Sublingual vaccination with influenza virus protects mice against lethal viral infection

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We assessed whether the sublingual (s.l.) route would be an effective means of delivering vaccines against influenza virus in mice by using either formalin-inactivated or live influenza A/PR/8 virus (H1N1). Sublingual administration of inactivated influenza virus given on two occasions induced both systemic and mucosal antibody responses and conferred protection against a lethal intranasal (i.n.) challenge with influenza virus. Coadministration of a mucosal adjuvant (mCTA-LTB) enhanced these responses and resulted in complete protection against respiratory viral challenge. In addition, s.l. administration of formalin-inactivated A/PR/8 plus mCTA-LTB induced systemic expansion of IFN-γ-secreting T cells and virus-specific cytotoxic T lymphocyte responses. Importantly, a single s.l. administration of live A/PR/8 virus was not pathogenic and induced protection mediated by both acquired and innate immunity. Moreover, s.l. administration of live A/PR/8 virus conferred heterosubtypic protection against respiratory challenge with H3N2 virus. Unlike the i.n. route, the A/PR/8 virus, whether live or inactivated, did not migrate to or replicate in the CNS after s.l. administration. Based on these promising findings, we propose that the s.l. mucosal route offers an attractive alternative to mucosal routes for administering influenza vaccines.

intranasal | mucosal adjuvant | mucosal immunity | redirection | secretory IgA

As the main entry site of most environmental pathogens, mucosal surfaces such as those of the respiratory, gastrointestinal, and genital tracts act as the first line of defense against pathogenic antigens (Ags) (1). Several recent studies have focused on development of mucosal vaccines capable of effectively inducing both mucosal and systemic immune responses, thereby resulting in two layers of host protection (2). Also, because the route of vaccine administration has a significant effect on the outcome of immune responses, a number of studies have attempted to develop mucosal vaccine delivery routes (3–5). In humans, the strongest response is elicited in mucosal tissues directly exposed to Ags, with the second strongest occurring in adjacent mucosa (5). These findings, coupled with recent outbreaks of highly pathogenic avian influenza virus infections in poultry and in humans, highlight the need for a new mucosal delivery system for an influenza virus vaccine.

Oral mucosa, including buccal (the cheek lining), sublingual (s.l.) (underside of the tongue), and gingival mucosa, have received attention as novel delivery sites for therapeutic drugs because they do not subject proteins and/or peptides to the degradation usually caused by gastrointestinal administration. Among oral mucosal routes, the s.l. route is commonly used for immunotherapeutic treatments of allergy because it quickly absorbs Ags and allows them to enter the bloodstream without passing through the intestine or liver, thereby eliciting allergen-specific tolerance (6). No cases of anaphylactic shock in humans were observed in recent studies of s.l. administered immunotherapy targeting allergies (7). These findings led us to ask whether the s.l. route might be useful for delivery of vaccines targeting infectious diseases. We have reported that s.l. administration of a prototype soluble protein together with a mucosal adjuvant induce a broad range of immune responses in mucosal and extramucosal tissues, including secretory and systemic antibody responses and mucosal and systemic cytotoxic T lymphocyte (CTL) responses (8).

In the current study, we assessed whether s.l. administration of live or inactivated influenza virions protects mice against influenza virus infection. Protection was associated with mucosal and systemic immune responses, including Ab production and CTL expansion. In contrast to intranasal (i.n.) immunization, s.l. vaccination is convenient and safe and poses no risk of Ag redirection to the CNS. Further, it not only induces humoral immune responses, but also protects against influenza virus infection.

Results

Sublingual Administration of Inactivated A/PR/8 Virus Induces Specific Systemic and Secretory Ab Responses. To determine the efficacy of s.l. vaccination for inducing systemic and mucosal Ab responses, BALB/c mice were immunized twice at 2-wk intervals by the s.l. route with formalin-inactivated A/PR/8 virus plus mCTA/LTB, a subunit of mutant cholera toxin (CT) E112K with the pentameric B subunit of a heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (LT). One week after the final immunization, the levels of A/PR/8 virus-specific Abs and the numbers of Ab-secreting cells (ASCs) were measured by ELISA and enzyme-linked immunospot (ELISPOT), respectively. Groups of mice receiving inactivated A/PR/8 virus either alone or together with mCTA/LTB by the s.l. route showed higher levels of A/PR/8-specific IgG and IgA Abs in serum, bronchoalveolar lavage (BAL) fluid, and nasal wash than found in control mice vaccinated with PBS (Fig. 1*A*). The profile of serum IgG1 and IgG2a Ab responses of immunized mice were parallel and not partial to IgG1 or IgG2a Ab responses. Thus, s.l. vaccination with inactivated A/PR/8 virus alone or together with mCTA/LTB could induce both Th1- and Th2-type responses. However, no significant levels of IgE Ab were elicited by s.l. vaccination of inactivated A/PR/8 virus with or without mCTA/LTB. These finding suggest that s.l. vaccination could avoid the danger of anaphylactic shock and/or allergic reactions provoked by IgE Abs. To further ascertain the levels of A/PR/8-specific IgA Abs in mucosal compartments, BAL fluid, nasal wash, saliva, and fecal extract were collected 1 wk after the final vaccination. Interestingly, groups of mice vaccinated with inactivated A/PR/8 alone or together with mCTA/LTB by the s.l. route showed significantly higher levels of A/PR/8-specific IgA Abs in mucosal secretions than the PBS-vaccinated animals (Fig. 1*A*). Because a portion of Abs

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The authors declare no conflict of interest.

