Review

Intranasal Inactivated Influenza Vaccines: a Reasonable Approach to Improve the Efficacy of Influenza Vaccine?

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SUMMARY: Influenza is a contagious, acute respiratory disease caused by the influenza virus. The mucosal lining in the host respiratory tract is not only the site of virus infection, but also the site of defense; it is at this site that the host immune response targets the virus and protects against reinfection. One of the most effective methods to prevent influenza is to induce specific antibody (Ab) responses in the respiratory tract by vaccination. Two types of influenza vaccines, intranasal live attenuated influenza virus (LAIV) vaccines and parenteral (injectable) inactivated vaccines, are currently used worldwide. These vaccines are approved by the European Medicines Agency (EMA) and the US Food and Drug Administration. Live attenuated vaccines induce both secretory IgA (S-IgA) and serum IgG antibodies (Abs), whereas parenteral vaccines induce only serum IgG Abs. However, intranasal administration of inactivated vaccines together with an appropriate adjuvant induces both S-IgA and IgG Abs. Several preclinical studies on adjuvant-combined, nasal-inactivated vaccines revealed that nasal S-IgA Abs, a major immune component in the upper respiratory tract, reacted with homologous virus hemagglutinin (HA) and were highly cross-reactive with viral HA variants, resulting in protection and cross-
Influenza viruses, which belong to the Orthomyxoviridae family, are classified as types A, B, and C based on antigenic differences in the NP and M proteins (1–4). The influenza A virus is an enveloped virus expressing two external glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and seven internal proteins (nucleoprotein [NP], three polymerase proteins [PA, PB1, and PB2], two matrix proteins [M1 and M2], and nonstructural proteins [NS2]) (1–3). The virus contains a negative-sense, single-stranded RNA genome, which encodes eight structural (PB1, PA, PB2, HA, NP, NA, M1, and M2) and two non-structural proteins (NS1 and NS2/NEP). The virus infects the respiratory tract epithelial cells via binding of HA to receptors (sialic acid) on cell surfaces. Several hours after infection, newly synthesized viruses bud from the infected cell surface and are released through the action of NA. Progeny viruses then infect neighboring cells, resulting in destruction of respiratory tract mucosa. This is the cause of respiratory symptoms associated with influenza.

1. INTRODUCTION

1–1. Characteristics of influenza

1–1–1. Respiratory disease

Influenza is a contagious, acute respiratory disease caused by the influenza virus (1–3). The disease is characterized by rapid onset of fever and other systemic symptoms, including common colds, pharyngitis, tracheobronchitis, and pneumonia. Most adults recover from influenza within a week, although the duration of illness depends on the severity of the disease. Disease severity, in turn, depends on a variety of viral factors (strain, virulence, inoculum size, and site of infection), host factors (prior immunity, genetic predisposition, underlying conditions, age, and gender) and other factors (access to care and antiviral therapy). The morbidity and mortality rates associated with seasonal influenza are estimated to be 5–20% and 0.1–1%, respectively. The host respiratory tract mucosa is both the site of virus infection and the site of host defense, as it is the site where protective immune responses are generated that facilitate recovery and protection against reinfection.

1–1–2. Virus structure and spread of infection

Influenza A (or B) virus is an enveloped virus expressing two external glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and seven internal proteins (nucleoprotein [NP], three polymerase proteins [PA, PB1, and PB2], two matrix proteins [M1 and M2], and nonstructural proteins [NS2]) (1–3). The virus contains a negative-sense, single-stranded RNA genome, which encodes eight structural (PB1, PA, PB2, HA, NP, NA, M1, and M2) and two non-structural proteins (NS1 and NS2/NEP). The virus infects the respiratory tract epithelial cells via binding of HA to receptors (sialic acid) on cell surfaces. Several hours after infection, newly synthesized viruses bud from the infected cell surface and are released through the action of NA. Progeny viruses then infect neighboring cells, resulting in destruction of respiratory tract mucosa. This is the cause of respiratory symptoms associated with influenza.

1–1–3. Classification and mutations

Influenza viruses, which belong to the Orthomyxoviridae family, are classified as types A, B, and C based on antigenic differences in the NP and M proteins (1–4). Influenza A viruses, which circulate in humans, are classified in two subtypes (H1N1 and H3N2) based on differences in the HA and NA molecules, while influenza B viruses are classified into two distinct lineages: B/Yamagata-like (Bv) and B/Victoria-like (Bv). The two subtypes of the influenza A virus are thought to be generated in pigs. This process involves rearrangement of viral gene segments (reassortment) in pig host cells infected with two different influenza viruses (from humans and poultry), a process known as “antigenic shift.” Influenza viruses identified in poultry are derived from aquatic birds, which harbor genes of almost all the subtypes of HA (H1–H16) and NA (N1–N9). Two subtype A viruses and two B-lineage viruses undergo continual antigenic changes by accumulating point mutations in the viral RNA during virus replication (referred to as “antigenic drift”). Consequently, a variant virus expressing antigenically different HA and NA molecules appears as an epidemic strain.

1–2. Immune responses against influenza virus infection

1–2–1. Innate immune responses

Studies of the immune mechanisms induced by natural viral infection show that influenza virus infection is initially eliminated from the respiratory tract in a nonspecific manner by innate immune mechanisms, including factors such as natural immunoglobulins and collectins, which inhibit the infection by blocking the binding of viral HA to its receptor (sialic acid) on epithelial cells (3,5–9). Once the virus spreads to the respiratory tract epithelial cells, it replicates and infects other cells, thereby destroying the epithelium and (sometimes) pneumocytes. During the early stages of influenza, several components of the innate immune system, such as epithelial cells, macrophages, dendritic cells (DCs), natural killer cells, various cytokines (type I interferons, interleukin [IL]-1, tumor necrotic factor -α, and IL-6), and complement, play roles in eliminating the virus from the respiratory tract. Viral antigens (e.g., single-stranded RNA and double-stranded RNA), known as pathogen-associated molecular patterns, are detected by pattern recognition receptors (PRRs), such as Toll-like receptors (TLR3, TLR7, and TLR8), retinoic acid-inducible gene I, and a nod-like receptor, which are expressed by epithelial cells, macrophages, and DCs. Upon ligation of PRRs, cells produce cytokines and chemokines to initiate various immune responses. For example, IFN-α produced by epithelial cells limits early viral replication, and cytokines produced by macrophages and DCs after uptake of the virus induce both local inflammatory reactions and the systemic symptoms associated with influenza infection, such as fever and myalgia. DCs take up and process virus-related materials and generate antigen-specific peptides, which are then presented on class I and class II MHC molecules and are presented to
The mucosal immune system in the respiratory tract can be divided into the inductive sites (nasopharynx-associated lymphoid tissue [NALT] and bronchus-associated lymphoid tissue [BALT]) and the effector sites (epithelial cell layer and the lamina propria). The respiratory tract mucosa is the site of influenza virus infection and the site of immune responses to the virus. Major effector molecules and cells involved in the adaptive immune responses against influenza virus infection are generated at the inductive site and act at the effector sites. 1. Viral neutralization by S-IgA Abs which are transported to the apical surface of epithelial cells via endosomes after formation of a complex between polymeric IgA and poly Ig receptor (pIgR) at the basolateral surface. IgG Abs move from serum to the apical surface of the epithelial cells by diffusion. 2. Intracellular neutralization by inhibition of virion assembly via binding of the pIgR-IgA complex to the newly synthesized viral glycoproteins. 3. Lysis of the infected cells by CTLs (CD8$^+$ cytotoxic T cells). 4. Blockade of virus replication by IFN-$\gamma$ produced by DTH-mediating T cells (Th1 cells).
1–2. Protective antigens

