiments to keep constant adjuvant activity.

Protective mucosal immunity against influenza virus infection in the nose and lung of mice inoculated intranasally with HA-Surfacten

The mucosal adjuvant effects of Surfacten in the protection conferred by the nasal influenza vaccine against influenza virus in BALB/c mice was compared with the effects of CTB*, one of the most effective mucosal adjuvants reported (12). The effectiveness of intranasal vs s.c. inoculation against viral infection was also examined. The mice received a primary intranasal or s.c. inoculation with HA-Surfacten (0.2 μg of each) or HA-CTB* (0.2 μg of each) and a boost 4 wk later. The mice were challenged with 7×10^4 PFU of IAV Aichi/2/68(H3N2) 2 wk after the second immunization. After virus infection for 2 days, significant loss of body weight was evident and logarithmic increase in the virus titer in lung wash was observed on days 3 and 4 (30). The virus titers in the nasal and lung washes were measured 3 days after the challenge (Fig. 2). Although separate intranasal administration of HA vaccine, Surfacten, and CTB* did not show significant protective mucosal immunity in the airway, HA-Surfacten or HA-CTB* markedly lowered the virus titers to <1/1000 in the nasal and lung washes (Fig. 2, *A* and *B*). It is particularly noteworthy that the virus titers were below the detection level in 81% of mice inoculated intranasally with HA-Surfacten, vs 72% of mice inoculated with HA-CTB*. There was no significant difference in adjuvanticity between 0.2 μg of Surfacten (consisting of ~97% lipid and 3% protein) and 0.2 μg of CTB* (consisting of protein only). In contrast with the effect of intranasal immunization, the s.c. administration of HA vaccine alone suppressed the virus titers by only ~10% in the nasal and lung washes, and no adjuvanticity was conferred by Surfacten or CTB* to the s.c. inoculation (Fig. 2, *C* and *D*). Although no loss of body weight was detected on day 3 only in the animals intranasally treated with HA-Surfacten and HA-CTB*, loss of body weight in the other groups, including animals treated with s.c. immunization, was evident (data not

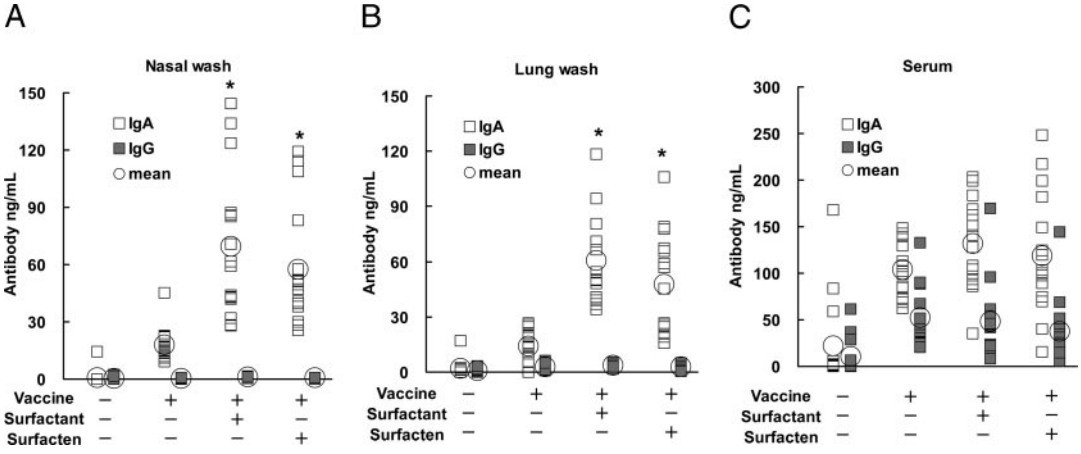

FIGURE 1. Effects of intranasal inoculation of pulmonary surfactant, or Surfacten combined with HA vaccine, on the production of Ag-specific IgA and IgG. Anti-IAV IgA (□) and IgG (▨) responses in the nasal wash (*A*), lung wash (*B*), and serum (*C*) 2 wk after boost intranasal inoculation of 0.2 μg of HA vaccine with or without 0.2 μg of pulmonary surfactant or Surfacten. Each circle represents the mean Ab concentration in each group of 12–15 mice. *, Significant ($p < 0.01$) increase in the Ab concentrations in comparison with those measured in mice inoculated with HA vaccine alone.

