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Review

Mechanisms of Broad Cross-Protection Provided by Influenza Virus Infection and Their Application to Vaccines

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SUMMARY: Mice recovered from influenza A virus infection have been shown to be cross-protected against challenge infection with either drift viruses within a subtype (subtype-specific immunity) or different subtype viruses (heterosubtypic immunity). The mechanisms of broad-spectrum cross-protection could be explained as follows. (i) Pre-existing S-IgA and IgG antibodies (Abs) induced by infection are involved in the elimination of challenge viruses by forming virus–Ig complexes shortly after re-infection. Due to their polymeric nature, the S-IgA Abs, existing more abundant on the mucosa than are IgG Abs, are strongly cross-reactive with challenge viruses, whereas the IgG Abs are weakly cross-reactive with challenge viruses, due to their monomeric nature. The specificity of Abs is directed mainly at hemagglutinin and neuraminidase. (ii) $CD8^+$ memory T cells induced by infection are involved in the elimination of challenge viruses by the accelerated killing of host cells infected with different subtype viruses from day 3 onwards after re-infection. The specificity of memory T cells is directed against viral internal proteins. (iii) The accelerated IgA and IgG Ab responses, produced by B memory cells after a challenge, are also involved in cross-protection from day 4 onwards after re-infection. (iv) In the epithelial cells of infected mice, dimeric IgA that is trafficked through the epithelial cells can contribute to the prevention of viral assembly by binding to newly synthesized viral proteins. Natural infection is well known to be superior to parenteral inactivated vaccines in inducing the broad-spectrum cross-protection. To improve the efficacy of current inactivated vaccines, many trials have been conducted to mimic natural infection, including intranasal or epidermal administration of inactivated vaccine with or without an adjuvant; such studies are still ongoing. In the near future, some of these trials may provide new, safer and more effective broad-spectrum vaccines than those currently available.

1. Introduction

1-1. Characteristics of influenza

One prominent characteristic of influenza is that it is a contagious, acute respiratory disease caused by an influenza virus infection, which attacks the host's respiratory tract (RT) mucosa (1,2) (Fig. 1A). The virus is an enveloped virus with seven internal proteins (nucleoprotein [NP], three polymerase proteins [PA, PB1, and PB2], two matrix proteins [M1 and M2], and nonstructural proteins [NS2]) and two external glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The viruses infect host epithelial cells by binding to receptors (sialic acid) on the cell surface via one of the major viral