The specific and protective Ab responses triggered by natural viral infection are directed mainly against the viral surface glycoproteins, HA and NA, which are the major protection-inducing antigens (3,10,26–28). Anti-HA Abs are primarily responsible for preventing infection by neutralizing (NT) influenza virus, while anti-NA Abs inhibit virus replication and contribute to recovery. Influenza viruses express about 5-fold more HA antigens than NA antigens, and anti-HA Ab responses are stronger than anti-NA Ab responses. Internal viral proteins generate only weak protective responses, although cytotoxic T cells specific for these proteins do inhibit virus replication by killing infected epithelial cells, thereby enabling recovery from infection. Thus, HA is the most powerful protection-inducing antigen expressed by influenza virus.

1–3. Current seasonal influenza vaccines

1–3–1. Characteristics and history

The most effective method for preventing influenza infection is to induce protective Ab responses by vaccination. Isolation of the influenza virus in 1933 led to the development of an influenza vaccine. Two types of influenza vaccines are currently used worldwide: parenteral vaccines (inactivated whole virion [WV] or split virion [SV] vaccines administered by subcutaneous or intramuscular injection) and intranasal live attenuated influenza virus (LAIV) vaccines (3,29–31). The earliest vaccines were bivalent (H1N1 + B) vaccines, which selects the most suitable vaccine strains for development of effective seasonal influenza vaccines (4,30). In the 1960s, parenteral inactivated WV preparations were first licensed for use in the USA in 1945. In the 1960s, parenteral inactivated WV vaccines were improved to minimize reactogenicity, leading to development of the inactivated SV preparations used today. LAIV vaccines were first prepared in the 1960s, but were not licensed for use in Russia until the 1980s and in the USA until 2003 (29,32).

The earliest vaccines were bivalent (H1N1 + B) vaccines (3,4). The subsequent discovery of “antigenic drift” led to annual immunization with vaccines derived from circulating strains to prevent epidemics. Since 1973, the World Health Organization (WHO) has issued annual recommendations for the composition of the influenza vaccine; these recommendations are based on the results of various surveillance systems (30). The first trivalent vaccine (H1N1 + H3N2 + B) and the first quadrivalent vaccine (H1N1 + H3N2 + Bv + Bv) were introduced in 1978 and 2014, respectively.

1–3–2. Evaluation of efficacy and effectiveness

The prophylactic effect (efficacy and effectiveness) of influenza vaccines is evaluated in double-blind, randomized, placebo-controlled field trials, and/or in placebo-controlled clinical trials designed to measure responses to laboratory-confirmed influenza virus infection with a challenge virus (29,33). The prophylactic effect of the vaccine (Ve) is represented mathematically as Ve = (1 – Rv/Rp) × 100%, where Rv and Rp are the attack rate in vaccinees and placebo recipients, respectively. Studies of vaccine efficacy (i.e., its ability to prevent illness in vaccinated persons in controlled trials) and effectiveness (i.e., its ability to prevent illness in the vaccinated population) have been evaluated by multiple case-finding methods, including protection against challenge with LAIV, prevention of seroconversion in response to circulating influenza virus strains, reduction of fever during the influenza season in all vaccinees compared with placebo recipients, and prevention of influenza or pneumonia-associated hospitalization or deaths.

Vaccine efficacy (and effectiveness) is affected by various host factors, including age, genetic differences in immune responsiveness, history of infection, and previous vaccination against influenza (i.e., immunological memory), gender, medical history, and health status (3,29). The most important factor among these is age, because the response of the host to the vaccine, which is probably maximal in young healthy adults, varies greatly with age. The lower efficacy of vaccines in younger children than in adults is explained by age-dependent immune competence, and by the fact that many children are naive in terms of infection and/or vaccination against influenza (i.e., they have no immunological memory for the virus).

Vaccine efficacy and effectiveness are also greatly affected by the antigenic relatedness of vaccine and circulating strains (3,24). Since 1973, the annual emergence of new strains is monitored by the WHO surveillance network of laboratories and international organizations, which selects the most suitable vaccine strains for development of effective seasonal influenza vaccines (4,30). In years when the vaccine strains are well matched to circulating strains, vaccine efficacy in healthy children and adults ranged from 60–90%; however, efficacy is likely to be lower in years in which the match is suboptimal.

Field and/or clinical trials to test the prophylactic effect of influenza vaccines require many volunteers. Therefore, most clinical trials designed to evaluate vaccine efficacy have used post-vaccination hemagglutination inhibition (HI) Ab titers as a surrogate for vaccine efficacy. This is because Abs against influenza virus HA are primarily involved in protection against influenza virus infection and because a serum HI titer of 1:40 is a clinically-validated Ab titer that affords 50% protection (34).
Inactivated influenza vaccines induce both S-IgA and IgG Abs in the respiratory tract when administered intranasally with an appropriate adjuvant (40). Several preclinical studies have shown that nasally administered inactivated vaccines, together with an extrinsic adjuvant, induce both cross-reactive S-IgA Abs (which prevent viral infection in the upper respiratory tract) and less cross-reactive IgG Abs (which provide anti-viral protection in the lower respiratory tract) (41–43). Our own recent clinical trials have shown that inactivated WV vaccines, which include a built-in adjuvant (viral single-stranded RNA), induced serum HI Ab responses that exceed the EMA criteria for determining vaccine efficacy (44). The nasally administered inactivated WV vaccines also induced high levels of HI and NT Ab titers, together with a weaker IgG response, in the upper respiratory tract; however, nasal HI titers have not yet been evaluated in terms of vaccine efficacy because of the lack of available criteria for assessment. In addition, one clinical study demonstrated that the ability of human S-IgA Abs to neutralize influenza viruses increased with increasing polymerization of IgA (IgA Abs can form dimers, trimers, tetramers, and larger polymers), suggesting that polymeric S-IgA plays a crucial role in protecting against both homologous and variant influenza viruses (45).