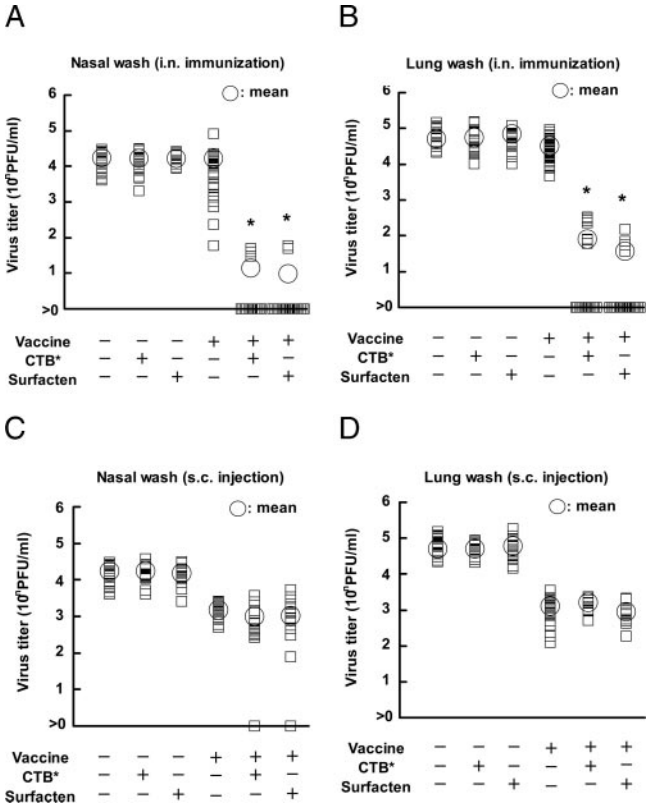

FIGURE 2. Effects of intranasal (i.n.) or s.c. administration of 0.2 μg of HA vaccine with or without 0.2 μg of Surfacten or of 0.2 μg of CTB* on IAV titers in the nasal and lung washes. Two weeks after the second vaccination, the mice were inoculated with 7×10^4 PFU of IAV. Three days after infection, virus titers (PFU/ml; □) in the nasal (*A* and *C*) and lung (*B* and *D*) washes were measured as described in *Materials and Methods*. CTB* was used as a positive control. Each circle represents the mean values of each group of 15–30 (intranasal administration) or 12–20 (s.c. administration) mice. *, Significant ($p < 0.01$) decrease in virus titers compared with titers measured in mice that received HA vaccine alone.

shown). These results agreed with the virus titers in nasal and lung washes.

Effects of Surfacten and CTB on the mucosal and systemic immune responses*

To compare the mucosal and systemic Ab responses of mice treated with intranasal vs s.c. administration of HA-Surfacten or HA-CTB*, we analyzed the concentrations of anti-HA IgA and IgG in the nasal and lung washes and in the serum 2 wk after the boost inoculation. CTB* is a mucosal adjuvant, which enhances both mucosal and systemic Ab responses (10). Intranasal instillation of HA vaccine (0.2 μg) alone induced a slight increase, whereas HA-Surfacten or HA-CTB* (0.2 μg of each) induced marked augmentation of mucosal anti-HA IgA in the nasal and lung washes (Fig. 3, *A* and *C*). In contrast, the s.c. inoculation of the HA vaccine alone increased the mucosal concentrations of both the IgA and IgG concentrations slightly, though significantly in the nasal and lung washes (Fig. 3, *B* and *D*). However, neither Surfacten nor CTB* increased the mucosal IgA and IgG concentrations in response to the s.c. inoculation. In contrast with the mucosal immune responses, the serum IgG and IgA were prominently induced by the s.c. inoculation of the HA vaccine alone, and Surfacten or CTB* had no additional adjuvant effects (Fig. 3*F*). A modest induction of serum IgG and IgA was also observed after the intranasal inoculation of the HA vaccine alone, and this induction was significantly enhanced by CTB*, but not by Surfacten (Fig. 3*E*). CTB* and Surfacten when administered alone did not affect the immune responses in the nasal and lung washes or in the serum. These observations indicate that the intranasal inoculation of HA-Surfacten (at a dose of 0.2 μg of each) specifically induced a marked mucosal IgA response, and that of HA-CTB* induced both mucosal and systemic IgG and IgA responses.

Neutralization of IAV by mucosal IgA

IgA is the prominent protective mucosal Ig fraction in the airway. A neutralization assay of the induced IgA was conducted in the nasal wash of mice immunized intranasally with HA-Surfacten (Fig. 4). Anti-HA IgA in doses of >50 ng neutralized IAV in the nasal wash significantly, and the neutralization reached its peak at doses $\geq$ 250 ng. No IgG was detected in the anti-HA IgA preparation. No neutralization activity was observed in the nasal wash of nonimmunized mice, which lack anti-HA Abs. The suppression of IAV titers in the lung wash was similar to that observed in the nasal wash (data not shown).

Dose-response curves of mucosal immunity conferred by Surfacten

The maximal adjuvant activity of Surfacten on the induction of HA-specific IgA and IgG in the nasal and lung washes, after its initial and boost intranasal inoculation combined with a fixed dose of 0.2 μg of HA vaccine, was examined by dose-response curves. In doses between 0.0 μg and 0.2 μg, Surfacten induced mucosal anti-HA IgA in a dose-dependent manner, reaching a maximal induction at doses > 0.2 μg in both washes (Fig. 5). However, in doses > 1 μg, Surfacten began to induce mucosal anti-HA IgG. This observation indicates that the induction of IgA immunity in the airway mucosa is more responsive to Surfacten, an adjuvant mimicking the natural pulmonary surfactant, than that of IgG.

Stimulation of nasal APCs by HA-Surfacten

The priming of naive $CD4^+$ T cells in the secondary lymphoid organs by APCs such as DCs is preceded by a modification of APCs upon exposure to the Ag. This modification is characterized by the regulation of MHC products and costimulatory molecules on the cell surface. Therefore, we measured the expression levels of MHC class II, CD40, CD80, and CD86 on DCs by FACS after vaccination with HA-Surfacten in vitro and in vivo. Incubation for 24 h of DCs isolated from the nose, lung, and spleen with HA-Surfacten induced a phenotypic change in the DCs (Fig. 6*A*). Compared with DCs pulsed with the HA vaccine alone, all DCs from various organs pulsed with HA-Surfacten enhanced the surface expression of MHC class II, CD40, and CD86 significantly, but not that of CD80. However, DCs pulsed with Surfacten alone did not change the expression of these molecules on the cell surface, compared with nonpulsed DCs. These observations suggest that treatment with HA-Surfacten stimulates the maturation of DCs isolated from mucosal tissues and spleen in vitro.