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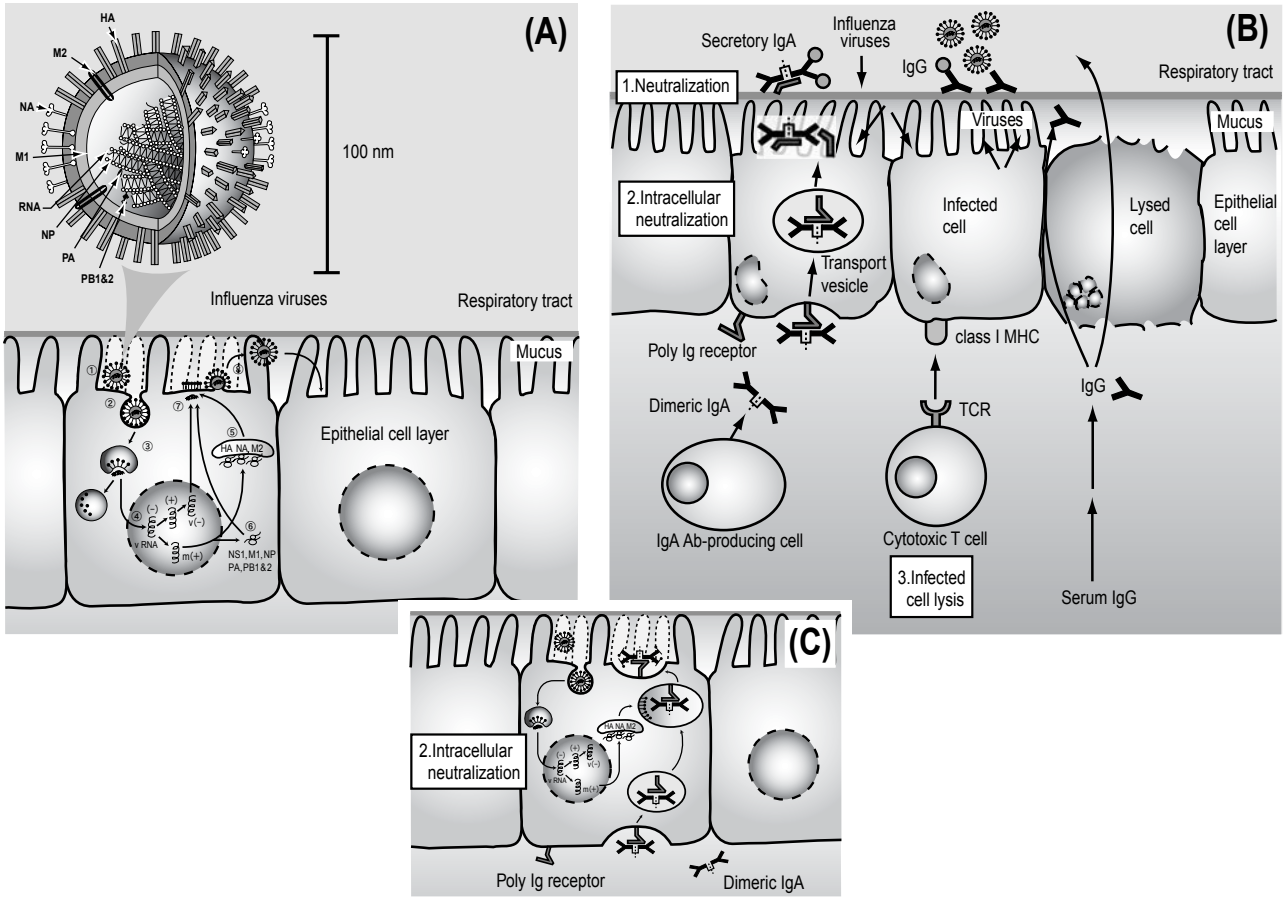

Fig. 1. Influenza virus infection and adaptive immune responses involved in the host defense in the respiratory tract. (A) Events following the infection of cells. ① Influenza viruses bind to the epithelial cell surface via HA and ② are rapidly endocytosed. ③ Following fusion between the viral and endosome membranes, ④ nucleocapsids move to the cell nucleus where transcription into viral (v) and messenger (m) RNA takes place. Translation of viral proteins occurs either ⑤ on membrane-bound ribosomes (HA, NA, and M2) or ⑥ on free ribosomes (NS1, M1, NP, PA, PB1, and 2). ⑦ Assembly of viral components occurs at the apical cell surface. ⑧ New virus particles, which are formed by budding, are released from the infected cell by cutting the binding between the cell and HA by NA. Cytoplasmic degradation of viral proteins is thought to result in the generation of peptides for recognition by cytotoxic T cells. (B) and (C) Major effector molecules and cells involved in the adaptive immune responses against influenza virus infection: 1. Viral neutralization by secretory IgA Abs which are transported to the apical surface of the epithelial cells by endosomes after the formation of a complex between dimeric IgA and the poly Ig receptor at the basolateral surface, and IgG Abs which transude from the serum to the apical surface of the epithelial cells by diffusion. 3. Lysis of the infected cells by CTLs ($CD8^+$ cytotoxic T cells), which are transiently generated, with a peak at around 7 days after primary infection; and acceleratedly after re-infectin. (C) 2. Intracellular neutralization by the prevention of virion assembly by the binding of pIgR-IgA complex to the newly synthesized viral glycoproteins.

surface glycoproteins, HA. They then replicate in the host infected cells. Several hours after infection, the newly synthesized viruses are released from the infected cells by the action of another major glycoprotein, NA. The degree of damage to epithelial cells by infection correlates well with the magnitude of the febrile response. Since flu symptoms emerge within a few days of infection, an acquired immunity raised around 7 days after infection cannot prevent the onset of respiratory symptoms. Therefore, an effective immunity must be induced in advance by natural infection or vaccination in order to prevent disease.