Adjuvant-combined nasal-inactivated vaccines have advantages over parenteral vaccines in that they induce both S-IgA and serum IgG Abs. In addition, nasal inactivated vaccines do not involve the pain associated with vaccine injection. Other advantage of nasal-inactivated vaccines over LAIV vaccines are that non-infectious, inactivated vaccines are suitable for high-risk groups and they probably induce stronger secondary antibody responses than LAIV vaccines.

Here, to demonstrate the utility of nasal-inactivated vaccines, we will present some of our own preclinical studies conducted in an influenza mouse model, along with the results of our clinical trials (43–45). Based on these studies, we propose that intranasal, inactivated vaccines should be developed to improve on influenza vaccine efficacy.
2. Utility of intranasal inactivated vaccines

2–1. Nasal-inactivated influenza vaccine-related knowledge through the 1980s

An intranasal, inactivated vaccine is yet to be licensed for clinical use. The earliest clinical trial of an intranasal inactivated influenza vaccine was conducted in children by Quillengen and Francis Jr (1947); however, the efficacy of the intranasal vaccine was inferior to that of the parenteral vaccine (46). Waldman et al. (1968) reported that repeated immunizations with an inactivated influenza virus induced specific IgA Ab responses that were detectable in human respiratory secretions (47). Preclinical studies comparing intranasal vaccines and parenteral vaccines were initiated by Schulman and Kilbourne (1965), who used mice to demonstrate that natural viral infection was more effective than a parenteral vaccine at providing cross-protection against variant virus infections (48). Later, Liew et al. (1984) demonstrated that mice infected with an influenza A virus produced S-IgA Abs in the lung, which prevented cross-infection by a variant virus of the same subtype (49). Thus, live attenuated virus vaccines, which mimic natural virus infection, would have advantages over parenteral vaccines in that they would induce higher levels of S-IgA Abs. Under these circumstances, Elson and Ealding (1984) demonstrated that cholera toxin (CT), a protein exotoxin produced by *Vibrio cholera*, was not only an effective intestinal immunogen, but also acted as an immunomodulating agent that stimulated the induction of both S-IgA and serum IgG Abs in response to an unrelated antigen when CT and the unrelated antigen were administered together in the intestine (50). The finding that CT is a strong mucosal adjuvant inspired us to develop an intranasal inactivated influenza vaccine. In 1988, we demonstrated that an inactivated influenza vaccine, when administered to BALB/c mice together with CT as an adjuvant, induced both nasal S-IgA and serum IgG Abs and protected them from infection (40).

2–2. Preparation of influenza mouse models for use in preclinical studies

Because influenza virus does not naturally infect mice, mouse models were established using mouse-adapted influenza A and B viruses that emerged during the serial passage of human influenza viruses in these animals (1,5,40,51,52). Briefly, an influenza model was prepared by infecting mice with a small volume (2 μl) of the mouse-adapted virus suspension. This caused a non-lethal upper respiratory tract infection. A viral pneumonia model was prepared by infecting mice with a large volume (20 μl) of the same virus suspension (total respiratory tract infection), which caused a lethal infection of both the upper and lower respiratory tract, along with viral pneumonia. The time-course of virus shedding (Fig. 2) and the histopathological changes in the nasal cavity and lung tissues were similar to those observed in humans with influenza and viral pneumonia (1,3,5,11).

These influenza mouse models were subsequently used for a preclinical study of the utility of nasal inactivated influenza vaccines. Mice were infected with a nasal inactivated influenza vaccine (an SV vaccine) together with the extrinsic adjuvant, CTB° (the cholera

![Fig. 2. Virus titers in naive BALB/c mice infected with a small volume (2 μl; upper respiratory infection) (A) or a large volume (20 μl; total respiratory tract infection) (B) of mouse-adapted A/PR8 virus suspension containing 10^4.1 EID₅₀ under light anesthesia. At specific intervals after infection, six mice from each group were sacrificed, and nasal wash (○) and 10% lung homogenate (●) were titrated. Virus titers were expressed in EID₅₀. Each point represents the mean ± SD of six mice per group. Upper respiratory tract infection caused nonlethal influenza, while total respiratory tract infection caused lethal viral pneumonia.](image)
Fig. 3. Protection against influenza A virus infection and production of cross-reactive anti-HA IgA and IgG Abs in the upper respiratory tract in vaccinated mice. BALB/c mice were immunized intranasally (i.n.) or subcutaneously (s.c.) with different CTB*-combined vaccines (0.5 mg vaccine + 1 µg CTB*). Four weeks later, the immunized mice were challenged with a small volume (1 µl × 2) of mouse-adapted A/Guizhou-X (H3N2) virus suspension. Three days later, virus titers and the concentration of IgA and IgG Abs against A/Guizhou-X viral HA were assayed in the nasal wash. Each column represents the mean ± SD of virus titers (EID₅₀ or Ab titer, ng/mouse) for six mice per group. A large asterisk (*) represents statistically significant difference from a non-immunized control (P < 0.05).
Fig. 4. Protection against influenza A virus infection and production of cross-reactive anti-HA IgA and IgG Abs in the lower respiratory tract of vaccinated mice. BALB/c mice were immunized intranasally (i.n.) or subcutaneously (s.c.) with different CTB*-combined vaccines (0.5 mg vaccine + 1 mg CTB*). Four weeks later, the immunized mice were challenged with a large volume (20 µl) of mouse-adapted A/Guizhou-X (H3N2) virus suspension. Three days later, virus titers and the IgA and IgG Abs against to A/Guizhou-X viral HA were assayed in lung homogenate. All other details are the same as described in the legend to Fig. 3.