To examine the mucosal adjuvanticity of Surfacten on APCs in vivo, the expressions of MHC class II and costimulatory molecules on the surface of DCs isolated from the nose, lung, and spleen were analyzed 48 h after primary intranasal inoculation with HA-Surfacten. Compared with the expressions observed in mice inoculated with the HA vaccine alone, this intranasal inoculation induced a modest up-regulation of MHC class II, CD40, and CD86, but not CD80, on the DCs harvested from the nose, but not from the lung or spleen. However, the intranasal inoculation of Surfacten alone did not change these expression levels (Fig. 6*B*). These results suggest that the intranasal inoculation of HA-Surfacten specifically stimulates the local maturation of nasal APCs,

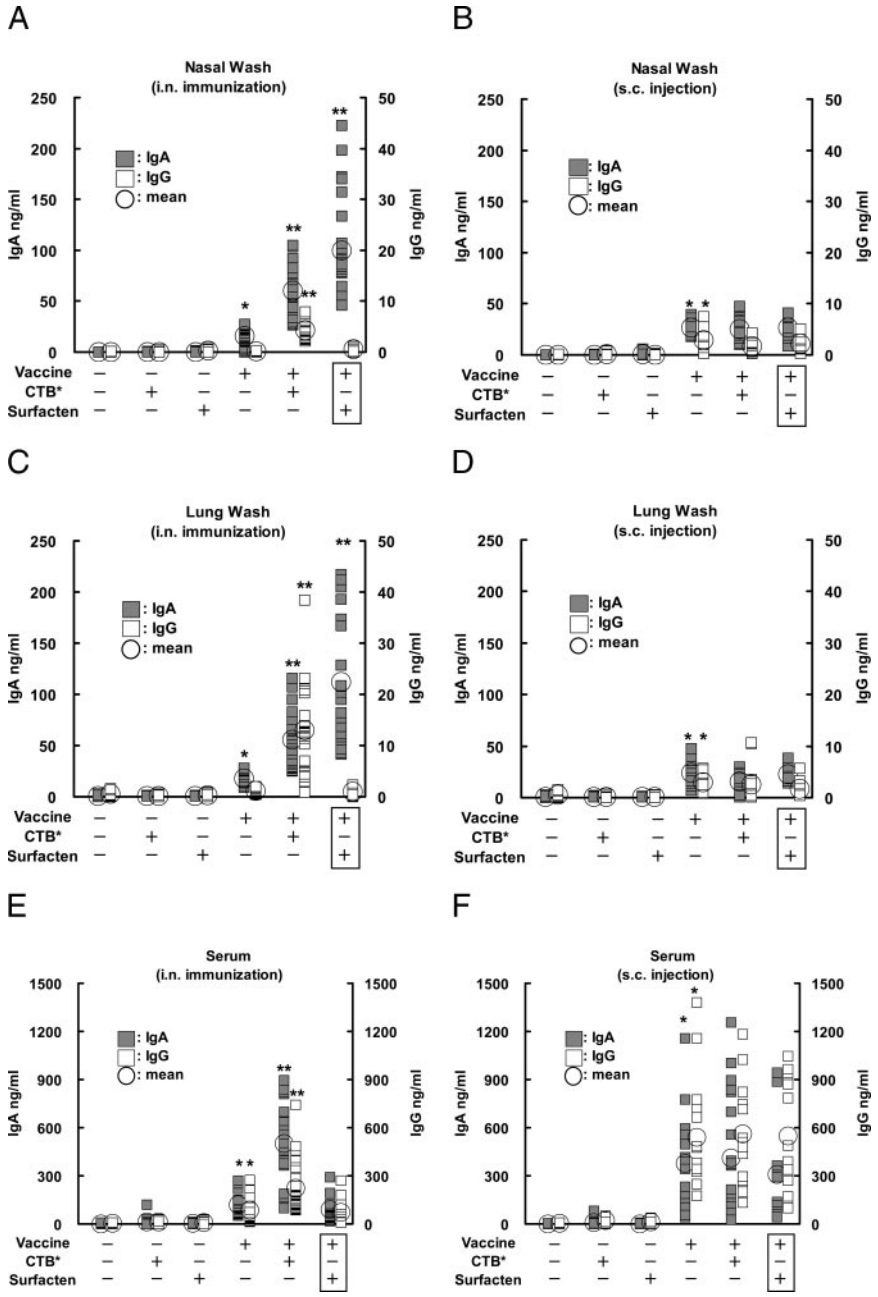

FIGURE 3. Effects of intranasal (i.n.) or s.c. administration of Surfacten and CTB* combined with the HA vaccine on mucosal and systemic immune responses. The concentrations of anti-IAV IgA (■) and IgG (□) in the nasal wash (*A* and *B*), lung wash (*C* and *D*), and serum (*E* and *F*) were measured after the secondary intranasal (*A*, *C*, and *E*) or s.c. (*B*, *D*, and *F*) inoculation of 0.2 μg of HA vaccine with or without 0.2 μg of Surfacten or of 0.2 μg of CTB* for 2 wk. Each circle represents the mean value in each group of 15–30 (intranasal) or 12–20 (s.c.) mice. *, Significant increase in the Ab concentrations ($p < 0.01$) in comparison with the concentrations measured in mice inoculated with HA vaccine alone. **, $p < 0.01$, in comparison with the concentrations measured in mice that received PBS as a vehicle.

though it does not affect the DCs from the lung and spleen at 48 h after primary immunization.

Release of TGF-β1 and Th2-type cytokines from the lymphocytes after stimulation by Ag in vitro

To clarify the mechanisms of induction of mucosal immunity by HA-Surfacten, the release profiles of cytokines from the lymphocytes of the nose, lung, and spleen of immunized mice were analyzed after stimulation by HA Ag in vitro (Table I). After stimulation in vitro by 1 μg/ml HA vaccine for 48 h, significant increases were observed in the release of TGF-β1, IL-5, and IL-6 from lymphocytes harvested from the nose and lung, but not from the spleen of mice inoculated intranasally by HA-Surfacten, compared with mice inoculated with the HA vaccine alone; however, the release of IL-4 and IL-13 (which are involved in systemic IgG and IgE responses) by HA Ag stimulation from lymphocytes harvested from these same organs of mice inoculated intranasally by HA-Surfacten, did not change significantly.