Another prominent characteristic of influenza is the emergence of variable HA and NA which results in pandemics or annual epidemics or local outbreaks of influenza. Influenza viruses are divided into types A, B, and C, based on the antigenic differences between NP and M proteins. Influenza A viruses are further subdivided into subtype viruses (H1N1, H3N2, etc.), based on the antigenic differences in the HA and NA molecules. These subtype viruses with marked antigenic changes are thought to have arisen due to genetic reassortant between human and animal viruses after a double infection of the host cells by such viruses (antigenic shift). These viruses may also arise in the course of the direct infection of animal viruses in humans. Antigenic shift is responsible for pandemics of influenza. The A subtype viruses and type B viruses undergo small changes in the HA and NA molecules every year (antigenic drift). The emergence of such variant virus strains is responsible for epidemics or local outbreaks of influenza.

1-2. Viral antigens involved in immunity

Influenza virus infection is initiated by the binding of the viruses to the host cell surface via HA (1) (Fig. 1A). The viruses are then endocytosed and fuse with the endosomal membrane by a conformational change in HA under low pH conditions, thereby everting the nucleocapsid (RNA, NP, PA, PB1, and PB2) into the cytoplasm. The nucleocapsids pass to the cell nucleus, where transcription into viral RNA and mRNA takes place. The translation of M1, NP, NS1 PA, PB1, and PB2 proteins occurs on free ribosomes, whereas that of HA, NA, and M2 proteins occurs on membrane-bound

ribosomes (3,4). Peptides are generated from these endogenous antigens synthesized within the infected cells by their cytoplasmic degradation and these peptides are then loaded on class I MHC molecules and are subsequently expressed on the cell surface. The peptide-class I MHC complexes presented on the infected cells are recognized by class-I MHC-restricted $CD8^+$ T cells (Tc cells), which destroy the infected cells. Among these endogenous antigens, internal antigens are major targets for Tc cells; NP is the strongest of these antigens (5). Since the structure of these antigens is conserved within the type of virus, Tc cells against these antigens are cross-reactive within the type of influenza.

Surface glycoproteins, HA and NA, are major antigens, which are taken up in an endocytic vesicle pathway of the antigen-presenting cells and are degraded; the peptides of these antigens are loaded on class-II MHC molecules and then are expressed on the antigen-presenting cells (4,6). The peptide-class II MHC complexes are recognized by class-II MHC-restricted $CD4^+$ T cells (Th cells). Th cell stimulation by antigen recognition results in the production of specific antibody (Ab) to the HA and NA molecules. Anti-HA Abs neutralize the infectivity of the virus, whereas anti-NA Abs prevent the release of viruses from infected cells (7). Thus, strain-specific or subtype-specific Abs against HA and NA molecules are major protective elements against virus infection. Abs to the M2 protein, which is conserved within the A-type viruses, are cross-protective between different subtype-virus infections, although the level of production is low (8). Abs to the conserved NP and M1 can be induced, although they fail to contribute to protection; passive transfer of anti-M1 or anti-NP monoclonal Abs (mAbs) fails to protect mice from challenge by a wild-type influenza virus (9).

These findings can be confirmed by results demonstrating that both HA and NA-expressing DNAs from the A or B virus provide the most effective protection against each lethal virus challenge among HA-, NA-, M1-, NP-, and NS1-expressing DNAs in mice (10-12); however, NP-expressing DNAs also provide protection when relatively large amounts of them are injected repeatedly (13). 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 for accelerating the recovery from influenza.

1-3. Defense mechanisms against influenza

The RT mucosa is not only the site of infection for influenza viruses, but also the site of defense against viral infection. Defense mechanisms against influenza virus infection comprise several effector cells and molecules (14,15). Viruses are initially detected and destroyed non-specifically by innate immune mechanisms, which 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 avoid the early defense mechanisms, they are detected and eliminated specifically by the following adaptive immune mechanisms (Fig. 1B and C), which could be augmented by influenza virus constituents via Toll-like receptors (TLRs) on macrophages and DCs in the RT (16-19).

(i) Secretory-IgA (S-IgA) and IgG Abs and CTLs ($CD8^+$ cytotoxic T lymphocytes) are involved in the recovery from influenza following the viral infection of naïve mice (20-28).

(ii) Preexisting specific S-IgA and IgG Abs in animals previously immunized by infection provide protection against challenge infection (29,30). The S-IgA Abs are carried to the mucus by transepithelial transport used for dimeric IgA (dIgA) (31,32). The IgG Abs, which are largely distributed on the alveolar epithelia to prevent influenza pneumonia, transude from the serum to the mucus by diffusion (33-35).

(iii) In the absence of Abs in 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 (9,36).

(iv) In the epithelial cells of infected animals, dIgA Abs being trafficked through the epithelial cells are involved in the prevention of viral assembly by binding to newly synthesized viral glycoproteins (37-39) (Fig. 1C). Thus, initially, the polymeric Ig receptor (pIgR)-IgA complex is delivered to early basolateral endosomes, but is later routed to apical recycling endosomes, by which viral glycoproteins, synthesized on the rough endoplasmic reticulum, are transported to the apical cell surface. The apical recycling endosomes are a potential location for IgA to intercept viral proteins (39).

(v) In the absence of Abs in pre-immunized animals, CTLs are also produced rapidly by $CD8^+$ memory Tc cells, and these CTLs are involved in killing the host cells infected from day 3 onwards after re-infection (9,25-28,36).

(vi) Similarly, memory Th1 cells that mediate an accelerated delayed-type hypersensitivity (DTH) response are involved in a blockade of virus replication by secreting IFN-γ (40).

1-4. Purpose of this review

Although it is difficult to control influenza due to the high variability of HA and NA, cross-protection against infection with a broad spectrum of viruses has been observed in infected mice. Mice recovered from infection with an influenza A virus are protected against challenge infection by drift viruses within the same subtype (subtype-specific immunity) and by other subtype viruses (heterosubtypic immunity) (29,41,42). Such subtype specific protection has also been shown in humans who recovered from natural infection, although heterosubtypic protection has only rarely been observed (43-45).

The ability of natural infection to induce subtype-specific and heterosubtypic immunity is superior to that of parenteral inactivated vaccines (41-45) (Table 1). This review first describes recent findings regarding the mechanisms by which subtype-specific or heterosubtypic immunity is induced in

Table 1. Comparison of cross-protective immunity induced by natural infection with that by parenteral inactivated vaccine

Immunity against challenge infection	Immunity induced by natural infection	Immunity induced by parenteral inactivated vaccine
Heterotypic Immunity (between A and B-type vaccines)	–	–
Heterosubtypic Immunity (among different subtypes within the same type)	+	–
Subtype-specific Immunity (among different strains within the same subtype)	++	+
Strain-specific Immunity	+++	+++

*Arbitrary units (+++, strong; ++, moderate; +, weak).

animals immunized previously by infection. It then addresses various vaccine trials for inducing subtype-specific or heterosubtypic immunity. These trials include the intranasal or epidermal administration of live or inactivated vaccine with or without an adjuvant and parenteral vaccination of conserved viral components, although not all trials are included. Finally, model experiments using mice are described that assessed the effectiveness of new influenza vaccines in humans. Some of these trials may lead to the development of new vaccines capable of providing safer and broader cross-protection than is achieved by the current vaccines.

2. Mechanisms inducing subtype-specific or heterosubtypic immunity

2-1. Mechanism of subtype-specific protection

Cross-protection against challenge infection by drift viruses within a subtype of the A-type virus (subtype-specific immunity) has been demonstrated in previously infected mice and humans (29,30,43-46). The cross-protection primarily results from the induction of cross-reacting IgA Abs, but not serum Abs or CTLs, in the RT of previously infected mice. Mice previously infected with the A/Rec 31 (H3N1) virus are strongly protected (lung virus titer reduction) against challenges by the A/Vic (H3N2) virus in parallel with the presence of cross-reactive anti-HA S-IgA Abs in the lung wash (29). Similarly, mice previously infected with A/Yamagata (H1N1) viruses (upper RT infection) were also protected against challenges by the A/PR8 (H1N1) virus (upper RT infection); these mice exhibited early virus clearance, i.e., within 3 days, in parallel with the production of cross-reactive anti-HA S-IgA Abs in the nasal wash (30). In addition, B-type viruses (upper RT infection) also provided cross-protection against challenges by other drift B viruses (upper RT infection), with specificity for either B/Victoria or B/Yamagata lineages, in parallel with the induction of nasal B virus-reactive anti-viral IgA Abs (46). Thus, the mechanisms of subtype-specific (or B virus lineage-specific) cross-protection are mediated by anti-HA or anti-viral S-IgA Abs in the RT.

The following findings have reinforced the roles of S-IgA Abs in subtype-specific cross-protection. Anti-HA S-IgA Abs, which were found to be prominent in the upper RT, cross-protected non-immune mice from a drift virus infection, when purified from the RT of mice immunized with HA molecules and when administered intranasally (47). Polymeric IgA and S-IgA Abs were highly cross-reactive; i.e., the activity of these Abs was several-fold higher than that of monomeric IgA (mIgA) and IgG in hemagglutination inhibition (HI) and virus neutralization (NT) which are derived from their polymeric nature (48). The interference of viral assembly with dIgA during transcytosis is also a potential mechanism of subtype-specific immunity that might be mediated by IgA Abs to viral glycoproteins (37-39). Thus, S-IgA Abs in the RT of infected mice play a causal role in providing cross-protection against infection with drift viruses within a subtype of the A-type virus, as well as within the B-type virus.

2-2. Mechanism of heterosubtypic protection

Cross-protection between the different subtypes of the influenza A virus has been reported in the absence of Abs of appropriate specificity in mice ("hetrosubtypic protection") (42); cross-protection against a challenge immunization with the A/USSR (H1N1) [or X-31 (H3N2)] virus correlated with the secondary CTL responses in mice immunized previously with a sublethal dose of the X-31 (H3N2) [or A/USSR (H1N1)] virus. Similarly, mice previously infected with the A/PR8 (H1N1) virus were cross-protected heterosubtypically against a lethal challenge by the X31 (H3N2) virus (49); in the nose, short-lived $CD8^+$ or $CD4^+$ T cells were involved in this heterosubtypic cross-protection, whereas in the trachea and lung, short-lived $CD8^+$ T cells were involved. In addition, in knockout mice lacking all Ig and B cells, lung virus titers four days after the H3N2 challenge were reduced significantly in previously H1N1 virus-immunized mice (50); the depletion of both $CD8^+$ and $CD4^+$ T cells from these Ig-deficient mice completely abolished the heterosubtypic protection. Thus, the mechanism by which $CD8^+$ T cells enhance viral clearance is most likely their CTL activity, whereas the role of $CD4^+$ T cells is to facilitate the CTL response in the absence of Ig.

On the other hand, the kinetics of lung virus replication in A/J mice immunized with the A/H3N2 virus and challenged with a lethal dose of the A/H1N1 virus one month later was examined under the depletion of both $CD4^+$ and $CD8^+$ T cells at the time of challenge (51); a highly significant reduction in challenge-virus replication was observed in the heterosubtypically immune mice, with a peak reduction occurring on day 4 from 2 to 7 days. Thus, an immune mechanism that pre-exists at the time of challenge or is activated by day 2 after a challenge may be involved in the induction of hetrosubtypic immunity without $CD4^+$ and $CD8^+$ T cells. Pre-existing Abs are one of mediators of immunity mechanisms. In this regard, pre-existing IgA Abs in the nasal secretions of mice immunized previously with the A/Guizhou (H3N2) virus were involved in cross-protection by forming immune complexes between IgA Abs and challenge viruses immediately after challenge infection with the A/PR8 virus (H1N1) (30); S-IgA Abs, which were more highly cross-protective than IgG and which pre-existed abundantly in the upper RT, were able to contribute to heterosubtypic cross-protection. In addition, Th cells, primed for conserved viral proteins (NP or M) in mice immunized previously with the H3N2 virus may aid in the induction of accelerated Ab responses to mismatched HA molecules in mice challenged with the H1N1 virus and result in heterosubtypic protection (52).

2-3. Kinetics of subtype-specific or heterosubtypic protection

To clarify the mechanisms by which Abs are involved in subtype-specific or hetrosubtypic protection, the time course of cross-protection after challenge infection was examined using the kinetics of PFU (infectious virus) and virus-Ig complexes following challenge infection with the A/PR8 (H1N1) virus (a relatively high-dose virus, 7.4×10^5) in the nasal secretions of mice immunized previously with A/PR8, A/Yamagata (H1N1), A/Guizhou (H3N2), and B/Ibaraki viruses (30). The viral numbers within virus-Ig complexes, captured in advance using an anti-mouse Ig-coated plate, were determined on the basis of the viral genome copy number using quantitative RT-PCR. The results are schematically represented in Figure 2, together with the kinetics of CTL reported elsewhere (28).

The kinetics of the PFU revealed that viral elimination from the nasal area occurs earlier in A/PR8-, A/Yamagata-, and A/Guizhou-X-virus-immunized mice, in that order, than in naïve mice and B/Ibaraki-immunized mice. The early viral elimination correlates with the amount of pre-existing A/PR8 virus-reactive Abs, in which the IgA Abs are more abundant than the IgG Abs. Moreover, the elimination is probably correlated with the amount of viral-Ig complexes shortly

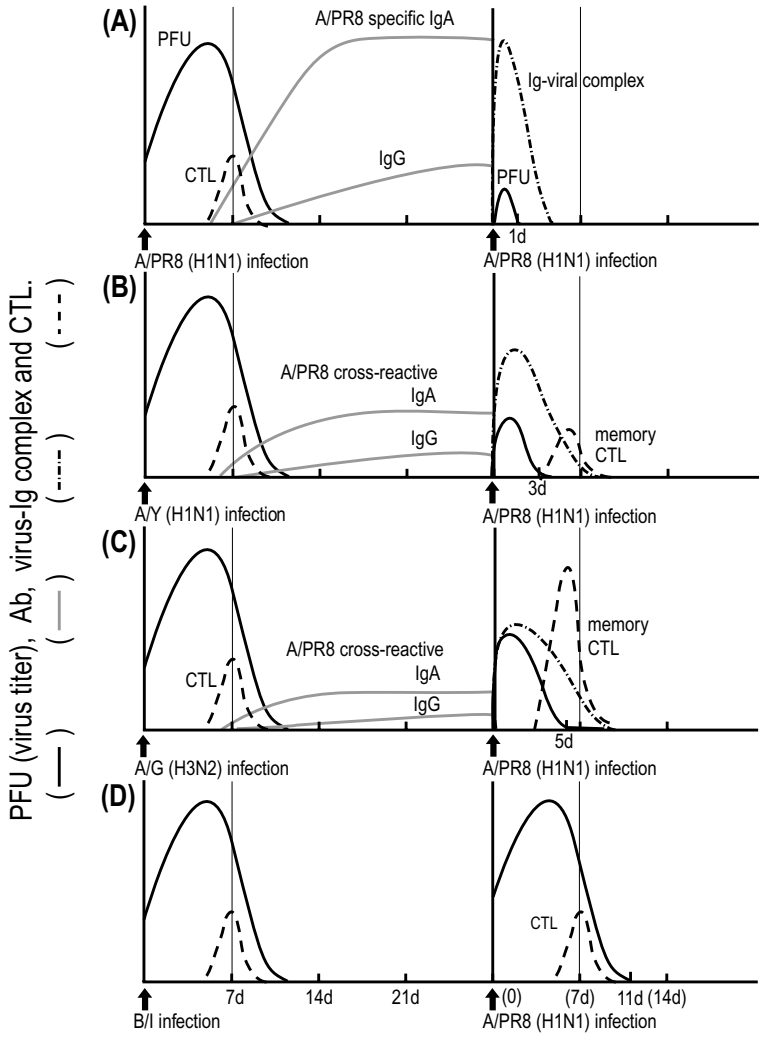

Fig. 2. Schematic representation of kinetics of PFU (as an index of infectious virus) and virus-Ig complexes following challenge infection with A/PR8 (H1N1) virus in nasal secretions of mice immunized intranasally with A/PR8, A/Yamagata (H1N1), A/Guizhou (H3N2) and B/Ibaraki viruses 4 weeks prior to the challenge. Simultaneously, kinetics of PFU, nasal anti-A/PR8 HA specific (A), or anti-A/PR8 HA cross-reactive IgA and IgG Abs following the primary infection with each virus, are represented schematically. As a reference, the kinetics of the primary and secondary CTL responses (accelerated and augmented memory CTL) in the nasal mucosa of mice immunized with X-31 (H3N2) virus 5 weeks prior to being challenged with A/PR8 virus (C) are also shown (28). The hypothetical kinetics of them in mice immunized with A/PR8, A/Yamagata, or B/Ibaraki viruses and challenged with A/PR8 virus (A, B, or D), are also shown. It is presumed that no memory CTL responses would be detected in the A/PR8 virus (A)- or B/Ibaraki virus (D)-immunized mice. The secondary accelerated responses showing only a small peak may be induced by a small portion of infectious viruses which are not eliminated by the cross-reactive Abs after challenge in the A/Yamagata virus-immunized mice (B).

after infection. Thus, in A/Yamagata (H1N1)-immunized mice, the relatively large amount of virus-Ig complexes between the A/PR8 virus and the cross-reactive Abs shortly after infection provides subtype-specific cross-protection, which was assessed by the low-level PFU response within 3 days after challenge (Fig. 2A and B). In A/Guizhou-X (H3N2)-immunized mice, a relatively small amount of the virus-Ig complexes provides partial heterosubtypic cross-protection, as assessed by the moderate extent of the PFU response within 5 days after a challenge by the A/PR8 virus (H1N1) (Fig. 2C). Absence of the cross-reactive Abs in B/Ibaraki-immunized mice failed to provide cross-protection (Fig. 2D). These results suggest that pre-existing Abs are heavily involved in subtype-specific protection, but are only weakly involved in heterosubtypic protection.

From the results described above, the process of viral clearance from the upper RT of mice immunized with a sublethal dose of virus and challenged with different A-type viruses could be divided into two phases after the challenge infection. In Phase 1, which extends from zero to 3 days after infection, strain-specific or subtype-specific protection is provided by pre-existing Abs. Thus, in Phase 1, the challenge viruses are eliminated solely by the formation of immune complexes between challenge viruses and pre-existing specific or cross-reactive Abs as the result of NT of the infectious viruses (Fig. 2A and B). In Phase 2, which extends from day 3 onwards after infection, those challenge viruses and their offspring viruses not initially eliminated by the formation of immune complexes are eliminated by CTLs, which are generated with increasing rapidity starting at 3 days after a challenge by memory cells (25-28). Thus, in Phase 2, secondary CTL responses are primarily involved in the mechanism of heterosubtypic protection in the absence of cross-reactive Abs.

2-4. Cross-protection against re-infection in humans infected previously with variant viruses

Natural influenza virus infection has been shown to be superior to inactivated vaccines at inducing cross-protection against challenge infection by drift viruses within a subtype of the A-type virus in humans (43-45). Children who previously experienced natural infection or who received live attenuated influenza A virus vaccine reduced the amount or duration of virus shedding by re-infection, when compared to subjects without prior infection (9). Re-infection results in a secondary IgA Ab response, which is characterized by a rapid rise in the IgA Ab titer, a higher peak titer, and the maintenance of detectable levels of Ab over a longer period of time. On the other hand, volunteers with high levels of CTL (class I MHC-restricted $CD8^+$ cytotoxic T lymphocyte) activity have been shown to shed fewer viruses than those with a low CTL activity, when experimentally administered wild-type influenza A virus (24). Thus, the CTL activity of memory T cells is also correlated with resistance to influenza.

However, the epidemiologic behavior of influenza viruses in humans suggests that infection with one subtype induces little or no immunity to other subtypes (9,45). Thus, the overall contribution of CTLs to providing heterosubtypic protection and disease reduction during re-infection with the influenza A virus is small, because repeated infection of humans with the influenza A virus bearing internal viral antigens provides little resistance to disease caused by new influenza variants. Similarly, the contribution of cross-reactive Abs to providing heterosubtypic cross-protection in humans may be small, judging from the reported epidemiologic behavior of influenza viruses in humans (9,45).

3. Cross-protection provided by current vaccines (Table 2)

3-1. Parenteral inactivated vaccine

Current inactivated vaccines include whole virion vaccines, split-product vaccines, and subunit vaccines (2). Split-product vaccines are obtained by treating purified whole virus particles with organic solvents, such as ether or detergents, to remove the lipid component of the virus (53), while subunit vaccines are highly purified vaccines mainly containing the HA and NA components (54-56). Immunogenicity increases in the order of subunits, split-products, and whole particles, whereas the safety of a vaccine containing these components decreases in that order. The superior immunogenicity of whole virus particles to split-product or subunit vaccines appears to be due to the adjuvant action of single-stranded RNA contained in the virus particles via TLR7 (17-19). These inactivated vaccines induce mainly serum IgG Abs that are weakly cross-protective among drift viruses within a subtype. Thus, inactivated vaccines can effectively protective against an epidemic of homologous viruses, but are relatively ineffective against an epidemic of heterologous viruses.

3-2. Live attenuated virus vaccine

A cold-adapted, live-attenuated virus vaccine (*ca*-virus vaccine) for intranasal vaccination has been licensed in Russia and in the USA (57-59). The *ca*-virus vaccine consists of reassortant viruses that contain the genes for HA and NA of the wild-type virus and the six non-surface genes from the *ca*-master virus. It is selected using plaque purification from tissue culture cells, which are coinfected with the *ca*-master virus and the wild-type virus and are passaged in the presence of antiserum to HA and NA of the *ca*-master virus. The *ca*-master viruses are prepared from wild-type viruses by serial passage under low-temperature conditions (25°C) (57). These *ca*-master strains have several genetic features that differ from those of the parent wild-type viruses and whose background of attenuation has not yet been elucidated.

The *ca*-virus vaccine can mimic natural infection and provide cross-protective immunity by inducing S-IgA Abs, serum IgG Abs, and CTLs (9,36). However, in the USA, the *ca*-vaccine is only approved for the age group of 5-49, thus excluding two major high-risk groups, infants and the elderly, in addition to immunodeficient patients and pregnant woman. This is because the *ca*-vaccine has the potential to

Table 2. Characteristics of current and developing or new vaccines

Vaccine	Immune responses against challenge infection			Secondary immune responses	Cross-protective immunity	References
	IgA	IgG	CTL			
Current Vaccines						
1. Patenteral Inactivated Vaccine						
*Split-product and Subunit vaccine	−	++	−	++	+	53 - 56
2. Live Virus Vaccine						
*Cold-adapted, live virus vaccine	+	+	+	+	++	57 - 59
Developing or New Vaccines						
1. Inactivated Vaccine						
(1) Adjuvant-combined Parenteral Vaccine	−	++	−	++	+	60 - 63
(2) Non-parenteral Vaccine						
i) nasal vaccine	++	+	−	++	++	64 - 70
ii) adjuvant-combined nasal vaccine	++	+	−	++	++	72 - 78
iii) epidermal vaccine	+	++	−	++	++	93 - 95
(3) Improvement of vaccine constituents						
i) neuraminidase (parenteral)	−	++	−	+	++	96 - 103
ii) conserved HA epitope (parenteral)	−	++	−	+	++	105 - 107
iii) M2 protein	−	++	−	+	++	108, 109
(4) DNA vaccine	−	+	++	++	++	10 - 13, 110
2. Live Virus Vaccine						
(1) Recombinant live virus vaccine	+	+	+	+	++	111 - 115
(2) Virus-vectored vaccine	+	+	−	+	+	116

produce some respiratory reactions.

4. Cross-protection provided by new vaccines (Table 2)

4-1. Inactivated vaccine

4-1-1. Parenteral adjuvant-combined vaccine

Virosomal subunit vaccines containing HA and NA incorporated into unilameller liposomes composed of phosphatidylcholine (egg lecithin) (Berna Biotech, Bern, Switzerland) are licensed as parenteral vaccines to elicit a stronger Ab response (60,61). Subunit vaccines emulsified with Microfluidized Emulsion 59 (MF59; emulsion containing 0.5% Tween 80, 0.5% sorbilin trioleate, and 4.3% squalene) (FLUAD, Chiron, Emeryville, Calif., USA) have also been developed to elicit a stronger Ab response by inducing Th2-type immune responses (62). