1. Introduction

1-1. Characteristics of influenza
Influenza is a contagious, acute respiratory disease caused by an influenza virus infection that attacks the host’s respiratory tract mucosa (1,2). The virus is an enveloped virus with two external glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and seven internal proteins (nucleoprotein [NP], three polymerase proteins [PA, PB1, PB2], two matrix proteins [M1 and M2], and nonstructural proteins [NS2]). The virus infects the host’s epithelial cells by binding to receptors (sialic acid) on the cell surface via one of the surface glycoproteins, HA. Several hours after infection, the newly synthesized viruses are released from the infected cells by the action of another surface glycoprotein, NA. Influenza is characterized by pandemics, annual epidemics, or local outbreaks, which are caused by the emergence of mutant viruses with variable HA and NA glycoproteins. Influenza viruses are divided into types A, B, and C, based on antigenic differences between the NP and M proteins. Influenza A viruses are further divided into subtypes (H1N1, H3N2, H5N1, etc.), depending on the antigenic differences in the HA and NA molecules. The subtype viruses, which are responsible for influenza pandemics, arise from genetic reassortment between human and animal viruses after a double infection of the host cells (antigenic shift). The A-subtype and B-type viruses undergo small changes in the HA and NA molecules every year (antigenic drift) and are responsible for annual epidemics or local outbreaks of influenza.

1-2. Immune responses following influenza virus infection in humans
The respiratory tract mucosa is not only the site of infection for influenza viruses but also the site of defense against virus infection. Viruses are initially detected and destroyed non-specifically by innate immune mechanisms; however, if the viruses escape the early defense mechanisms, they are detected and eliminated specifically by adaptive immune mechanisms. The major adaptive immune mechanisms in-
volved in the systemic and mucosal antibody (Ab) responses after infection with live attenuated influenza A virus have been investigated in humans (3,4). Mucosal immunoglobulin (Ig) A1 and serum IgG Abs are involved in the immunity induced by influenza virus infection. Secondary nasal wash anti-HA IgA Ab responses are involved in this protection and are manifested by accelerated virus shedding from the upper respiratory tract. On the other hand, an association between cell-mediated immune responses and resistance to the influenza virus in humans has also been reported (5). The antigens (on the NP and M1 proteins) recognized by class I restricted cytotoxic T cells (CTLs) in humans are highly conserved among influenza A viruses. However, the epidemiologic behavior of influenza viruses in humans suggests that the overall contribution of CTLs to the reduction in disease during re-infection with the influenza virus is small because repeated infection with the virus, which bears common internal viral antigens, provides little resistance to disease caused by new influenza variants (3.6). Further investigation into the mechanisms of mucosal immune responses following influenza virus infection or vaccination in humans have been carried out using animal models of influenza.

1-3. Mouse model of influenza

Although mice are not natural hosts of the influenza virus, they can be infected experimentally with either influenza A or B virus mutants (mouse-adapted influenza viruses) that emerge during serial passaging of human influenza viruses and cause pathological changes similar to those that occur during human infection (2,7). The mouse model of influenza has played an important role in analyzing the details of the defense mechanisms against influenza virus infection and in developing an effective vaccine to prevent influenza (8,9). Some advantages of the mouse model over other animal models are the easy breeding and availability of inbred strains, the extensive background knowledge of the murine immune system, which involves the major histocompatibility complex (MHC) molecules, and the availability of well-defined reagents for characterizing components of the immune response. Moreover, the easy availability of transgenic mice, in which the gene for a certain molecule can be deleted, overexpressed, or expressed in a mutated form, for investigating the function of a particular molecule is also one of the advantages of the mouse model (10).

1-4. Defense mechanisms against influenza

The defense mechanisms have primarily been analyzed using a mouse model of influenza. The mechanisms comprise several effector cells and molecules (2,3,8,9). Viruses are initially detected and destroyed non-specifically by innate immune mechanisms that are not antigen-specific and do not require a prolonged period of induction. Several components such as mucus, macrophages, dendritic cells (DCs), natural killer (NK) cells, interferon (IFN) α, β, and other cytokines, and complement components are involved in the innate immune system. However, if the viruses manage to avoid the early defense mechanisms, they are detected and eliminated specifically by adaptive immune mechanisms in which secretary-IgA (S-IgA), IgG Abs, and CTLs (CD8+ cytotoxic T lymphocytes) are involved in the recovery from influenza following viral infection of naïve mice. Preexisting specific S-IgA and IgG Abs in animals immunized previously against infection provide protection against challenge infection. In the absence of Abs in the pre-immunized animals, the production of S-IgA and IgG Abs is accelerated and augmented by B memory cells after re-infection, and these Abs play a role in viral elimination from day 3 onwards after re-infection. In infected animals, the dimeric IgA Abs are transported through the epithelial cells, where they are involved in the prevention of viral assembly by binding to newly synthesized viral glycoproteins. In the absence of Abs in pre-immunized animals, CTLs are also produced rapidly by CD8+ memory T cells and these CTLs are involved in killing the host cells infected from day 3 onwards after re-infection. Induction of the adaptive immune mechanisms, including IgA/Ab production, is mediated by mucosal DCs, which are functionally distinct from DCs in other tissues (11,12).

1-5. Influenza vaccine

To prevent influenza, protective immunity must be induced in advance by the administration of a vaccine. Currently available inactivated vaccines, which are composed of either formalin-inactivated whole-virus (WV) vaccines, subvirion (SV) vaccines (detergent disrupted split-virus proteins, which usually contains 30% HA antigen), or surface antigen vaccines (purified glycoproteins), are injected parenterally (2,9). The major protective immunity induced by influenza virus infection is provided by S-IgA and IgG Abs in the respiratory tract. However, current parenteral inactivated vaccines mainly induce serum IgG Abs, rather than mucosal IgA Abs, which are cross-reactive among drift viruses within a subtype. Thus, the inactivated vaccines, injected parenterally, are effective in protecting against an epidemic of homologous viruses, but are relatively ineffective against an epidemic of heterologous viruses. We therefore, have advocated an intranasal administration of inactivated vaccine to elicit S-IgA Ab induction to improve the protective efficacy of the current inactivated vaccines (8,9). In this regard, the Food and Drug Administration (FDA) recently approved a cold-adapted, live-attenuated vaccine (Flumist; MedImmune Vaccines, Inc., Gaithersburg, Md., USA) for intranasal administration (2,13–15). This live-virus vaccine can induce IgA and IgG Abs and CTLs. In addition to the nasal-spray seasonal flu vaccine (live attenuated influenza vaccine; FluMist®), the nasal spray vaccine for 2009 H1N1 influenza is currently used in the United States. However, the live-virus vaccine is not licensed for vaccination of children aged less than 2 years, or for adults greater than 49 years (thus excluding two major high-risk groups, infants and the elderly), in addition to immunodeficient patients and pregnant women, because of side effects, such as coryza, sore throat, and febrile reactions. An advantage of the nasal-inactivated influenza vaccine over the live-virus vaccine would be the availability for all age groups, including high-risk groups.

1-6. Viral antigens involved in protection

Virus strain-specific or subtype-specific Abs against HA and NA molecules are major protective elements against virus infection. Abs against the M2 protein, which is conserved within A-type viruses, are cross-protective between different subtype-viral infections, although the production levels are low (16). Abs against the conserved NP and M1 proteins can be induced, although they fail to contribute to protection; passive transfer of anti-M1 or anti-NP monoclonal Abs (mAbs) have failed to protect mice from challenges by a wild-type influenza virus (3). These findings can be confirmed by results indicating that vaccination with plasmid DNAs expressing both HA and NA viral proteins from A or B viruses provide the most effective protection against a lethal virus challenge among HAs, NA, M1-, NP-, and NS1-expressing DNAs in mice (17–19), although NP-expressing DNAs also
provided protection when relatively large amounts of them are injected repeatedly (20). Thus, anti-HA Abs are primarily responsible for preventing infection, while anti-NA Abs and CTLs specific for viral core proteins are responsible for reducing viral spread and thereby accelerating recovery from influenza.

1-7. Purpose of this review

To improve the efficacy of current inactivated vaccines, many trials that include intranasal or epidermal administration of inactivated vaccine with or without an adjuvant have been conducted and are still being conducted. We have demonstrated that intranasal immunization with inactivated vaccines used in conjunction with the cholera toxin B subunit adjuvant (CTB*), the cholera toxin B subunit (CTB) containing a trace amount (0.1%) of cholera toxin (CT) (or LTB*, the Escherichia coli heat-labile enterotoxin B subunit [LTB]) containing a trace amount [0.5%] of the heat-labile toxin [LT]), provides effective cross-protection in the upper respiratory tract against variants (drift viruses) within the influenza A virus subtypes or B virus variants (21,22). The strong cross-protection in the upper respiratory tract is provided mainly by S-IgA Abs, whereas the weak cross-protection in the lower respiratory tract is provided by IgG Abs (22-24). However, the use of CTB* or LTB* as an adjuvant with the nasal influenza vaccine may not be clinically safe because the use of an intranasal virosomal vaccine together with LT (Nasalflu; Berna Biotech, Bern, Switzerland), following licensing in 2001, has been linked to several cases of transient Bell’s palsy (facial paralysis) (25,26). Thus, clinically safer and more effective adjuvants are required for the nasal-inactivated influenza vaccine. In the process of developing the adjuvant-combined vaccine, we have discovered a means of estimating the effective doses of the nasal-inactivated influenza vaccine required in humans from mouse-model experiments. This process is also useful in assessing the efficacy of a new unknown adjuvant for the nasal vaccine in humans. In this review, we describe the data and explain the rationalization behind the method for estimating the effective vaccine dose in humans. We also describe a non-specific suppression of influenza virus replication as a means of assessing the efficacy of unknown adjuvants and the development of other adjuvants for nasal vaccines that should be clinically safer than CTB*.

2. Estimation of an effective dose of nasal vaccine in humans from BALB/c mice

2-1. Minimal effective dose of nasal vaccine in BALB/c mice

The minimal effective doses of nasal SV vaccine required to provide complete protection against infection by a lethal dose of homologous virus were examined in BALB/c mice. When the naïve mice were anesthetized and then infected by dropping 20 μl of PBS containing the virus suspension with 40 LD₅₀ into a right nostril, the viruses (detected by the virus titer expressed in PFU/ml) appeared on day 1 at a high level (around 10⁶ PFU/ml in the lung wash) and reached the highest level (around 10⁷ PFU/ml in the lung wash) on days 3–5. Almost all mice died from pneumonia around day 7 with a rapid decrease in body weight (around 40%). The mice were immunized intranasally with CTB*-combined SV vaccine from the PR8 virus (A/PR/8/34, H1N1) and were boosted with the same dose of the PR8 vaccine 4 weeks later (a two-dose regimen) (27,28). The minimal effective dose that provided complete protection (less than 10³ PFU/ml from day 1 to day 3 without the body weight reduction) against the lethal virus infection was 0.1 μg of the SV vaccine (containing approximately 30 ng HA) (together with 0.1 μg of CTB*, 1 μl/nostril × 2). The highly cross-protective IgA Abs in the upper respiratory tract and the less cross-protective IgG Abs in the lower respiratory tract were induced to provide complete protection. A single dose of the adjuvant-combined vaccine was not enough to provide complete protection. In addition, CTL responses and delayed-type hypersensitivity-related T-cell responses did not play major roles in the induction of complete protection.

2-2. Effectiveness of nasal adjuvant-combined vaccine in humans

The first field trial to evaluate the efficacy of the LTB*-combined nasal SV vaccine was conducted during the winter season of 1993 to 1994 (29,30). A trivalent SV vaccine (total 140 μg protein) composed of three monovalent vaccines (each containing approximately 15 μg HA) from A/Yamagata (H1N1), A/Kitakyushu (H3N2), and B/Bangkok virus strains with or without recombinant LTBI* (100 μg) (125 μl × 2) was administered intranasally to volunteers by spraying twice with a 4-week interval. Salivary IgA and serum hemagglutination-inhibition (HI) Abs were measured before and 8 weeks after the primary vaccination. The results showed that in the group given the nasal LTBI*-combined vaccine, 37 (50.7%) and 35 (49.3%) of 72 vaccinees were positive for IgA and HI Ab responses, respectively, to one or more of these vaccine strains. In the control group given the vaccine alone, 16 (32.7%) and 15 (30.6%) of 49 vaccinees showed positive IgA and HI Ab responses. There was a statistically significant difference between these two groups (Student t test: P < 0.05). Thus, the nasal LTBI*-combined SV vaccine (approximately 50 μg for the monovalent vaccine) could induce higher responses for both nasal IgA and serum IgG Abs than the SV vaccine alone. Whether the increase in the salivary IgA Ab response was effective in preventing influenza could not be determined in the present trial because there was no epidemic of influenza during the test period.

In the winter season of 1994 to 1995, a second field trial was conducted to evaluate the efficacy of the nasal LTBI*-combined SV vaccine (30,31). The vaccination conditions in the second field trial were the same as those in the first field trial, except that B/Mie/1/93 was used instead of B/Bangkok/163/90. Contraction of influenza was followed for up to 8 weeks after the second vaccination. During the season, we had outbreaks of H3N2-subtype virus and B-type virus. The results showed that in the group given the LTBI*-combined vaccine, 3 (16.7%) of 18 vaccinees contracted an influenza, while in the non-vaccinees, 6 (42.7%) of 14 subjects contracted an influenza. These data suggest that the nasal LTBI*-combined SV vaccine is effective in preventing influenza (effectiveness, 61%), although there was no statistically significant difference between the two groups. In these studies, the LTBI*-combined nasal vaccine induced slight side-effects (running nose, stuffy nose, headache, etc.) in some of the vaccinees, which subsided within a few days after vaccination.

2-3. Relationship of the effective dose of nasal vaccine between BALB/c mice and humans

The minimal SV vaccine dose required for the induction of complete protection against a lethal virus infection was 0.1 μg of monovalent vaccine/approximately 20 g mouse in BALB/c mice immunized with the CTB*-combined SV vaccine twice in a 4-week interval (27,28). The vaccine dose
in BALB/c mice corresponds to the ratio of 100 μg monovalent SV vaccine/20 kg of body-weight. On the other hand, in the human vaccination trials conducted using a similar schedule to that in BALB/c mice, the effective SV vaccine dose, which induced significantly high IgA and IgG Ab responses in humans (approximately 50% of vaccinees), was approximately 50 μg monovalent vaccine/approximately 50 kg person in the immunized volunteers (29-31). Thus, the minimal effective dose in BALB/c mice is very close to that in humans when expressed as the dose/body weight ratio. These results suggest that the minimal effective dose in humans could be estimated using the dose/body weight ratio from the BALB/c mouse model experiments: the minimal effective SV vaccine dose (approximately 0.1 μg) in BALB/c mice corresponds to around 100 μg vaccine proteins containing approximately 30 μg HA in humans.

2-4. Rationale for estimation of the effective nasal vaccine dose in humans from BALB/c mice

The responsiveness to the vaccine could vary greatly from individual (mouse inbred strain) to individual (strain) because it is controlled by multiple MHC genes that direct the immune responsiveness of a certain individual to a certain viral antigen, and this is true in humans as well as in mice (3,32). MHC molecules, encoded by the MHC genes, bind peptides from viral antigens synthesized within the infected cells and displayed on the antigen-presenting cell surface. The MHC-peptide complex on the antigen-presenting cell is recognized by the appropriate T cells, which activate B cells, which in turn produce the antigen-specific Ab and CTL (CD8+ cytotoxic T lymphocyte) responses (3,8). Thus, in theory, estimation of the effective dose of nasal vaccine from the dose/body weight ratio between BALB/c mice and humans could not be applied to other inbred strains of mice and humans (33).

In this regard, we have examined the MHC-linked differences in anti-HA Ab responses using various inbred and congenic strains of mice immunized intranasally with the PR8 SV vaccine (45 μg) in the presence or absence of a CTB* adjuvant (1 μg). The responsiveness to HA was related to the H-2 haplotype: H-2^a and H-2^d strains, H-2^b strains, and H-2^k strains were high, intermediate, and low responders, respectively (32). We have also examined the MHC-linked strain differences in Ab responses to the influenza viral HA protein (A/Beijing/262/95, H1N1) in B10 congenic mouse strains, immunized intranasally with 0.1, 0.3, or 1 μg of HA together with CTB* and boosted with 0.3 μg of CTB*-combined HA 4 weeks later (34). The HA immunization induced HA-specific IgG Ab responses, which increased with an increase in HA doses used for primary immunization and depended on the H-2 haplotype of the strain (Fig. 1). Thus, the B10.A (H-2^a), B10.D2 (H-2^b), B10.BR (H-2^k), and B10 (H-2^d) strains were high, intermediate (upper and lower), and low responders, respectively. These results demonstrate that mice of the B10.D2 strain, which carries the H-2^d haplotype, are intermediate responders to HA, just as those of the BALB/c strain.

The distribution of each strain in the population of a particular mouse species, with regard to the responsiveness to HA, seems to follow a statistically normal distribution, in which low, intermediate, and high responders are included (Fig. 2). Based on these results, the mouse strains protected by the minimal effective vaccine dose (0.1 μg) would be the intermediate and high responders, which make up a considerable proportion of mouse species. The distribution of individuals in a human population, with regard to the responsiveness to HA, would also be a statistically normal distribution. Thus, estimation of the effective dose of nasal vaccine in humans from BALB/c mice (H-2^d) can be rationalized by the hypothesis that a BALB/c mouse corresponds roughly to an average individual (an intermediate responder to HA) in the human population, and that a considerable proportion of the human population is made up of intermediate and high responders for the responsiveness to HA in a statistically normal distribution.

3. Effect of the adjuvant on inactivated WV vaccines or SV vaccines

The WV vaccine is different from the SV vaccine with respect to the ability of BALB/c mice to induce anti-HA Ab responses to the intranasal immunization of the vaccine alone. When BALB/c mice were immunized intranasally with various doses of the WV vaccine (0.01 to 10.0 μg) from A/New Caledonia/20/99 (H1N1) in a two-dose regimen 3 weeks apart, nasal anti-HA IgA Ab responses were induced, appearing first at 0.1 μg and increasing with increases in the vaccine doses. In addition, serum IgG Ab responses appeared first at 0.01 μg, increased with increases in the vaccine doses, and reached a maximum at 0.1 μg (Fig. 3). In contrast, BALB/c mice, when immunized intranasally with various doses of the SV vaccine (0.1 to 1.0 μg), showed serum IgG Ab responses that appeared clearly at 1.0 μg, but only a marginal level of

![Graph](image-url)

**Fig. 1.** A/Beijing (A/Beijing/262/95/H1N1) viral HA-reactive serum IgG Ab responses induced in B10 (H-2^a), B10.BR (H-2^b), B10.D2 (H-2^d), and B10.A (H-2^d) mice. Serum samples were obtained from each group of mice, which were immunized intranasally with 0.1, 0.3, or 1 μg of HA, together with CTB* (0.3 μg), and then boosted with 0.3 μg of the CTB*-combined HA 4 weeks later. HA molecules were purified from detergent-disrupted A/Beijing viruses. Each bar represents the mean of A/Beijing HA-reactive serum IgG ELISA titers ± S.D. from each group of five mice.
nasal anti-HA IgA Ab responses even at 1.0 μg. Thus, the WV vaccine itself seems to be a promising nasal vaccine if higher doses (around 10.0 μg) can be used. The higher immunogenicity of the WV vaccine than that of the SV vaccine could be explained by the adjuvant action of single-stranded RNAs contained in the virus particle, via Toll-like receptor (TLR) 7 (33,35–37). Some clinical trials have been reported in which a threefold strength of commercially available SV vaccine or the WV vaccine was administered intranasally, resulting in enhanced production of both local HA-specific IgA Abs and serum HI Abs (38–41).

The addition of CTB* to the WV vaccine (0.1 μg) slightly enhanced the upper levels of the IgA and IgG Ab responses, induced by the WV vaccine alone at 0.1 μg, resulting in the highest Ab responses. On the other hand, the addition of CTB* to the SV vaccine greatly enhanced the lowest levels of IgA and IgG Ab responses induced by 0.1 μg of the SV vaccine alone, resulting in the highest Ab response levels (Fig. 3). These results suggest that the addition of adjuvant to either the WV vaccine or the SV vaccine at the lower dose levels (around 0.1 μg) is important for providing the highest level of protective Ab responses.

4. Mechanisms by which a nasal adjuvant enhances adaptive Ab responses

To clarify the mechanisms by which the CTB* adjuvant enhances the specific IgA and IgG Ab responses against influenza viral antigens when administered intranasally to mice together with the vaccine, we examined the effects of intranasal administration of CTB* alone on the influenza virus infection (42). Mice were infected with a nasal cavity-restricted
volume of the PR8 virus on days 0, 3, 5, 7, 14, and 21 after CTB* administration. Three days after the infection, the nasal wash samples were obtained for the virus titration. As a positive control, mice were administered a CTB*-combined vaccine on day 0 and the nasal wash samples for the virus titration were obtained on days 0, 3, 5, 7, 14, and 21 after the CTB*-combined vaccine administration. The virus titer 3 days after the vaccination was plotted against days after the administration of CTB* or CTB*-combined vaccine (Fig. 4). The figure shows that CTB* immunization induces a transient reduction in the virus titer, which reaches a maximum on day 5, while the CTB*-combined vaccine induces a further reduction in the virus titer to the lowest level 7 days after the vaccination, which correlates inversely with the increase in anti-vaccine Ab responses. These results suggest that intranasal immunization with CTB* causes a non-specific reduction in influenza virus replication in the upper respiratory tract, since there is no antigenic relationship between CTB* and the influenza virus. On the other hand, immunization with the CTB*-combined vaccine induces specific Ab responses, resulting in a complete elimination of the virus from the upper respiratory tract by day 7. As immunization with the vaccine alone does not induce specific Ab responses, the addition of CTB* to the vaccine stimulates the adaptive immune system non-specifically to respond to the vaccine. Thus, the non-specific suppression of influenza virus replication by CTB* and the transient suppression, which reaches a maximum within 5 days after CTB* administration, can be explained by the non-specific stimulation of the innate immune system. In addition, stimulation of the innate immune system by CTB* was accompanied by the activation of non-T cells (macrophages, DCs, and others) in the nasal-associated lymphoid tissues (NALT) and promotion of interleukin (IL)-1β secretion by NALT cells in vitro (8,42,43). These results suggest that the efficacy of an unknown material as a nasal adjuvant could also be assessed simply by the strength of its ability to induce nonspecific suppression of influenza virus replication in the nasal cavity of BALB/c mice.

5. Perspectives

The current trivalent SV vaccine used for subcutaneous or intramuscular injection contains a total of 45 µg HA (15 µg HA for each monovalent vaccine) in the total vaccine protein of less than 240 µg. In the present review, the effective SV monovalent vaccine dose (together with CTB*) was estimated to be around 100 µg SV vaccine proteins containing approximately 30 µg HA (together with 100 µg CTB*) from the mouse experiments (27–31). Although the LT-adjuncted nasal trivalent vaccine (Nasalflu) is no longer in clinical use, the dose of the inactivated vaccine (together with LT) was 7.5 µg HA for each of the three monovalent vaccines (total 22.5 µg) (together with 2 µg LT) (25,26). The inactivated vaccine in the Nasalflu was a virosome-based surface antigen vaccine for facilitating the immune response, in which the purified HA and NA antigens were inserted in the phospholipid bilayer. Thus, the estimated effective dose (approximately 30 µg HA) of the SV vaccine in the CTB*-adjuncted vaccine was close to the vaccine dose used in the Nasalflu (7.5 µg HA), although the forms of inactivated vaccine and adjuvant in the CTB*-adjuncted SV vaccine were different from those in the Nasalflu. The effective doses of both nasal vaccines were very close to the dose of the current injection vaccine (15 µg HA).

Clinically safe and effective adjuvants other than CTB*, LTB*, or LT are required for the nasal-inactivated influenza vaccine because an LT-adjuncted nasal vaccine (Nasalflu) has been linked to several cases of transient Bell’s palsy (25,26). Thus, the enterotoxin adjuvants cannot be used clinically as an adjuvant of a nasal-influenza vaccine because of the neurotoxic effects when it is administered intranasally together with the inactivated vaccine. The search for new and more effective nasal adjuvants becomes increasingly possible as new discoveries are made. For example, the recognition of influenza virus by the cells involved in the innate immune system (including DCs) via several classes of pattern-recognition receptors (including TLRs) leads not only to the induction of innate immune responses for clearing the virus, but also to the induction of adaptive immune responses (44,45). Thus, ligands for several TLRs could provide new candidate adjuvants (35–37,46–57). Their efficacy could be detected by their ability to suppress the replication of influenza viruses non-specifically in the upper respiratory tract (8,42), as well as by the ability of the adjuvant-combined vaccine to stimulate anti-HA nasal IgA and serum IgG Ab responses (9). The usefulness of a new nasal adjuvant in humans would be assessed by the degree of the Ab responses in BALB/c mice, immunized intranasally with approximately 0.1 µg SV vaccine, together with a new adjuvant. The protective Ab responses in BALB/c mice, immunized intranasally with 0.1 µg SV vaccine together with CTB*, could be used as a positive control for assessing the effectiveness of new unknown adjuvants.

By estimating the efficacy of a new nasal adjuvant, the usefulness of synthetic RNA poly (I:C), ligands of TLR3, has been noted (56,57). On the other hand, the potential toxic effects of double-stranded RNA on age-related macular degeneration (the common cause of irreversible visual impairment) have been reported (58). Whether there is a causal relationship between the use of poly (I:C) as an adjuvant of nasal-influenza vaccine and the development of age-related macular degeneration remains to be determined. From the standpoint of safety of influenza vaccine, special care must be exercised to avoid the potential neurotoxic effects of a new nasal adjuvant. Powerless use of an adjuvant should also be avoided. For example, in the two-dose regimen for providing effective protection against infection, CTB* together with the vaccine is required for the primary immunization, which induces strong memory activities for anti-HA Ab responses, but not for the booster immunization (27,28). Critical parameters affecting the outcome of a nasal influenza vaccine are dose (including volume and concentration) and vaccine form. Generally speaking, the higher the doses of vaccines used for the immunization, the better the immune responses induced in mice; better immune responses usually provide more cross-protective immune responses. However, the maximum dose of vaccine in humans would be restricted not only from the standpoint of safety, but also from the standpoint of cost performance. A new adjuvant-combined effective vaccine with a vaccine dose of less than 15 µg HA in humans, which should lead to a clinically safer and lower-cost vaccine, could be developed in the near future.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to all their colleagues for their contributions to the works cited in this review and apologize to those whose work could not be cited.