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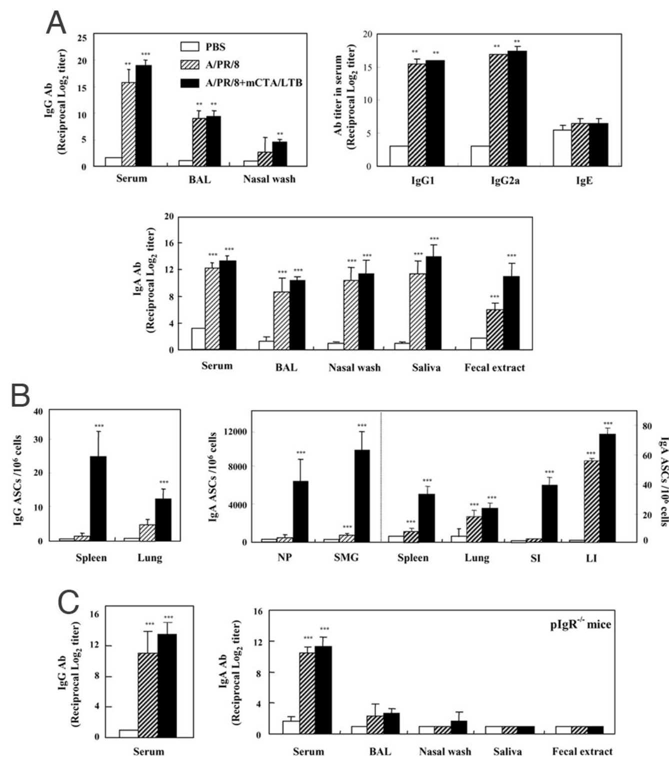

Fig. 1. A/PR/8 virus-specific Ab responses induced in serum and mucosal secretions after s.l. administration of inactivated A/PR/8/34 with or without adjuvant. Mice were vaccinated s.l. with inactivated A/PR/8 (20 μg) and mCTA/LTB (5 μg) as adjuvant at days 0 and 14. At 1 wk after the second immunization, serum and mucosal secretions were collected and A/PR/8 virus-specific Ab levels assessed by ELISA. (*A*) Anti-A/PR/8 virus-specific IgG and IgA Ab responses measured in sera and other mucosal secretions. Ab production levels are shown as reciprocal $\log_2$ titer. (*B*) Mononuclear cells from spleen, lung nasal passage (NP), submandibular gland (SMG), lung, and small and large intestines (SI, LI) were prepared from s.l. immunized mice given formalin-inactivated virus with or without adjuvant. ELISPOT was used to count A/PR/8 virus-specific ASCs. (*C*) Levels of anti-A/PR/8 virus-specific Ab responses in $pIgR^{-/-}$ mice immunized s.l. with inactivated A/PR/8 and mCTA/LTB. **, $P < 0.01$; ***, $P < 0.001$ vs. PBS-vaccinated group. Each group had five to seven mice. Data are representative of three separate experiments.

detected in secretions may originate from plasma by transudation of Ig, ELISPOT assays were carried out to determine the contribution of the local plasma cell pool after s.l. immunization. A/PR/8-specific ASCs were counted in cell suspensions from spleen, lung, nasal passage, submandibular gland, and lamina propria of small and large intestines 1 wk after the final booster vaccination. Vaccination s.l. with formalin-inactivated A/PR/8 virus plus mCTA/LTB elicited higher numbers of A/PR/8-specific IgG ASCs in the spleen and lung than did PBS or inactivated A/PR/8 virus alone (Fig. 1*B*). High numbers of A/PR/8-specific IgA ASCs were detected in the nasal passage, submandibular gland, and small and large intestines of mice vaccinated with formalin-inactivated A/PR/8 virus plus mCTA/LTB given by the s.l. route (Fig. 1*B*). Previous studies have shown that secretory IgA (SIgA) may play an important role in protecting against variant influenza virus infections in the upper respiratory tract (9). Thus, we used $pIgR^{-/-}$ mice in which the transepithelial transport of dimeric IgA is blocked to measure whether IgA Abs in the mucosal compartments induced by s.l. vaccination are secretory in nature. There were significantly fewer A/PR/8 virus-specific IgA Abs in the mucosal secretions of $pIgR^{-/-}$ mice (Fig. 1*C*) than in wild-type mice (Fig. 1*A*), indicating that s.l. vaccination induces both systemic IgG and mucosal SIgA Ab responses.