Discussion

This study demonstrates that the intranasal inoculation of influenza virus HA Ag entrapped with low doses of <0.2 μg of Surfacten, a modified natural pulmonary surfactant used as a mucosal adjuvant, induced a prominent production of mucosal neutralizing IgA in the airway of mice, without inducing systemic IgA and IgG responses. Surfacten, in a phospholipid dose of 0.2 μg, induced maximal mucosal protective immunity of IgA, its efficacy being equivalent to 0.2 μg of CTB* protein, one of the most effective mucosal toxin-based adjuvants, which stimulates mucosal as well as systemic immune responses (10). In contrast, the s.c. inoculation of HA-Surfacten augmented neither the mucosal nor the systemic IgA and IgG responses induced by treatment with the HA vaccine alone.

Pulmonary surfactant, a complex mixture of proteins and phospholipids, lowers the surface tension of the air-liquid interface and prevents collapse of the alveoli. The natural surfactant contains

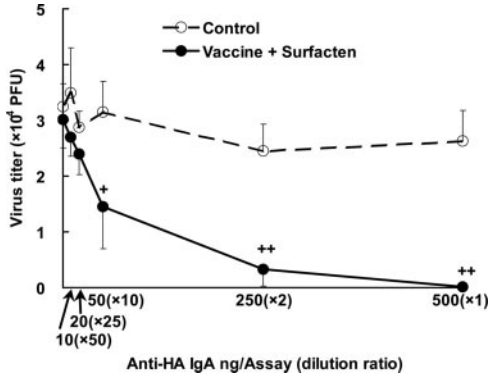

FIGURE 4. Neutralization of IAV by anti-HA IgA in the nasal wash of mice immunized intranasally with the HA vaccine combined with Surfacten. Various dilutions (1- to 50-fold) of the nasal wash of mice with (solid line) or without (dashed line) intranasal inoculation of HA-Surfacten were incubated on ice for 1 h with 5×10^4 PFU of IAV. The original nasal wash of immunized mice contained 500 ng of anti-HA IgA/3 ml of assay volume. After incubation, the virus titers (PFU/ml) in the neutralization mixture were determined (●). The neutralizing activity of a series of diluted (1- to 50-fold) nasal wash from nonimmunized mice were also tested as negative control experiments (○). Data represent the mean (± SD) of six experiments. +, $p < 0.05$; ++, $p < 0.01$; indicate a significant suppression of virus titers in comparison with the controls.

four different types of proteins, including the hydrophilic C-type lectins SP-A and SP-D, which play a role in the innate defense system (31), and the small hydrophobic proteins SP-B and SP-C, which play a role in lowering the surface tension and in the formation of a stable surfactant monolayer. In the present study, we used the modified pulmonary surfactant Surfacten instead of the natural product because their adjuvanticities are nearly equivalent (Fig. 1) and because Surfacten is used clinically in neonates. Surfacten contains SP-B and SP-C, representing ~3% of its weight, but not antigenic SP-A and SP-D. The addition of synthetic lipids adjusts its content in unsaturated phosphatidylcholine, fatty acids, and triacylglycerols to 47, 7, and 7% of its weight, respectively (24, 25).

Mucosal adjuvants assist in the delivery of vaccine into mucosal APC cells (e.g., macrophages and DCs) or function as mucosal immunostimulants or act as both. The pulmonary surfactant is rapidly turned over and recycled in vivo by alveolar cells, macrophages, and probably by DCs, with a half-life of 6–7 h (19, 32), and SP-B and SP-C proteins stimulate the uptake of surfactant-like liposomes by these cells (33). These observations suggest that SP-B and SP-C in Surfacten efficiently promote the delivery of the HA vaccine combined with the liposome into the nasal APCs, after the intranasal inoculation of the liposome adjuvant and the Ag.

Surfacten also acts as a mucosal immunostimulant. The in vitro studies showed that HA-Surfacten modestly stimulated the expression of MHC class II, CD40, and CD86 of DCs from the nose, lung, and spleen (Fig. 6*A*). The up-regulation of MHC class II is a marker of DC maturation after an immune response by increasing the efficiency of $CD4^+$ T cell priming (34). In addition, the up-regulation of costimulatory molecules on APCs, especially CD86, is required for the induction of mucosal IgA responses (35, 36). In vivo, the observation of similar stimulatory effects of HA-Surfacten on DCs was limited to DCs from the nose and did not include DCs from the lung and spleen, probably because of the small volume of HA-Surfacten used for limited inoculation into the nostrils (Fig. 6*B*).

Microfold epithelial cells and DCs of the NALT are the major sites of Ag uptake and immune responses in the nose (37). Although an endogenous pulmonary surfactant may play a role in mucosal immunity in the lung, it does not play a role in the nose under physiological conditions because pulmonary surfactant is secreted only by alveolar type II cells in the lungs and by Clara cells in the terminal and respiratory bronchioles (17). The present study proposes application of Surfacten as a mucosal adjuvant to the NALT.

Intranasal inoculation of HA-Surfacten sensitized the nasal and lung lymphocytes of immunized mice, but not the spleen lymphocytes, to produce TGF-β1, IL-5, and IL-6 after their stimulation with Ag in vitro, in comparison with those of mice pretreated with HA vaccine alone. TGF-β1 is a pleiotropic cytokine known to affect T cell proliferation (38, 39), differentiation (40), Ag presentation, effector functions of macrophages, and the expression of