Table 1. Serum cross-reactive HI Abs in the mouse immunized intraperitoneally with inactivated influenza viruses

| Antigen                  | Mouse antisera |
|--------------------------|----------------|
| A/PR/8/34 (H1N1)         | Anti-PR8 Abs   |
|                          | Anti-A/ Guizhou Abs |
|                          | Anti-B/ Ibaraki Abs |
| A/Bangkok/10/83 (H1N1)   | <16            |
| A/Yamagata/120/86 (H1N1) | 16             |
| A/Fukuoka/C29/85 (H3N2)  | <16            |
| A/Sichuan/2/87 (H3N2)    | 16             |
| A/Guizhou/54/89-X (H3N2) | 16             |
| B/Nagasaki/1/87          | 16             |
| B/Aichi/5/88             | 16             |

Pooled mouse antisera were obtained from 20 mice that received a single intraperitoneal injection of 0.5 ml of formalin-inactivated whole influenza virus suspension (2,000–5,000 HA titters), prepared from PR8, A/Guizhou-X, or B/Ibaraki virus, 3 weeks previously.

Table 2 shows that the intranasal trivalent inactivated vaccines induced high levels of cross-protection against PR8, A/Guizhou (H3N2), and B/Ibaraki. Three days after challenge, virus and anti-HA Ab titers were measured in nasal washes and lung homogenates. When the ability of each virus to induce serum HI Abs was compared in mice immunized intraperitoneally with each formalin-inactivated whole virus preparation, we found that they differed from one another in terms of HA antigenicity (Table 1).

Table 2. Serum cross-reactive HI Abs in the mouse immunized intraperitoneally with inactivated influenza viruses

| Immunization                  | Challenge with virus, A/Guizhou-X (4 weeks) | A/Guizhou-X HA-reactive Ab (ELISA ng/mouse) in lung wash | Virus titers (EID<sub>50</sub>10<sup>3</sup>) in lung homogenate |
|------------------------------|---------------------------------------------|-----------------------------------------------------------|-------------------------------------------------------------|
| Vaccine with CTB<sup>*</sup>  | Route                                      | 0 200 400 600 800                                         | 0 1 2 3 4 5 6                                              |
|                              |                                             |                                                           |                                                            |
| -                            |                                             |                                                           |                                                            |
| A/Guizhou-X (H3N2)           | i.n.                                        |                                                           |                                                            |
| A/Fukuoka (H3N2)            | i.n.                                        |                                                           |                                                            |
| A/Sichuan (H3N2)            | i.n.                                        |                                                           |                                                            |
| A/PR8                        | i.n.                                        |                                                           |                                                            |
| B/Ibaraki                    | i.n.                                        |                                                           |                                                            |
|                              | s.c.                                        |                                                           |                                                            |
| A/Guizhou-X (H3N2)           | s.c.                                        |                                                           |                                                            |
| A/Fukuoka (H3N2)            | s.c.                                        |                                                           |                                                            |
| A/Sichuan (H3N2)            | s.c.                                        |                                                           |                                                            |
| A/PR8                        | s.c.                                        |                                                           |                                                            |
| B/Ibaraki                    | s.c.                                        |                                                           |                                                            |

were equally as effective as the subcutaneous vaccines in terms of providing cross-protection in the lower respiratory tract.

Next, the efficacy of CTB<sup>*</sup>-combined trivalent inactivated influenza vaccines derived from various virus strains was compared in mice immunized intranasally and subcutaneously (51,55). BALB/c mice were immunized using a two-dose regimen: a primary trivalent SV vaccine containing A/Yamagata/120/86 (H1N1), A/Sichuan/2/87 (H3N2), and B/Nagasaki/1/87 virus split-products plus CTB<sup>*</sup>, and a secondary trivalent vaccine containing A/Bangkok/10/8 (H1N1), A/Fukuoka/C29/85 (H3N2), and B/Aichi/5/88 virus split-products alone. There was a 4-week interval between vaccinations. Two weeks after the second immunization, the immunized mice were challenged with a small volume (influenza model) or large volume (viral pneumonia model) of mouse-adapted virus suspension derived from A/PR/8/34 (H1N1), A/Guizhou-X (H3N2), or B/Ibaraki. Days after challenge, virus and anti-HA Ab titers were measured in nasal washes and lung homogenates. When the ability of each virus to induce serum HI Abs was compared in mice immunized intraperitoneally with each formalin-inactivated whole virus preparation, we found that they differed from one another in terms of HA antigenicity (Table 1).

Table 2 shows that the intranasal trivalent inactivated vaccines induced high levels of cross-protection against PR8, A/Guizhou (H3N2), and B/Ibaraki. Days after challenge, virus and anti-HA Ab titers were measured in nasal washes and lung homogenates. When the ability of each virus to induce serum HI Abs was compared in mice immunized intraperitoneally with each formalin-inactivated whole virus preparation, we found that they differed from one another in terms of HA antigenicity (Table 1).

Table 2. Serum cross-reactive HI Abs in the mouse immunized intraperitoneally with inactivated influenza viruses

| Antigen                  | Mouse antisera |
|--------------------------|----------------|
|                          | Anti-PR8 Abs   |
|                          | Anti-A/ Guizhou Abs |
|                          | Anti-B/ Ibaraki Abs |
| A/PR/8/34 (H1N1)         | 2,048          |
| A/Bangkok/10/83 (H1N1)   | <16            |
| A/Yamagata/120/86 (H1N1) | 16             |
| A/Fukuoka/C29/85 (H3N2)  | <16            |
| A/Sichuan/2/87 (H3N2)    | 16             |
| A/Guizhou/54/89-X (H3N2) | 16             |
| B/Nagasaki/1/87          | 16             |
| B/Aichi/5/88             | 16             |

Pooled mouse antisera were obtained from 20 mice that received a single intraperitoneal injection of 0.5 ml of formalin-inactivated whole influenza virus suspension (2,000–5,000 HA titters), prepared from PR8, A/Guizhou-X, or B/Ibaraki virus, 3 weeks previously.

Thus, these experiments based on a single- or two-dose regimen showed that nasal-inactivated vaccines were superior to subcutaneous vaccines in terms of providing cross-reactive Abs, which are associated with cross-protection in the upper respiratory tract. In addition, the ability of the nasal vaccine to provide cross-
from infection by PR8 virus when transferred to the respiratory tract of the host after transfer. These observations revealed that S-IgA Abs were polymeric (dimeric and pentameric) than purified IgG Abs. Reactive to H1N1 subtype variant viruses (and only HA-specific Ab activity by ELISA, were more cross-activities against the PR8 virus, together with high PR8 protection in the lower respiratory tract was equivalent to that of the subcutaneous vaccine. Furthermore, cross-protection against variant virus infection was dependent on the production of cross-reactive anti-HA S-IgA Abs in the respiratory tract, while lower levels of cross-protection in the respiratory tract resulted from the production of less cross-reactive anti-HA IgG Abs.