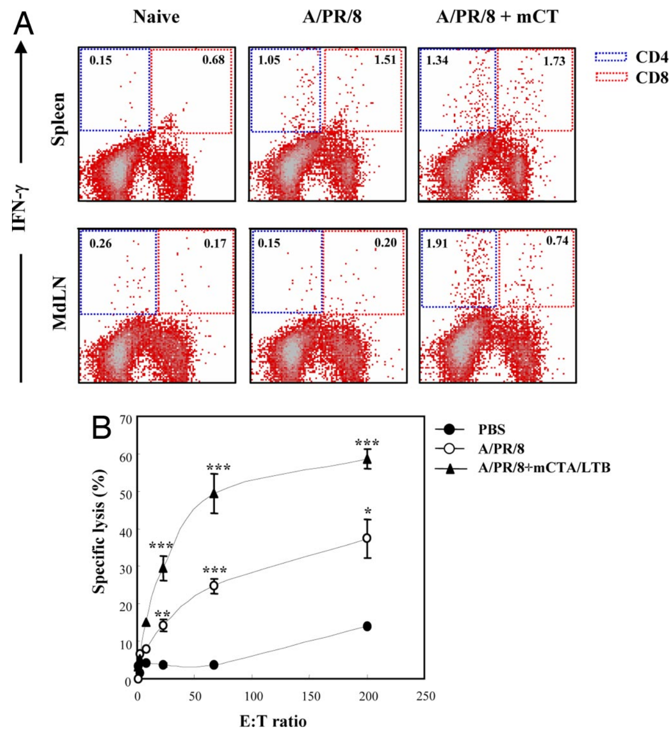

Fig. 2. Induction of the IFN-γ-secreting T cells and influenza virus-specific CTL responses in mice after s.l. vaccination. (*A*) Analysis of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in spleen after two s.l. vaccinations with formalin-inactivated A/PR/8 virus with/without mucosal adjuvant (mCTA/LTB). Splenocytes from each vaccinated group were cultured with inactivated A/PR/8 for 4 days and T cells were gated out by anti-CD3 mAb. Then, IFN-γ-producing T cells were stained by anti-CD8 mAb. (*B*) At 2 wks after the second s.l. vaccination with killed influenza virus, splenocytes were cocultured with A/PR/8 virus-infected autologous splenocytes for 5 days. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. PBS group. E:T, effector-to-target.

Vaccination s.l. Leads to $CD4^+$ and $CD8^+$ T Cell IFN-γ Secretion. To determine whether s.l. vaccination with killed A/PR/8 could elicit Th1-type immune responses, the numbers of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the spleen and mediastinal lymph nodes (MdLNs) were assessed after two s.l. administrations of formalin-inactivated A/PR/8 virus with/without mucosal adjuvant (mCTA/LTB). Significantly more IFN-γ-producing $CD4^+$ and $CD8^+$ T cells were detected in the spleens and MdLNs of mice coadministered inactivated A/PR/8 virus and mCTA/LTB than in mice given inactivated A/PR/8 virus alone (Fig. 2*A*). Moreover, s.l. vaccination with inactivated A/PR/8 plus mCTA/LTB elicited significantly more virus-specific CTL responses than did vaccination with PBS or A/PR/8 virus alone (Fig. 2*B*). Taken together, these results demonstrate that s.l. vaccination with a nonreplicating virus coadministered with a pertinent adjuvant can induce Th1-type cell-mediated responses, such as IFN-γ-producing T cell activation and CTL responses.

Administration s.l. of Inactivated A/PR/8 Virus Protects Mice Against Lethal Respiratory Challenge with Influenza Virus. We next addressed the efficacy of s.l. administration of inactivated A/PR/8 virus plus mCTA/LTB on induction of protective efficacy after challenge with a lethal dose of A/PR/8 virus. Two weeks after the second immunization with inactivated A/PR/8 virus alone or with mCTA/LTB, groups of mice were inoculated i.n. with 20 LD_{50} of live A/PR/8 influenza virus and monitored daily. As shown in Fig. 3*A*, the PBS-treated control mice progressively lost weight and died within 9 days after i.n. challenge. In contrast, s.l. vaccination with A/PR/8 alone resulted in 80% survival, despite transient weight loss.

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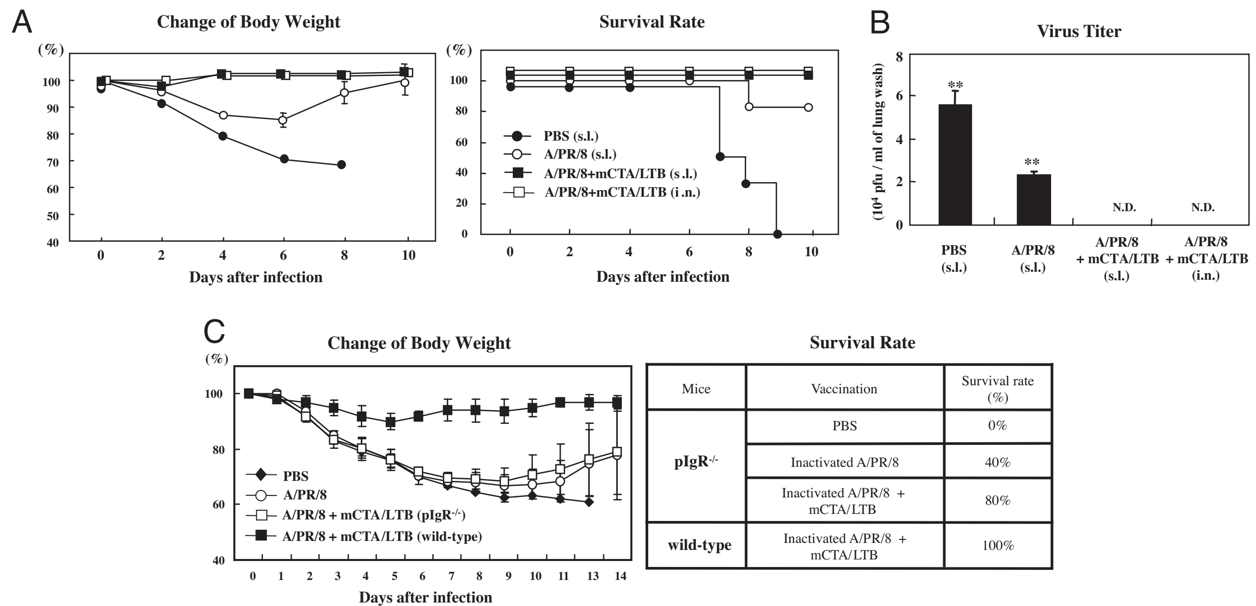