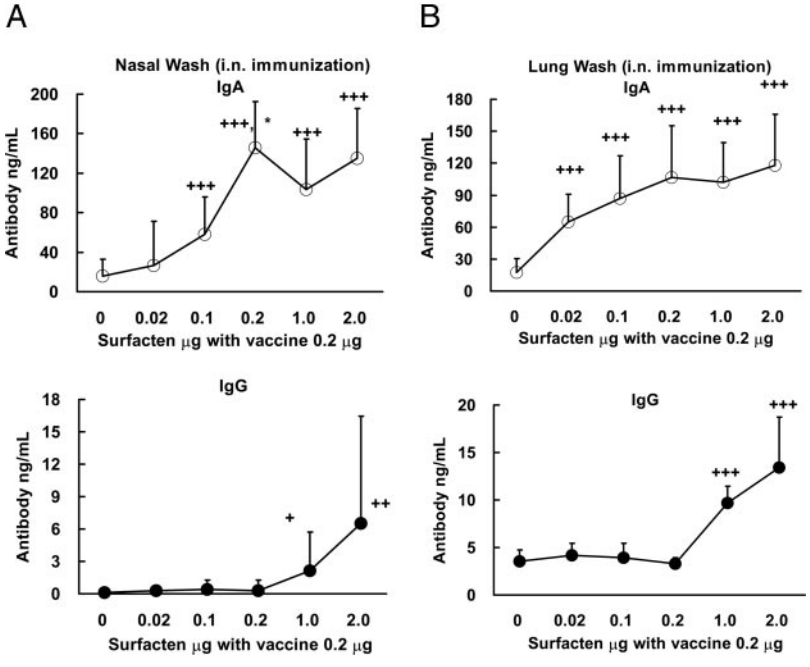

FIGURE 5. Dose-response curves of Surfacten with respect to anti-IAV IgA and IgG production in the nasal wash (*A*) and lung wash (*B*) after the intranasal (i.n.) secondary immunization of HA-Surfacten. Mice were immunized intranasally with the HA vaccine (protein dose = 0.2 μg) combined with a 0- to 2-μg phospholipid dose of Surfacten as described in *Materials and Methods*. Data are the mean values of IgA (○) and IgG (●) ± SD in each group of 8–12 mice. +, $p < 0.08$; ++, $p < 0.05$; +++, $p < 0.01$; indicate a significant increase in the Ab concentration in comparison with the concentrations measured in mice that received vaccine alone. *, $p < 0.05$, in comparison with the concentrations measured in mice that received vaccine combined with 0.1 μg of Surfacten.

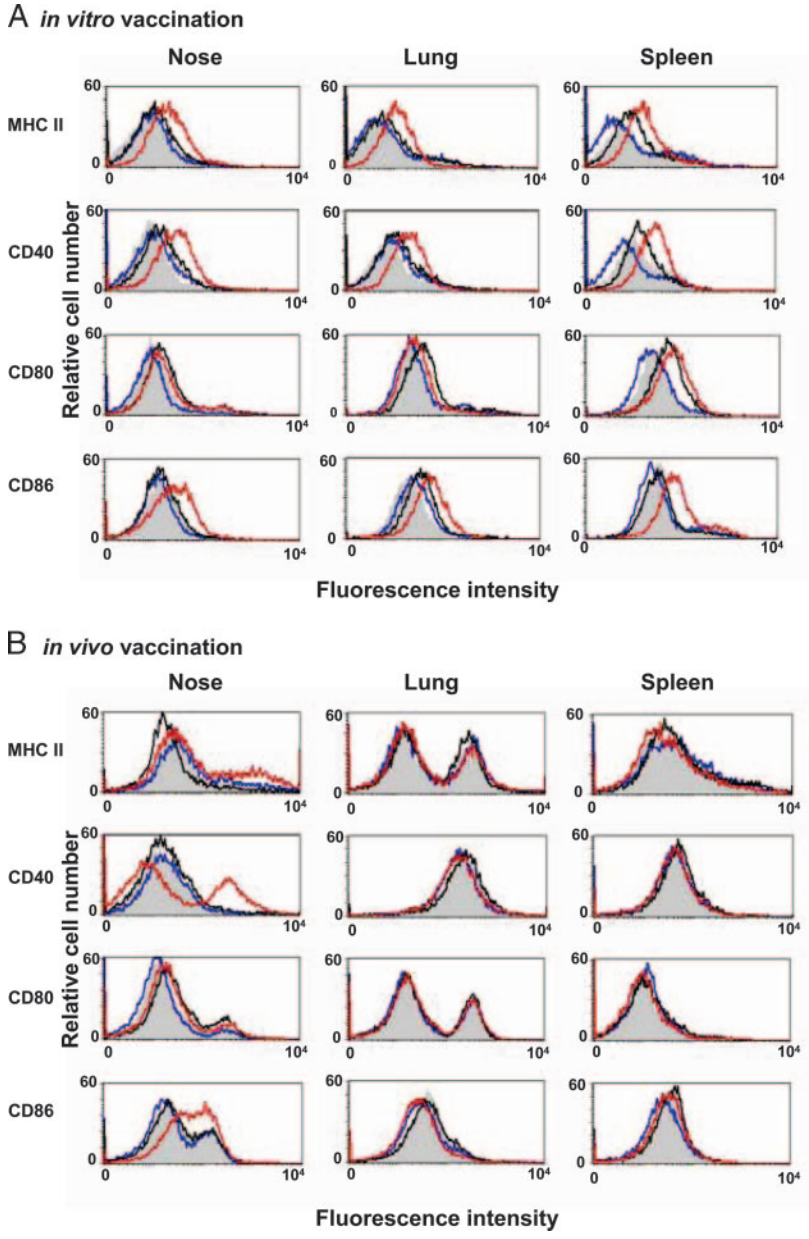

FIGURE 6. Activation of DCs by HA vaccine with or without Surfacten in vitro (*A*) and in vivo (*B*). *A*, $CD11c^+$ cells isolated from the nose, lung, and spleen of nonimmunized mice were plated in the absence or presence of 1 μg/ml HA vaccine with or without 1 μg/ml Surfacten for 24 h. Fluorescent intensity data were recorded for nonpulsed (shaded area), Surfacten-pulsed (blue line), HA vaccine-pulsed (black line), and HA-Surfacten-pulsed (red line) experiments. Data are representative of at least three experiments. *B*, $CD11c^+$ cells were isolated from mice that had been primarily inoculated for 2 days with PBS or with 0.2 μg of HA vaccine with or without 0.2 μg of Surfacten. The expressions of MHC class II, CD40, CD80, and CD86 on DCs were assessed by flow cytometry. Data were recorded for $CD11c^+$ cells from unvaccinated mice (shaded area) and mice inoculated intranasally with Surfacten (blue line), HA vaccine (black line), and HA-Surfacten (red line). Data are representative of at least three experiments.