Characteristics of HA-specific S-IgA Abs

The functional roles and characteristics of the anti-HA S-IgA Abs derived from BALB/c mice immunized intranasally with CTB*-combined PR8 (H1N1) SV vaccines using a two-dose regimen were examined after isolating these Abs from nasal and lung washes (56). Purified S-IgA Abs, which showed high NT and HI activities against the PR8 virus, together with high PR8 HA-specific Ab activity by ELISA, were more cross-reactive to H1N1 subtype variant viruses (and only partially to H3N2 viruses) than purified IgG Abs. Sephacryl column size-exclusion chromatography revealed that S-IgA Abs were polymeric (dimeric and larger polymers). In addition, they protected naive mice from infection by PR8 virus when transferred to the respiratory tract. The degree of this protection depended on the amount of the anti-HA S-IgA Abs remaining in the respiratory tract of the host after transfer. These results suggest that HA-specific, polymeric IgG Abs in the respiratory tract are more cross-protective against variant viruses than IgG Abs. Also, they protected the host by binding directly to viruses, thereby eliminating them from the respiratory tract.

Distribution of specific IgA and IgG Abs at different sites within the respiratory tract

BALB/c mice were immunized with a primary intranasal CTB* (0.1 μg)-combined PR8 SV vaccine (0.1 μg) followed by a secondary SV vaccine alone (0.1 μg) 4 weeks later. The distribution and concentration of PR8 HA-specific IgA and IgG Abs in the mucus or serous fluid within the entire respiratory tract were then examined (57,58). The vaccine doses used in the two-dose regimen were the minimal doses required to provide complete protection against a lethal PR8 challenge infection 2 weeks after the secondary immunization (59). Examination of the total respiratory tract revealed that 74% of anti-HA IgA Abs and about 6% of

### Table 2. Cross-reactive anti-HA IgA and IgG Ab responses and cross-protection against different virus infection in mice having received a primary and a secondary intranasal inoculation of different trivalent virus vaccines

| Primary | Secondary | Virus* used for challenge infection | Nasal wash | Lung |
|---------|-----------|------------------------------------|------------|------|
|         |           |                                    | Virus titer | Cross-reactive Abs<sup>c</sup> to challenge viral HA | Cross-reactive Abs<sup>c</sup> to challenge viral HA |
| Vaccine + CTB<sup>*b</sup> | Vaccine | Virus titer | IgA | IgG | Virus titer | IgA | IgG |
|         |           |                                    |            |      |            |      |      |
| —       | —         | PR8                                | 4.4 ± 0.4 | 5 ± 2 | <1         | 6.6 ± 0.3 | 44 ± 7 | 3 ± 1 |
| Y + S + N | B + F + A | PR8                                | 1.7 ± 0.7 | 280 ± 191 | 18 ± 9 | 4.9 ± 0.8 | 137 ± 35 | 219 ± 76 |
| —       | —         | A/Guizhou-X                        | 3.8 ± 0.5 | 3 ± 1 | 1 ± 1     | 5.5 ± 0.4 | 19 ± 5 | 7 ± 2  |
| Y + S + N | B + F + A | A/Guizhou-X                        | 0.6 ± 0.2 | 178 ± 136 | 45 ± 37 | 0.9 ± 0.2 | 67 ± 19 | 1,586 ± 846 |
| —       | —         | B/Ibaraki                          | 3.3 ± 0.3 | ND | ND        | 5.4 ± 0.2 | ND | ND |
| Y + S + N | B + F + A | B/Ibaraki                          | 1.0 ± 0.6 | ND | ND        | 2.9 ± 1.0 | ND | ND |

*<sup>a</sup>: Mice received a primary intranasal inoculation of one trivalent vaccine (Y + S + N) composed of A/Yamagata (H1N1), A/Sichuan (H3N2), and B/Ibaraki virus vaccine together with CTB, and 4 weeks later, a second intranasal inoculation of another trivalent vaccine (B + F + A) composed of A/Bangkok (H1N1), A/Fukuoka (H3N2), and B/Aichi virus vaccine.  
*<sup>b</sup>: Two weeks after the second vaccination, mice were infected intranasally with a small (2 μl) or a large volume (20 μl) of PR8 (H1N1), A/Guizhou-X (H3N2), or B/Ibaraki virus. Three days later, their nasal or lung virus titers were assayed.  
*<sup>c</sup>: Two weeks after the second vaccination, nasal or bronchoalveolar washes were collected from the vaccinated mice. The Abs bound to PR8 HA or A/Guizhou-X HA were assayed by ELISA.

### Table 3. Cross-reactive anti-HA IgA and IgG Ab responses and cross-protection against different virus infection in mice having received a primary and a secondary subcutaneous inoculation of different trivalent virus vaccines

| Subcutaneous vaccine*<sup>a</sup> | Primary | Secondary | Virus used for challenge infection | Nasal wash | Lung |
|----------------------------------|---------|-----------|------------------------------------|------------|------|
| Vaccine + CTB<sup>*b</sup>       | Vaccine | Virus titer | Cross-reactive Abs<sup>c</sup> to challenge viral HA | Cross-reactive Abs<sup>c</sup> to challenge viral HA |
| —                                | —       | PR8       | 4.5 ± 0.3 | 3 ± 1 | <1 | 6.3 ± 0.2 | 19 ± 3 | 3 ± 1 |
| Y + S + N | B + F + A | PR8       | 4.0 ± 0.8 | 3 ± 1 | 28 ± 11 | 5.6 ± 0.4 | 30 ± 7 | 375 ± 258 |
| —                                | —       | A/Guizhou-X | 4.1 ± 0.4 | 2 ± 1 | <1 | 5.5 ± 0.6 | 14 ± 3 | 3 ± 1 |
| Y + S + N | B + F + A | A/Guizhou-X | 3.6 ± 0.3 | 1 ± 1 | 14 ± 8 | 2.8 ± 1.7 | 26 ± 4 | 298 ± 127 |
| —                                | —       | B/Ibaraki | 3.8 ± 0.3 | ND | ND | 5.6 ± 0.5 | ND | ND |
| Y + S + N | B + F + A | B/Ibaraki | 3.3 ± 0.2 | ND | ND | 3.0 ± 0.9 | ND | ND |

*<sup>a</sup>: Mice received a primary subcutaneous inoculation of one trivalent vaccine (Y + S + N) composed of A/Yamagata (H1N1), A/Sichuan (H3N2), and B/Ibaraki virus vaccine together with CTB, and 4 weeks later, a second subcutaneous inoculation of another trivalent vaccine (B + F + A) composed of A/Bangkok (H1N1), A/Fukuoka (H3N2), and B/Aichi virus vaccine. For other details, see footnote for Table 2.
anti-HA IgG Abs were present in the nasal mucosa. On the other hand, about 4% of anti-HA IgA Abs and about 91% of anti-HA IgG Abs in the respiratory tract were found in the alveolar serous fluid. The ratio of mucosal IgA Abs to serum IgA Abs was less than 0.5. This suggests that mucosal IgA Abs are secreted actively, whereas mucosal IgG Abs diffuse from the serum to the mucus (12). These results also support previous findings that S-IgA Abs play a primary role in preventing influenza virus infection in the upper respiratory tract, whereas serum IgG Abs play a predominant role in preventing the progression to lethal influenza-induced pneumonia in the lower respiratory tract.