Mice	Vaccination	Survival rate (%)
pIgR-/-	PBS	0%
	Inactivated A/PR/8	40%
	Inactivated A/PR/8 + mCTA/LTB	80%
wild-type	Inactivated A/PR/8 + mCTA/LTB	100%

Fig. 3. Vaccination s.l. with inactivated A/PR/8/34 with adjuvant protects mice against lethal challenge with homologous virus. BALB/c mice were immunized s.l. or i.n. with inactivated A/PR/8 (20 μg) plus mCTA/LTB (5 μg) as adjuvant at days 0 and 14. At 2 wks after immunization, mice were challenged i.n. with 20 LD_{50} of influenza A/PR/8 virus. (*A*) Body weights and survival were monitored daily after challenge. (*B*) At day 3 after challenge, virus titers in BAL fluid were measured by plaque assay. **, $P < 0.01$ vs. mice vaccinated with A/PR/8 virus with adjuvant by i.n. (*C*) Protective efficacy against influenza infection in s.l. immunized wild-type and $pIgR^{-/-}$ mice. N.D., not detectable. Each group consisted of five to seven mice. Data are representative of three separate experiments.

Moreover, addition of mCTA/LTB adjuvant to the inactivated whole virus Ag resulted in 100% survival. Consistent with these findings, viral titers in the BAL fluid from mice vaccinated s.l. with inactivated A/PR/8 virus plus mucosal adjuvant showed complete clearance of A/PR/8 virus at 72 h after i.n. challenge (Fig. 3*B*). To compare the effectiveness of s.l. and i.n. routes, mice were vaccinated i.n. with inactivated A/PR/8 virus alone or with mCTA/LTB. There were no significant differences between routes for protective efficacy (Fig. 3 *A* and *B*).

To assess the role played by SIgA in the protection afforded by s.l. vaccination, wild-type and $pIgR^{-/-}$ mice were administered inactivated A/PR/8 virus plus mCTA/LTB twice and then challenged i.n. with 20 LD_{50} of live A/PR/8 influenza virus. When vaccinated s.l. with A/PR/8 virus alone or A/PR/8 virus plus mCTA/LTB, $pIgR^{-/-}$ mice lost significant weight, had ruffled fur and hunched posture, and gradually became lethargic after i.n. challenge. They also had only partial protection similar to that seen in the control group treated with PBS. In contrast, wild-type mice had 100% protection (Fig. 3*C*). In our study, s.l. vaccination with inactivated A/PR/8 virus plus mucosal adjuvant significantly enhanced protective immune response and resulted in effective protection against subsequent influenza virus challenge. This protection appeared to require intact transport of SIgA.

Live A/PR/8 s.l. Vaccination Induces Mucosal and Systemic Immune Responses and Protects Mice Against Lethal Challenge with Influenza Virus. To address the safety and efficacy of the s.l. route for delivery of live influenza virus vaccines, groups of mice were administered a single inoculum of a 0.2-, 2-, or 20-fold higher dose of LD_{50} live A/PR/8 virus and were monitored for up to 14 days. In contrast to i.n.-administered mice, which all died after doses of 2 or 20 LD_{50}, none of the three doses proved lethal to s.l.-administered mice (data not shown). We further tested the immunogenicity and protective efficacy of an 800-pfu (2 LD_{50}) s.l. dose of live A/PR/8 virus. A single s.l. administration with live A/PR/8 virus resulted in significantly higher levels of A/PR/8 virus-specific IgG Abs in serum and IgA Abs in mucosal secretions than observed in the PBS-treated control group (Fig. 4*A*). Furthermore, s.l. administration with live A/PR/8 virus efficiently cleared the influenza virus from the BAL fluid (Fig. 4*B*) and elicited 100% protection against lethal i.n. challenge with A/PR/8 virus (Fig. 4*B*). These results demonstrate that s.l. administration with live A/PR/8 virus is safer than i.n. inoculation and is highly effective in protecting mice against lethal respiratory challenge with influenza virus.

To further address the efficacy of the s.l. route for the induction of innate immunity, mice were challenged i.n. with a lethal dose of A/PR/8 virus 3 days after s.l. vaccination with live A/PR/8 virus. We found that s.l. administration of live A/PR/8 conferred complete protection against A/PR/8 virus infection (Fig. 4*C*). To determine whether this protection was mediated by innate immune responses, $MyD88^{-/-}$ mice, which are defective in toll-like receptor (TLR) signaling, were immunized s.l. with live A/PR/8 and challenged with a lethal dose of live A/PR/8 3 days later, a time preceding development of effective adaptive immune responses. In contrast to wild-type mice, all vaccinated $MyD88^{-/-}$ mice died after i.n. challenge (Fig. 4*C*). These results clearly indicate that early protection against murine influenza conferred by s.l. vaccination requires intact innate immune responses.