accessory molecules on APCs (41, 42). Administration of Ag to mucosal tissue often generates Ag-specific, TGF-β1-secreting Th3 regulatory T cells and induces immune tolerance without any increase in IgG response (43, 44). In addition, TGF-β1 activates B cells and Ig isotype switch to IgA^+ B cells (45). Furthermore, IL-5 and IL-6 are important IgA-enhancing cytokines for the preferential activation and clonal expansion of IgA^+ B cells and their terminal differentiation into IgA plasma cells (7, 46). These findings

Table I. *HA vaccine-induced stimulation of cytokine production by cultured lymphocytes from the nose, lung, and spleen of mice intranasally immunized with HA vaccine-Surfacten or HA vaccine alone*[a]

	IL-4	IL-5	IL-6 (pg/10^6 cells)	IL-13	TGF-β1
Vaccine					
Nose	2.1 ± 3.0	26.4 ± 19.1	15.0 ± 26.3	5.0 ± 7.2	627.1 ± 377.4
Lung	1.6 ± 1.1	11.9 ± 7.5	2.9 ± 5.7	3.0 ± 6.3	289.7 ± 428.9
Spleen	<0	10.3 ± 20.1	57.1 ± 27.4	1.7 ± 4.1	752.6 ± 381.5
Vaccine + Surfacten					
Nose	8.7 ± 7.1	187.6 ± 52.1^{++}	271.2 ± 72.4^{++}	1.6 ± 3.9	1874.4 ± 372.8^{+++}
Lung	7.1 ± 8.4	102.0 ± 61.9^{+}	269.4 ± 181.5^{++}	2.2 ± 4.7	1559.2 ± 340.4^{+++}
Spleen	6.4 ± 6.4	30.0 ± 48.6	31.4 ± 50.7	7.2 ± 16.0	900.5 ± 284.1

[a] Data are expressed as a net increase in the cytokine levels after treatment with HA vaccine or HA-Surfacten after calculation by subtracting the cytokine levels in the conditioned medium of untreated lymphocytes from those of treated lymphocytes. Values are mean ± SD of three or four separate experiments in each group.
+, $p = 0.06$; ++, $p < 0.05$; +++, $p < 0.01$ compared with the respective cytokine level for HA vaccine alone.

suggest that up-regulation by intranasal administration of HA-Surfacten of TGF-β1, IL-5, and IL-6 in mucosal lymphocytes plays a crucial role in the production of mucosal Ag-specific IgA.

It has been reported that induction of various cytokines are triggered by bacterial components, such as LPS and peptidoglycans (47). To exclude the possibility of LPS contamination in the samples of HA vaccine, surfactant, and Surfacten, we measured LPS levels in those samples. LPS level was <1 ng/mg of each sample and presumed LPS amounts administrated to animals and in cell culture were 10 pg/kg and 1 pg/ml, respectively. These data suggest that tracing amounts of LPS in the samples has no stimulatory effects on cytokine release from lymphocytes and the up-regulation of cell surface molecules of DCs (48).

It is noteworthy that the intranasal inoculation of low doses of Surfacten ≤ 0.2 μg combined with 0.2 μg of HA vaccine specifically enhanced the mucosal IgA response without influencing systemic IgG (Fig. 5), although all reported potent toxin-based adjuvants, such as CT and HLT, promote both mucosal and systemic immunity to coadministered Ags (2, 10–13). However, distinct adjuvant effects of Surfacten in doses $>$ 1–2 μg were observed, by the induction of mucosal IgG in the nasal and lung washes. The IgG response to >1 μg of Surfacten may be partly related to the previously reported induction of a systemic immune response in the spleen and serum by intratracheal instillation of an extremely high phospholipid dose of 300 μg of surfactant-like liposome combined with Ag, in animals depleted of their alveolar macrophages (49). However, that study did not report the observation of mucosal immunity.

Mucosal IgA represents the first immunological barrier to pathogens that infect the epithelial surface, with cross-protection against variant virus infection (4), resulting in an overall higher protective activity than with systemic IgG. In addition, IgA is a noninflammatory Ab, whereas the binding of several systemic Abs, such as IgE and IgG, to Ags initiates an inflammatory response. In our experiments, the magnitude of the response of the Ag-specific mucosal IgA in the nasal and lung washes induced by intranasal inoculation of HA-Surfacten was 6-fold higher than the response to vaccine without Surfacten and ~2-fold higher than the response to the vaccine with CTB*. The magnitude of mucosal immunological response to intranasal inoculation was 4-fold higher than that observed after s.c. vaccination (Fig. 3). In addition, the intranasal and s.c. inoculation of Surfacten alone did not induce inflammatory cell migration in vivo (data not shown). Our data suggest that Surfacten, by mimicking the natural surfactant, is a safe and effective mucosal adjuvant that enhances the protective mucosal immunity without incurring a risk of inflammation. This is the first report of the experimental application of Surfacten for the purpose of nasal vaccination against IAV.

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Disclosures

The authors have no financial conflict of interest.

References

1. Murphy, B. R., and R.G. Webster. 1996. Orthomyxoviruses. In *Virology.* B. N. Field and D. N. Knipe, eds. Lippincott-Raven, Philadelphia, pp. 1397–1445.
2. Tamura, S. I., H. Asanuma, Y. Ito, Y. Hirabayashi, Y. Suzuki, T. Nagamine, C. Aizawa, T. Kurata, and A. Oya. 1992. Superior cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza hemagglutinin vaccine. *Eur. J. Immunol.* 22: 477–481.
3. Waldman, R. H., and R. Ganguly. 1974. Immunity to infections on secretory surfaces. *J. Infect. Dis.* 130: 419–440.
4. Liew, F. Y., S. M. Russell, G. Appleyard, C. M. Brand, and J. Beale. 1984. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA Ab rather than serum Ab or cytotoxic T cell reactivity. *Eur. J. Immunol.* 14: 350–356.
5. Murphy, B. R., and M. L. Clements. 1989. The systemic and mucosal immune response of humans to influenza A virus. *Curr. Top. Microbiol. Immunol.* 146: 107–116.
6. Renenger, K. B., and P. A. Small, Jr. 1994. Passive immunization: systemic and mucosal. In *Handbook of Mucosal Immunology*. P. L. Ogra, M. E. Lamm, J. R. McGhee, J. Mestecky, W. Strober, and J. Bienenstock, eds. Academic Press, San Diego, pp. 333–343.
7. Yuki, Y., and H. Kiyono. 2003. New generation of mucosal adjuvants for the induction of protective immunity. *Rev. Med. Virol.* 13: 293–310.
8. Greenbaum, E., A. Furst, A. Kiderman, B. Stewart, R. Levy, M. Schlesinger, A. Morag, and Z. Zakay-Rones. 2001. Serum and mucosal immunologic responses in children following the administration of a new inactivated intranasal anti-influenza vaccine. *J. Med. Virol.* 65: 178–184.
9. O'Hagan, D. T., M. L. MacKichan, and M. Singh. 2001. Recent developments in adjuvants for vaccines against infectious diseases. *Biomol. Eng.* 18: 69–85.
10. Tamura, S., A. Yamanaka, M. Shimohara, T. Tomita, K. Komase, Y. Tsuda, Y. Suzuki, T. Nagamine, K. Kawahara, and H. Danbara. 1994. Synergistic action of cholera toxin B subunit (and *Escherichia coli* heat-labile toxin B subunit) and a trace amount of cholera whole toxin as an adjuvant for nasal influenza vaccine. *Vaccine* 12: 419–426.
11. Gluck, R., R. Mischler, P. Durrer, E. Furer, A. B. Lang, C. Herzog, and S. J. Cryz, Jr. 2000. Safety and immunogenicity of intranasally administered inactivated trivalent virosome-formulated influenza vaccine containing *Escherichia coli* heat-labile toxin as a mucosal adjuvant. *J. Infect. Dis.* 181: 1129–1132.
12. Wilson, A. D., C. J. Clarke, and C. R. Stokes. 1990. Whole cholera toxin and B subunit act synergistically as an adjuvant for the mucosal immune response of mice to keyhole limpet haemocyanin. *Scand. J. Immunol.* 31: 443–451.
13. Tamura, S., H. Asanuma, T. Tomita, K. Komase, K. Kawahara, H. Danbara, N. Hattori, K. Watanabe, Y. Suzuki, and T. Nagamine. 1994. *Escherichia coli* heat-labile enterotoxin B subunits supplemented with a trace amount of the holotoxin as an adjuvant for nasal influenza vaccine. *Vaccine* 12: 1083–1089.
14. de, B., A. Zanasi, S. Ragusa, R. Gluck, and C. Herzog. 2002. An open-label comparison of the immunogenicity and tolerability of intranasal and intramuscular formulations of virosomal influenza vaccine in healthy adults. *Clin. Ther.* 24: 100–111.
15. Durrer, P., U. Gluck, C. Spyr, A. B. Lang, R. Zurbriggen, C. Herzog, and R. Gluck. 2003. Mucosal antibody response induced with a nasal virosome-based influenza vaccine. *Vaccine* 21: 4328–4334.
16. Mutsch, M., W. Zhou, P. Rhodes, M. Bopp, R. T. Chen, T. Linder, C. Spyr, and R. Steffen. 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N. Engl. J. Med.* 350: 896–903.
17. Crouch, E., and J. R. Wright. 2001. Surfactant proteins A and D and pulmonary host defense. *Annu. Rev. Physiol.* 63: 521–554.
18. Kudo, K., H. Sano, H. Takahashi, K. Kuronuma, S. Yokota, N. Fujii, K. Shimada, I. Yano, Y. Kumazawa, D. R. Voelker, S. Abe, and Y. Kuroki. 2004. Pulmonary collectins enhance phagocytosis of *Mycobacterium avium* through increased activity of mannose receptor. *J. Immunol.* 172: 7592–7602.
19. Gurel, O., M. Ikegami, Z. C. Chroneos, and A. H. Jobe. 2001. Macrophage and type II cell catabolism of SP-A and saturated phosphatidylcholine in mouse lungs. *Am. J. Physiol.* 280: L1266–L1272.
20. Konishi, M., S. Chida, S. Shimada, T. Kasai, Y. Murakami, K. Cho, Y. Fujii, H. Maeta, and T. Fujiwara. 1992. Surfactant replacement therapy in premature babies with respiratory distress syndrome: factors affecting the response to surfactant and comparison of outcome from 1982–86 and 1987–91. *Acta Paediatr. Jpn.* 34: 617–630.
21. Davenport, F. M., A. V. Hennessy, F. M. Brandon, R. G. Webster, C. D. Barrett, Jr., and G. O. Lease. 1964. Comparisons of serologic and febrile responses in humans to vaccination with influenza A viruses or their hemagglutinins. *J. Lab. Clin. Med.* 63: 5–13.
22. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150: 76–85.
23. Hawgood, S., B. J. Benson, and R. L. Hamilton, Jr. 1985. Effects of a surfactant-associated protein and calcium ions on the structure and surface activity of lung surfactant lipids. *Biochemistry* 24: 184–190.
24. Tanaka, Y., T. Takei, T. Aiba, K. Masuda, A. Kiuchi, and T. Fujiwara. 1986. Development of synthetic lung surfactants. *J. Lipid. Res.* 27: 475–485.
25. Tanaka, Y., and T. Takei. 1983. Lung surfactants. I. Comparison of surfactants prepared from lungs of calf, ox, dog and rabbit. *Chem. Pharm. Bull. (Tokyo).* 31: 4091–4099.
26. Watanabe, I., Y. Hagiwara, S. E. Kadowaki, T. Yoshikawa, K. Komase, C. Aizawa, H. Kiyono, Y. Takeda, J. R. McGhee, J. Chiba, et al. 2002. Characterization of protective immune responses induced by nasal influenza vaccine containing mutant cholera toxin as a safe adjuvant (CT112K). *Vaccine* 20: 3443–3455.
27. Tobita, K., A. Sugiura, C. Enomote, and M. Furuyama. 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med. Microbiol. Immunol.* 162: 9–14.

28. Asanuma, H., A. H. Thompson, T. Iwasaki, Y. Sato, Y. Inaba, C. Aizawa, T. Kurata, and S. Tamura. 1997. Isolation and characterization of mouse nasal-associated lymphoid tissue. *J. Immunol. Methods* 202: 123–131.
29. Gonzalez-Juarrero, M., and I. M. Orme. 2001. Characterization of murine lung dendritic cells infected with *Mycobacterium tuberculosis*. *Infect. Immun.* 69: 1127–1133.
30. Yang, B., D. F. Yao, M. Ohuchi, M. Ide, M. Yano, Y. Okumura, and H. Kido. 2002. Ambroxol suppresses influenza-virus proliferation in the mouse airway by increasing antiviral factor levels. *Eur. Respir. J.* 19: 952–958.
31. Sano, H., and Y. Kuroki. 2005. The lung collectins, SP-A and SP-D, modulate pulmonary innate immunity. *Mol. Immunol.* 42: 279–287.
32. Wright, J. R., and J. A. Clements. 1987. Metabolism and turnover of lung surfactant. *Am. Rev. Respir. Dis.* 136: 426–444.
33. Poelma, D. L., L. J. Zimmermann, W. A. van Cappellen, J. J. Haitsma, B. Lachmann, and J. F. van Iwaarden. 2004. Distinct effects of SP-B and SP-C on the uptake of surfactant-like liposomes by alveolar cells in vivo and in vitro. *Am. J. Physiol.* 287: L1056–L1065.
34. Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat. Rev. Immunol.* 3: 331–341.
35. Yamamoto, M., H. Kiyono, S. Yamamoto, E. Batanero, M. N. Kweon, S. Otake, M. Azuma, Y. Takeda, and J. R. McGhee. 1999. Direct effects on antigen-presenting cells and T lymphocytes explain the adjuvanticity of a nontoxic cholera toxin mutant. *J. Immunol.* 162: 7015–7021.
36. Cong, Y., C. T. Weaver, and C. O. Elson. 1997. The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 expression. *J. Immunol.* 159: 5301–5308.
37. Davis, S. S. 2001. Nasal vaccines. *Adv. Drug Deliv. Rev.* 51: 21–42.
38. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, M. B. Sporn, and A. S. Fauci. 1986. Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth. *J. Exp. Med.* 163: 1037–1050.
39. Schmitt, E., P. Hoehn, C. Huels, S. Goedert, N. Palm., E. Rude, and T. Germann. 1994. T helper type 1 development of naive $CD4^+$ T cells requires the coordinate action of interleukin-12 and interferon-γ and is inhibited by transforming growth factor-β. *Eur. J. Immunol.* 24: 793–798.
40. Ludviksson, B. R., D. Seegers, A. S. Resnick, and W. Strober. 2000. The effect of TGF-β1 on immune responses of naive versus memory $CD4^+$ Th1/Th2 T cells. *Eur. J. Immunol.* 30: 2101–2111.
41. Geiser, A. G., J. J. Letterio, A. B. Kulkarni, S. Karlsson, A. B. Roberts, and M. B. Sporn. 1993. Transforming growth factor β1 (TGF-β1) controls expression of major histocompatibility genes in the postnatal mouse: aberrant histocompatibility antigen expression in the pathogenesis of the TGF-β1 null mouse phenotype. *Proc. Natl. Acad. Sci. USA* 90: 9944–9948.
42. Takeuchi, M., P. Alard, and J. W. Streilein. 1998. TGF-β promotes immune deviation by altering accessory signals of antigen-presenting cells. *J. Immunol.* 160: 1589–1597.
43. Fukaura, H., S. C. Kent, M. J. Pietrusewicz, S. J. Khoury, H. L. Weiner, and D. A. Hafler. 1996. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-β1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. *J. Clin. Invest.* 98: 70–77.
44. Iijima, H., I. Takahashi, and H. Kiyono. 2001. Mucosal immune network in the gut for the control of infectious diseases. *Rev. Med. Virol.* 11: 117–133.
45. Cazac, B. B., and J. Roes. 2000. TGF-β receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity* 13: 443–451.
46. Coffman, R. L., B. W. Seymour, D. A. Lebman, D. D. Hiraki, J. A. Christiansen, B. Shrader, H. M. Cherwinski, H. F. Savelkoul, F. D. Finkelman, and M. W. Bond. 1988. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev.* 102: 5–28.
47. Allison, A. C., J. C. Lee, and E. M. Eguchi. 1995. Pharmacological regulation of the production of inflammatory cytokines TNF-α and IL-1-β. In *Human Cytokines: Their Role in Disease and Therapy.* B. Aggarwal and R. K. Puri, eds. Blackwell Science Oxford, pp. 689–713.
48. Maino, V. C., and L. J. Picker. 1998. Identification of functional subsets by flow cytometry: intracellular detection of cytokine expression. *Cytometry* 34: 207–215.
49. van Iwaarden, J. F., E. Claassen, S. H. Jeurissen, H. P. Haagsman, and G. Kraal. 2001. Alveolar macrophages, surfactant lipids, and surfactant protein B regulate the induction of immune responses via the airways. *Am. J. Respir. Cell. Mol. Biol.* 24: 452–458.