2–3–4. Protection against viral infection in the absence of S-IgA Abs

Polymeric immunoglobulin receptor (pIgR)-knockout (KO) BALB/c mice were immunized intranasally with various CTB*-combined B type SV vaccines and then challenged with a small volume of B/Ibaraki virus suspension 4 weeks later. The ability of B/Ibaraki virus-specific S-IgA Abs to provide cross-protection against upper respiratory tract infection with a variant virus was then examined (57,60). Immunized control wild-type mice showed various levels of cross-protection in the upper respiratory tract, depending on the amounts of cross-reactive S-IgA Abs induced by variant B virus vaccines. On the other hand, blockade of the transcytosis of polymeric IgA Abs in immunized pIgR-KO mice resulted in the absence of S-IgA Abs from the upper respiratory tract and a subsequent lack of cross-protection. These observations clearly showed that S-IgA Abs provided cross-protection against variant virus infection in the upper respiratory tract.

2–4. Properties of adjuvants for nasal-inactivated influenza vaccines

2–4–1. Enhancement of vaccine-specific Ab responses by extrinsic adjuvants

The mechanisms by which CTB*, together with nasal-inactivated PR8 SV vaccines, enhance vaccine-specific Ab responses, were examined in BALB/c mice immunized intranasally with an adjuvant-combined vaccine, an SV vaccine alone, or CTB* alone, followed by infection with a small volume of PR8 virus suspension on days 0, 3, 5, 7, 14, and 21 post-immunization (5,61,62). Virus replication (viral titers) was monitored in nasal washes 3 days after immunization. Small-volume virus infection alone induced the maximal nasal wash virus titer 3 days after infection (Fig. 2). The SV vaccine alone failed to prevent viral replication after the immunization, suggesting that it failed to induce an immune response. CTB* alone partially inhibited PR8 virus replication on days 3, 5, 7, and 14, with the maximum inhibition on day 5. This suggests that CTB* induces a non-specific innate immune response during early stages post-immunization. The CTB*-combined vaccine partially inhibited PR8 virus replication on days 3 and 5, but completely inhibited replication on day 14. These results suggest that mice immunized with the adjuvant-combined nasal-inactivated vaccine mount a CTB*-induced non-specific innate immune response that activates APCs to generate a bystander effect, thereby enhancing vaccine-specific Ab responses. Taken together, these observations suggest that, in addition to stimulating vaccine-specific Ab responses, the efficacy of a nasal vaccine adjuvant candidate can be assessed based on its ability to non-specifically suppress influenza virus replication in the nasal cavities of BALB/c mice.

The finding that a nasal influenza vaccine mixed with LT (NasalFlu, Berna Biotech; licensed in 2001) was linked to several cases of transient Bell’s palsy (facial paralysis) led to a ban on the clinical use of CT or LT-related materials as nasal vaccine adjuvants (63). Thus, safer adjuvants for clinical use as nasal vaccine adjuvants are required, and many adjuvants are currently being developed to improve influenza vaccine efficacy (15,64). We note the usefulness of synthetic RNA poly (I:C), a ligand for TLR 3, as a nasal vaccine adjuvant instead of CT or LT-related materials (15,65,66). However, pathologic effects of double-stranded RNA with respect to age-related macular degeneration (a common cause of irreversible visual impairment) have been reported (67). Whether there is a causal relationship between the development of age-related macular degeneration and the use of poly (I:C) as an adjuvant for nasal-inactivated influenza vaccines remains to be determined. From the standpoint of safety, potential neurotoxic effects must be considered when developing new nasal adjuvants.

2–4–2. Enhancement of specific Ab responses by whole inactivated virus vaccines

Intranasal immunization of mice with formalin-inactivated WV vaccines, but not SV vaccines, induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice (68). The effects of adjuvants on nasal anti-HA S-IgA and IgG Ab responses induced by SV or WV vaccines derived from A/New Caledonia/20/99 (H1N1) viruses were compared in BALB/c mice that received primary and secondary intranasal administrations with 0.1 μg of SV vaccine alone, 0.1 μg of SV vaccine plus CTB*, 0.1 μg of WV vaccine alone, or 0.1 μg of WV vaccine plus CTB* with an interval of 3 weeks (62). The WV vaccine alone induced a stronger Ab response than the SV vaccine alone, which showed the weakest Ab responses. However, while the CTB*-combined WV vaccine induced slightly stronger Ab response than the WV vaccine alone, the CTB*-combined SV vaccine induced a much stronger Ab response than the SV vaccine alone. The maximal level of Ab responses induced by the CTB*-combined WV vaccine was equivalent to that induced by the CTB*-combined SV vaccine. These observations suggested that the higher immunogenicity of the WV vaccine alone might be due to the intrinsic adjuvant effect of single-stranded RNAs within the influenza virus particle; indeed, single-stranded RNAs derived from influenza viruses act as an adjuvant via TLR 7 (69,70).

3. Clinical studies on the intranasal inactivated vaccines

3–1. Clinical trials for nasal-inactivated influenza vaccines from the 1990s

Several clinical trials aimed at improving the efficacy of vaccines via the induction of both S-IgA and IgG Abs in the respiratory tract have been conducted in vaccinees who received nasal-inactivated vaccines either with or
without an extrinsic adjuvant. Kuno-Sakai et al. (1994) evaluated the immunogenicity and efficacy of aerosol (intranasal) trivalent SV vaccines and trivalent LAIV vaccines in young volunteers (aged 16 or 17 years) (71). Volunteers received two doses of the intranasal SV vaccine with an interval of 1 week between doses, and each dose contained 3 times more than that of the parenteral vaccine. First, either the SV vaccine or LAIV vaccine was administered, followed by the other 6 weeks later. The immunogenicity and efficacy of each vaccine was then evaluated by measuring serum HI and nasal anti-HA IgA Ab responses at 6 and 12 weeks post-vaccination. The live virus vaccine was deemed to trigger specific immune responses if it induced a 4-fold or greater increase in serum HI levels. The results showed that the intranasal SV vaccine stimulated serum HI and nasal IgA Ab responses more strongly than the live virus vaccines.

Hashigucci et al. (1996) conducted clinical trials to evaluate the efficacy of an LTβr-combined nasal SV vaccine during the winter season from 1993 to 1994 (72). A trivalent SV vaccine (140 μg of protein, total) comprising three monovalent vaccines (each containing 15 μg of HA) derived from A/Yamagata (H1N1), A/Kitakyushu (H3N2), and B/Bangkok strains, either with or without recombinant LTβr (100 μg) was administered intranasally (125 μl to each nostril) to volunteers. The vaccine was given in two doses, with a 4-week interval in between. Salivary IgA and serum HA Abs were measured before and at 8 weeks after each vaccination. The results showed that 50.7% and 49.3% of 73 vaccinees receiving the nasal LTβr-combined vaccine were positive for IgA and HI Ab, respectively, against one or more of three vaccine strains. Only 32.7% and 30.6% of 49 vaccinees in the control group given the vaccine alone showed positive IgA and IgG Ab responses, respectively. Thus, the nasal LTβr-combined SV vaccine induced stronger responses (in terms of nasal IgA and serum IgG Abs) than the nasal SV vaccine alone. In the winter season from 1994 to 1995, a second similar clinical trial was conducted, except that this time B/Mie/1/93 was used instead of B/Bangkok/163/90. Influenza infections were monitored for up to 8 weeks after the second vaccination. During the season, many people suffered from infection by H3N2-subtype and B-type viruses. Thus, the preventive efficacy of the LTβr-combined SV vaccine was 61%, although there was no statistically significant difference between the vaccinated and control groups. The nasal LTβr-combined SV vaccine induced mild side effects (running nose, stuffy nose, headache, etc.) in some vaccinees; however, these subsided within a few days after vaccination.

In 1999, a research group in Switzerland evaluated the safety and comparative immunogenicity of a trivalent virosomal influenza vaccine, administered either with or without *Escherichia coli* heat-labile toxin (HLT), in healthy working adults. The vaccines were given once or twice by intranasal spray, with an interval of 1 week between vaccinations in the latter case (73–75). They found that the use of HLT as a mucosal adjuvant was necessary to induce a humoral immune response comparable with that observed after parenteral vaccination. Serial studies confirmed that the intranasal HLT-combined virosome vaccine induced strong HI Ab responses in both young adults and elderly subjects. They also confirmed that both humoral and cell-mediated responses and IgA NT Ab responses were elicited in the mucosa of healthy adults. However, the use of CT or LT-related materials as an adjuvant for nasal inactivated vaccines is not clinically safe (63).

Research by GlaxoSmithKline Biologicals revealed that an intranasally administered proteasome-based influenza vaccine comprising detergent-split influenza and proteosome adjuvants derived for *Neisseria meningitidis* outer membrane proteins induced production of both serum HI and nasal S-IgA Abs, and determined its safety and immunogenicity in healthy adults and its efficacy in preventing clinical illness (76–78). The vaccines demonstrated 56–68% efficacy against influenza-like illness and laboratory-confirmed influenza infection, as assessed by positive virus culture or reverse transcriptase PCR.

Another research group in Israel evaluated the immune responses induced by a novel inactivated trivalent WV vaccine (formaldehyde and β-propiolactone free), which was given intranasally to children (2001), young adults (2004), or the elderly (2000, 2003) either once or twice (with an interval of 3 weeks in the latter case), and compared the responses with those induced by a commercial intramuscular trivalent vaccine (79–82). Although the difference in the seroconversion rate between intramuscular vaccination and intranasal vaccination was not statistically significant, mucosal IgA Ab titers were significantly higher after intranasal vaccination than after intramuscular vaccination.

### 3-2. Our recent clinical trials of nasal-inactivated influenza vaccines

#### 3-2-1. Nasal wash-NT Abs from adults immunized with nasal-inactivated vaccines

In one of our recent clinical trials, we examined NT Ab levels in nasal washes and sera from five healthy adults immunized intranasally with an SV vaccine containing a dose of 45 μg of HA (a dose 3-fold higher than that of the SV vaccine developed for parenteral use), which was derived from the A/Uruguay/716/2007 (H3N2) virus. Individuals received five doses, with an interval of 3 weeks between each (83). The concentrated SV vaccine was chosen because a 3-fold higher dose of trivalent SV vaccine induced S-IgA Ab responses in the nasal wash after intranasal vaccination in humans (71). Usually, HI Ab titers are used to evaluate vaccine efficacy, despite their instability; however, nasal wash NT Ab titers are considered a more important criterion than the serum HI Ab titers when assessing the protective efficacy of a vaccine (84,85). Therefore, we measured the NT activity in nasal mucus, which is physiologically comparable to serum. The nasal mucus samples were prepared by concentrating nasal wash samples to yield 1 mg/ml of total mucus protein, containing approximately 1/10 of the total IgA Abs found in undiluted mucus (86). When nasal mucus-NT Ab responses were compared with serum NT Ab responses, the nasal NT Ab responses increased more efficiently than the serum responses (and in line with the number of vaccinations). Thus, a greater than 4-fold increase in the nasal-NT Ab titers was observed after the second vaccination, whereas an increase in serum-NT Ab titers was observed only after the fifth vaccination.
NT Ab titers were 4-fold that of serum HI Ab titers. Gel filtration chromatography revealed that nasal and serum NT Abs were mainly found in the polymeric IgA and monomeric IgG fractions. These results suggest that a 3-fold-concentrated nasal SV vaccine induces high levels of nasal-NT Abs and lower levels of serum-NT Abs when administered five times with a 3 week-interval (a five-dose regimen). When used as a two-dose regimen, the intranasally administered SV vaccine failed to induce serum HI Ab titers that fulfilled the EMA criteria.

Next, we obtained nasal wash samples from adult volunteers immunized intranasally in a five-dose regimen with an inactivated WV vaccine derived from an A/Victoria/210/2009 (H3N2)-like virus and examined the relationship between the structure of human S-IgA Abs and NT activity against influenza virus (45). The nasal wash samples were concentrated, separated by gel filtration chromatography, and then NT activity was measured. In addition, the quaternary structures of the nasal IgA were determined by biochemical analyses and high-speed atomic force microscopy (AFM). The results showed that human nasal IgA comprised at least five quaternary structures: monomers, dimers, trimers, tetramers, and polymers. Direct and real-time visualization of S-IgA by AFM revealed that trimeric and tetrameric S-IgA had six and eight antigen-binding sites, respectively, and that these structures exhibited large-scale asynchronous conformational changes when capturing influenza HA antigen in solution. Among these structures, the polymeric form showed higher NT activity against various seasonal influenza viruses (H3N2) and highly pathogenic influenza virus (H5N1) than the dimeric form. Thus, the ability of S-IgA Abs to provide cross-protection depends on polymeric structures, which displayed increasing NT activity with increasing polymerization. These results suggested that the presence of large polymeric S-IgA Abs with higher NT activity in the respiratory tract play a crucial role in providing protection against homologous and variant influenza viruses.

**3–2–2. Enhancement of nasal and serum Ab responses by nasal-inactivated WV vaccines**

Several reports suggest that intranasal immunization of mice and humans with inactivated WV vaccines induces more effective immune responses than immunization with SV vaccines alone (62,65,73–75,83). In our second clinical trials, 50 healthy adults were vaccinated intranasally with a 3-fold strength WV vaccine (containing 45 μg of HA/dose) derived from an A/Victoria/210/2009 (H3N2) virus and using a two-dose regimen, with an interval of 3 weeks in between doses. The protective efficacy of the intranasal inactivated vaccine was then assessed by examining HI and NT Ab responses in serum and nasal mucus samples obtained 3 weeks after the second immunization (Table 4) (44,83). Serum HI titers after two doses of the nasal vaccine showed a 4.25-fold rise in the ratio of geometric mean titers upon vaccination, 43.5% of subjects showed a ≥4-fold increase in HI titer, and 76.1% of subjects showed a titer of ≥1:40. Thus, the intranasal inactivated WV vaccine induced high levels of serum HI Ab, fulfilling all of the EMA criteria for evaluating vaccine efficacy. The serum HI Ab responses correlated with NT Ab responses, which were about 2-fold higher than the HI titers. These strong serum responses were accompanied by strong HI and NT Ab responses in nasal mucus. Serum and nasal HI and NT Ab comprised HA-specific IgG and IgA Abs, with IgG and IgA Abs being dominant in serum and nasal washes, respectively. In addition, the intranasal WV vaccine induced partially cross-reactive HI and NT Abs to A/Sydney05/1997 (H3N2) in both serum and nasal mucus. Taken together, these results suggest that the intranasal WV vaccine is a promising candidate.

However, before nasal-inactivated WV vaccines can be applied, the efficacy of the WV vaccine needs to be improved by reducing the vaccine dose (from 45 μg of HA/dose to less than 15 μg of HA/dose) to cut vaccine costs. Future studies are likely to yield further improvements (87). Cutting-edge research into a quadrivalent intranasal inactivated WV seasonal influenza vaccine, which incorporates a carboxy-vinyl polymer as a mucoadhesive excipient, is ongoing. Another issue that must be resolved prior to practical application is confirmation of the efficacy and safety of the nasal inactivated vaccine in the infants, children, and the elderly (88).

**4. Perspectives**

Here, we showed that current vaccination programs can be improved by the introduction of intranasally inactivated vaccines, such as the WV vaccine (containing 45 μg of HA/dose). The advantages of nasal-inactivated vaccines over the current vaccines are as follows: (i) The nasal vaccine induces both S-IgA Ab (mainly responsible for preventing virus infection in...
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...the upper respiratory tract) and serum IgG Ab (mainly responsible for providing protection against serious illness due to infection in the lower respiratory tract) responses; (ii) nasal S-IgA Abs are not only reactive with homologous virus HA but are also highly cross-reactive with variant viral HA, thereby providing protection and cross-protection against homologous and variant viruses, respectively, whereas serum-derived IgG Abs are less cross-reactive and less cross-protective. Thus, nasal-inactivated vaccines are expected to be more effective than parenteral vaccines when the immunogenicity of the vaccine virus strain is different from that of the epidemic virus strain; (iii) the nasal-inactivated vaccine will alleviate the phenomenon of “original antigenic sin” more effectively than current parenteral and live attenuated vaccines (21–25,54,55); (iv) the nasal-vaccinated non-infectious preparation) is expected to be available for high-risk subjects; (v) since the vaccine is sprayed into the nasal cavity, there is no pain involved. This may increase the vaccination rate.

Vaccine efficacy can be affected by many factors, including vaccine constituents, conformation, manufacturing method, dosage, storage conditions, use of adjuvants, and route of administration. The efficacy of inactivated vaccines that are administered through the parenteral (subcutaneous or intramuscular) route can be improved by changing to nasal administration.

The efficacy of current vaccines can be improved by cell culture-derived vaccines (89–92). Current vaccines are prepared from viruses grown in chicken eggs; however, vaccine viruses sometimes mutate during proliferation. The multiple passages required for virus proliferation in eggs (to generate a vaccine virus from a seed virus) may result in a variant vaccine virus that has immunogenic properties different from that of an epidemic virus due to the accumulation of mutations. This suggests that a variant virus vaccine that is not effective against an epidemic virus might be administered. This problem can be solved using mammalian cell culture-derived vaccines, in which the frequency of mutation is very low. The efficacy of culture-derived vaccines could be further improved by nasal inoculation.

The efficacy of SV or inactivated WV vaccines can be further improved using recombinant protein vaccines (e.g., a virosome vaccine comprising rHA and rNA) or virus-like particle (VLP) vaccines (e.g., a vaccine that combines HA-M1 VLPs with NA-M1 VLPs), which are produced based on the genetic information of predicted epidemic virus strains (93–98). The use of recombinant protein or VLP vaccines may prevent a mutation-induced reduction in vaccine efficacy during virus proliferation and strengthen the role of HA and NA molecules as protective antigens. Administration of these vaccines via the nasal route would further improve their protective capacity.

Generally speaking, the higher the doses of vaccine or adjuvant the better the immune response. However, lower doses of the vaccine or adjuvant are obviously preferable to higher doses from the standpoint of safety and cost. Therefore, the quality of newly developed nasal inactivated vaccine will be evaluated according to the improvements in efficacy, safety, and cost. In particular, nasal-inactivated vaccines are expected to be superior to the current injectable vaccines in terms of efficacy, safety, and cost, regardless of whether they are used in infants, children, or the elderly. On the other hand, measurement of serum HI titers is not an accurate method to assess the efficacy of nasal vaccines (38,85). Novel criteria for the assessment of influenza vaccines in which nasal and serum NT Abs are taken into account should be established to better assess the utility of nasal-inactivated vaccines in development.

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