Live Influenza Virus s.l. Vaccination Induces Heterosubtypic and Homologous Protection. We next evaluated whether s.l. vaccination with live virus could elicit cross-protective efficacy against heterosubtypic and homologous viral challenge. Mice were immunized with mouse-adapted live A/Philippine influenza virus (H3N2) or a human isolate of A/Chile (H1N1) virus by the s.l. or i.n. routes; all vaccinated mice were challenged i.n. 4 wks later with 20 LD_{50} of A/PR/8 virus (H1N1). As expected, all control mice given PBS alone died within 6–9 days after i.n. challenge with A/PR/8 virus (Table 1). Confirming the results in ref. 10, i.n. vaccination with a sublethal dose (10^2 pfu) of A/Philippines (H3N2) elicited effective heterosubtypic immunity against A/PR/8 (H1N1). Of interest, mice vaccinated s.l. with 10^2 pfu or 10^4 pfu of A/Philippine virus, a dose 100 times higher than the sublethal dose for the i.n. route, showed strong protection against A/PR/8 viral challenge. In the group vaccinated s.l. with A/Chile virus (H1N1) (a human isolate), all mice survived. However, mice given A/Chile virus (H1N1) i.n. had

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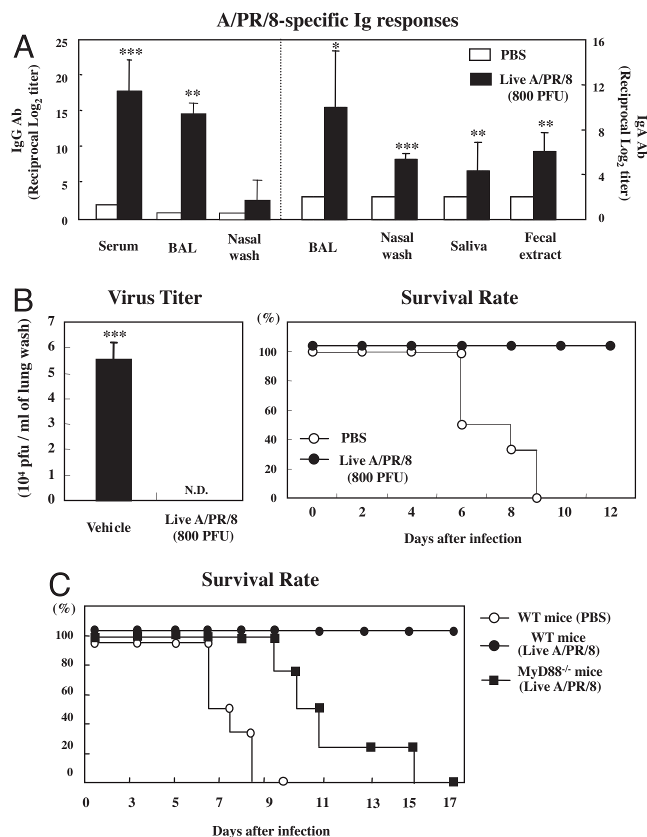

Fig. 4. The s.l. route is safe and effective for vaccination with live A/PR/8 virus. Mice were vaccinated once s.l. with 800 pfu (2 LD_{50}) of live A/PR/8 virus. Two weeks later, mice were challenged i.n. with 20 LD_{50} of influenza A/PR/8 virus. Virus titers and survival were monitored daily. (*A*) Levels of anti-A/PR/8 virus-specific IgG and IgA Ab responses in serum and mucosal secretions were determined by ELISA 2 wks after s.l. vaccination with live A/PR/8 virus. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. PBS-vaccinated group. (*B*) Virus titers were measured 3 days after i.n. challenge. Survival was monitored for 14 days. (*C*) Three days after s.l. administration of 800 pfu (2 LD_{50}) of live A/PR/8, wild-type mice or MyD88$^{-/-}$ mice were challenged i.n. with 20 LD_{50} of A/PR/8 virus. Survival after i.n. challenge: ***, $P < 0.001$ vs. mice vaccinated with 800 pfu (2 LD_{50}) of live influenza virus. Each group had five to seven mice. Data are representative of three separate experiments.

greater weight loss and signs of disease at day 5 after challenge than did those vaccinated s.l. (data not shown). These results clearly demonstrate that the s.l. route can be used to provide efficient heterosubtypic and homologous protective immunity without severe illness.

Administration s.l. of Inactivated and Live A/PR/8 Virus Does Not Redirect Ags to the CNS. Because trafficking of Ags and adjuvants into the CNS has been reported after i.n. administration, raising serious safety issues, we next sought to determine whether s.l. administered vaccines could be redirected to the CNS. Acridinium-labeled inactivated A/PR/8 virus was administered either s.l. or i.n. to separate groups of mice. As shown in Fig. 5*A*, i.n. administration of labeled A/PR/8 virus resulted in the accumulation of Ags in the olfactory bulbs (OBs) and brain within 24 h. In contrast, labeled A/PR/8 virus was undetectable in OB and brain tissues after s.l. administration (Fig. 5*A*). Further, i.n. administered acridinium-labeled, inactivated A/PR/8 was readily detected in the lungs and at levels higher than those seen after s.l. administration (Fig. 5*A*).

We used real-time quantitative PCR to measure the levels of viral RNA in several tissues after i.n. or s.l. administration of live A/PR/8 viral infection (20 LD_{50}) (11). Viral RNA was strongly expressed in both lung and OB tissues from mice infected i.n. with the A/PR/8 virus (Fig. 5*B*). However, the A/PR/8 virus gene was not detected in lung, OB, and brain isolated from mice administered s.l. (Fig. 5*B*). These results demonstrate that inactivated and live A/PR/8 virus can be transported into CNS tissues after i.n. but not after s.l. administration.

Viral titers in BAL specimens and histopathological analyses of lung tissues were determined after infection i.n. or s.l. with live A/PR/8 virus (20 LD_{50}). At 24 h postinfection, viral titers remained high in mice exposed i.n. to live A/PR/8 virus but had disappeared from the BAL fluid of s.l. exposed mice (Fig. 5*C*). Lung sections from s.l. exposed mice showed modest alterations in lung tissue morphology compared with mice exposed i.n. In the i.n. exposed animals, thickening of the bronchi, inflammatory infiltrates, and alveoli destruction were common as early as 24 h after infection (Fig. 5*C*).

Discussion

Our results provide the first evidence that the s.l. route is highly efficient for influenza vaccine. Importantly, in contrast to the i.n. route, s.l. administration of either formalin-killed or live influenza virus did not redirect viral Ag into the CNS. Therefore, we anticipate that s.l. vaccine delivery should not raise the same safety concerns as i.n. delivery, an issue that is being addressed in an ongoing human trial.

One major advantage of s.l. vaccination against influenza virus is its ability to induce SIgA Abs in the respiratory tract, the major target organ of influenza virus infection. SIgA Abs are considered major effectors in adaptive immune defense of the respiratory mucosa (12). Although parenteral influenza vaccines protect against homologous virus infection by inducing serum IgG Abs to the viral hemagglutinin (13), i.n. administered influenza vaccine appears more effective for inducing cross-protection, probably as a result of enhanced SIgA responses in the respiratory mucosa (14,

Table 1. Sublingual vaccination can induce heterosubtypic and homologous protection

Protection	Immunization: Virus	Immunization: Dose, pfu	Immunization: Route	Challenge	Survival rate, %
Heterosubtypic protection	PBS			A/PR/8(H1N1),20×LD_{50}	0
	A/Philippine (H3N2)	10^2	i.n.		100
	A/Philippine (H3N2)	10^2	s.l.		100
	A/Philippine (H3N2)	10^4	s.l.		100
Homologous protection	PBS				0
	A/Chile (H1N1)	10^3	i.n.		100
	A/Chile (H1N1)	10^3	s.l.		100
	A/Chile (H1N1)	10^2	s.l.		100

Mice were immunized with live mouse-adapted A/Philippine (H3N2) or homologous human isolated A/Chile (H1N1) influenza virus by different doses and routes. Four weeks later, the mice were challenged with the heterosubtypic or homologous mouse-adapted A/PR/8/34 strain (H1N1) by the i.n. route and their mortality was monitored daily. Each group consisted of five to seven mice and the experiment was repeated three times.

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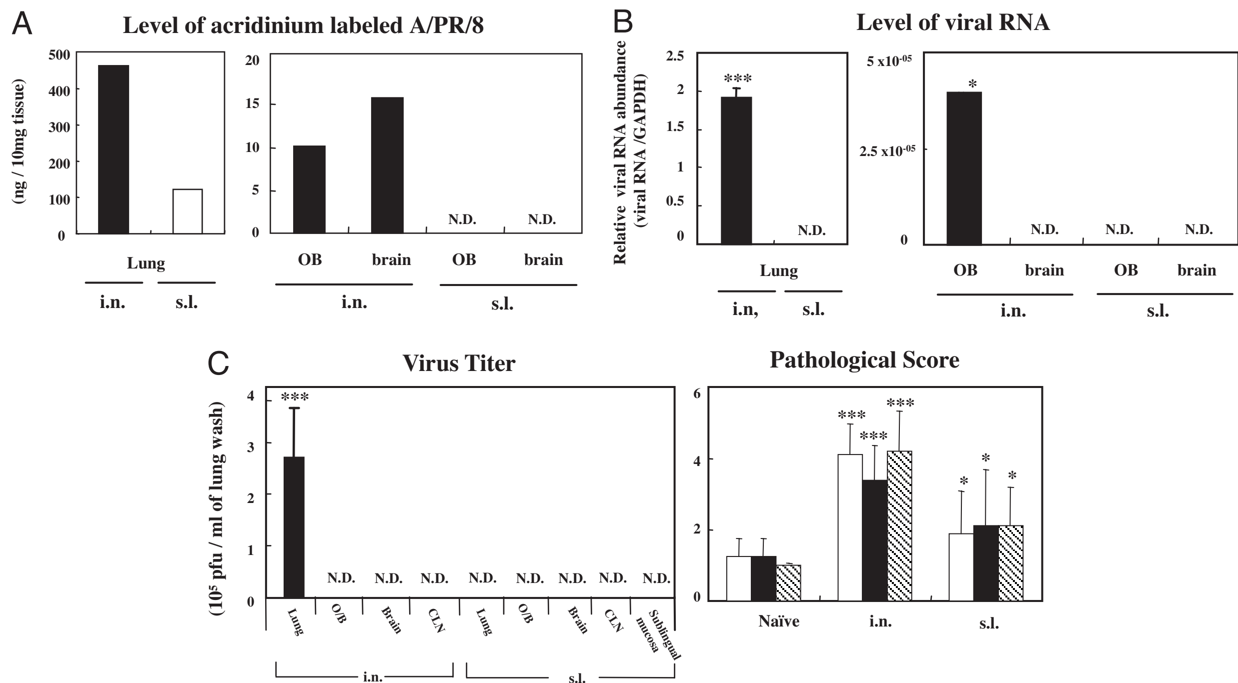

Fig. 5. Live and inactivated A/PR/8 viruses are not transported into the CNS after s.l. administration. (*A*) BALB/c mice were immunized i.n. or s.l. with acridinium-labeled, inactivated A/PR/8 virus 24 h before killing. All tissues were collected, homogenized, and analyzed for acridinium activity. Data are representative of three separate experiments, all of which showed the same pattern. (*B*) BALB/c mice were infected i.n. or s.l. with 20 LD_{50} of live A/PR/8 virus. Viral RNA was detected 24 h later by RT-PCR with M2-specific primers (see *Materials and Methods*). ***, $P < 0.001$ vs. s.l. vaccinated group. (*C*) At 24 h after infection, lungs of mice challenged i.n. or s.l. with 20 LD_{50} of live A/PR/8 were removed to determine virus titers (*Left*) and histopathological changes after H&E staining (*Right*). Pathological score was determined by using a blinded test (□, damage to epithelial cell layers; ■, infiltration of inflammatory mononuclear cells; ▧, alveolitis). *, $P < 0.1$; ***, $P < 0.001$ vs. naïve mice or s.l. vaccinated group. Each group had five to seven mice. Data are representative of two separate experiments.

15). Consistent with this view, Asahi and colleagues (9, 16) demonstrated that SIgA plays a role in protecting against heterologous influenza virus strains. In our current study, s.l. vaccination with inactivated or live A/PR/8 virus elicited high levels of SIgA in various mucosal compartments, including respiratory tissues and secretions. Such SIgA Ab responses could play a major role in preventing entry and replication of influenza virus in the respiratory tract.

Previous studies have suggested the essential role of mucosal immunity in protecting against influenza virus, which mainly infects and provokes inflammation at respiratory mucosal sites (5, 17). The i.n. and the pulmonary route (aerosol delivery), which target nasopharynx-associated lymphoid tissue and/or the lung mucosa, have been the preferred routes for inducing immunity in the respiratory tract (18). In a Swiss clinical trial, i.n. administration of inactivated influenza vaccine together with mucosal adjuvant (i.e., LT) elicited brisk levels of systemic and mucosal immunity, but also led to some cases of Bell's palsy (19). Murine studies have demonstrated that CT, when administered i.n. as a mucosal adjuvant, can redirect coadministered vaccine Ag into the CNS (e.g., into the olfactory nerves/epithelium, OB, and brain) (20). Facial nerve fibers might adsorb the adjuvant, leading to retrograde transport and neuronal damage. Such safety concerns appear to limit the usefulness of the i.n. route in humans. CNS involvement is a rare complication after natural infection with influenza virus during human epidemics (21); however, the relevance of our findings using a mouse-adapted virus remains unknown. In contrast, when delivered by the s.l. route, killed and live A/PR/8 virus did not migrate to or replicate in the CNS, theoretically making the s.l. route a safer alternative than the i.n. route for mucosal delivery of influenza virus vaccines.

Sublingual administration has been successfully used for allergen-specific desensitization in humans and its safety is now well established. Several studies have suggested that s.l. administered Ags are captured locally by Langerhans cells and probably dendritic cells (DCs) or as a result of phagocytosis, macropinocytosis, or receptor-mediated endocytosis (22, 23). We recently showed that the s.l. epithelium harbors a dense lattice of DCs and that CT adjuvant mobilizes DCs within the sublingual epithelium (8). These cells migrate to the proximal draining lymph node (e.g., submaxillary, superficial cervical, and internal jugular), which represent specialized microenvironments favoring the induction of mucosal tolerance (8). Immune responses in mucosal tissues are determined by the route of delivery, by the nature of the Ag, by the type of Ag-presenting cells (APCs), and by the local microenvironment (2, 5). APCs recognize pathogenic viral or bacterial Ags as danger signals (24, 25). This recognition leads to proinflammatory conditions and then to stronger and broader humoral and cellular immune responses, but does not lead to tolerance. Given the difference in outcome (immunity/tolerance), it can be assumed that viral nucleic acids can be the ligands for some TLRs (e.g., TLR3 and/or TLR7) on mucosal APCs (26, 27).

The results reported here suggest that the s.l. delivery route could be highly effective and safe. Immunization with inactivated or live A/PR/8 virus by the s.l. mucosa induced protective immune responses, increased mucosal SIgA Ab levels, and enhanced virus-specific CTL responses without posing the risk of damage to the CNS. Many issues regarding s.l. administration remain to be resolved, including the development of mucosal adjuvants and the improvement of formulations that would enable enhanced efficacy and lowered dose. Nonetheless, our findings strongly suggest that s.l. delivery could be a more effective avenue than traditional approaches for vaccinating against both seasonal and pandemic flu.

Experimental Procedures

Mice. Female BALB/c mice aged 5–6 wks were purchased from Charles River Co. Polymeric Ig receptor knockout ($pIgR^{-/-}$) mice were provided by Masanobu

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Nanno of Yakult Central Institute (Tokyo, Japan), and MyD88$^{-/-}$ mice were provided by Shizuo Akira of Osaka University (Osaka, Japan). Mice were maintained in the International Vaccine Institute animal facility (Seoul, Korea) under specific pathogen-free conditions and received sterilized food (certified diet MF; Orient Co.) and water ad libitum.

Immunization Protocols. Mice were anesthetized by i.p. injection of ketamine (100 mg/kg of body weight; Yuhan Co.) and xylazine hydrochloride (10 mg/kg of body weight; Bayer). For s.l. immunization, forceps were placed under the tongue of the anesthetized mouse and its mouth was stretched open; Ag was administered by micropipette. The total volume of Ag plus adjuvant was kept to $<7\ \mu l$ to avoid swallowing effects. Mice were immunized s.l. on days 0 and 14 with 20 μg of inactivated A/PR/8 [A/Puerto Rico/8/34 (H1N1)] alone or together with a well established mucosal adjuvant, that is, mCTA/LTB (5 μg) (28, 29). The mCTA/LTB were kindly provided by Hiroshi Kiyono of Tokyo University (Tokyo, Japan) In some experiments, mice received live A/PR/8 virus s.l. once without any boosting. For homologous or heterosubtypic protection assays, anesthetized mice were immunized with 10^2 pfu or 10^4 pfu of A/Philippines (H3N2) virus or 10^2 pfu or 10^3 pfu of A/Chile (H1N1) i.n. or s.l., respectively (10).

ELISPOT Assay. Ninety-six-well nitrocellulose microplates (MilliporeA) were coated with inactivated A/PR/8 virus overnight at 4°C and blocked as described in ref. 30. In brief, serially diluted cells in complete medium were applied to plates and incubated for 4 h in a 37°C, 5% CO_2 incubator. Then, either HRP-conjugated goat anti-mouse IgG or IgA Abs (Southern Biotechnology Associates) were added and incubated overnight at 4°C. To develop color, peroxidase substrate reagents (Moss) were added to the plates, which were left at room temperature until spots could be visualized.

Analysis of CTL Responses. To measure *in vitro* CTL responses, 5×10^7 spleen cells were isolated from mice 2 wks after s.l. vaccination and then cocultured with A/PR/8 virus-pulsed autologous splenocytes (1×10^7) for 5 days. To prepare the stimulator cells, spleen cells from naïve autologous mice were irradiated at 2,200 rad (22 Gy) and then pulsed with 10 multiplicity of infection (MOI) units of the A/PR/8 virus as described in ref. 31.

Protection Assay Against Influenza Virus A/PR/8/34. Two weeks after the final vaccination, anesthetized mice were challenged i.n. with 20 μl (10 μl per nostril) of live A/PR/8 virus suspension (20 LD_{50}; 8×10^3 pfu). In some experiments, mice were challenged i.n. with live A/PR/8 virus suspension 3 days after s.l. vaccination with live A/PR/8 virus (800 pfu). Animals were monitored daily for weight loss and survival for 14 days. For heterosubtypic and homologous protection, groups of mice were inoculated i.n. or s.l. with A/Philippine (H3N2) virus or A/Chile (H1N1) virus, respectively. At 4 wks after vaccination, all groups of mice were challenged i.n. with a suspension of live A/PR/8 virus (20 LD_{50}, 8×10^3 pfu per head).

Tracking of s.l. Administered Ag. To determine whether s.l. administered Ag could be redirected to the CNS, formalin-inactivated A/PR/8 virus was labeled with the chemoluminescent tag acridinium as described in ref. 32. The specific activity of acridinium-labeled, inactivated A/PR/8 virus was 6.7×10^5 relative light units per nanogram of tissue. Mice were given 10 μg of the acridinium-labeled, inactivated A/PR/8 virus i.n. or s.l., and lung, OB, and brain were removed 24 h after immunization as described in ref. 33. Tissues were weighed, and 200 μl of CellLytic MT lysis buffer (Sigma) was added per 10 mg of tissue wet weight. The tissues were homogenized and frozen at −20°C. After thawing, the homogenates were centrifuged at $10,000 \times g$ for 10 min, and the supernatants were tested for light activity by using the LMax II^{384} system (Molecular Devices).

Real-Time Quantitative PCR for A/PR/8 Virus. Primers and probes for influenza virus A were selected as described in ref. 11. In brief, the primers (INFA-1 and INFA-2) were based on genomic regions highly conserved in various subtypes and genotypes of influenza A (matrix protein gene). Influenza virus RNA levels were normalized to the corresponding GAPDH mRNA levels. Water controls and samples without PCR mixtures were set up to eliminate the possibility of significant DNA contamination.

Data and Statistical Analysis. Ab titers, defined as the reciprocal of the sample dilution giving an absorbance value 0.1 higher than the PBS-vaccinated control group, were expressed as geometric mean ± SD and compared by *t* test (SigmaPlot; Systat). Each experiment was repeated at least three times with use of five to seven mice per group.

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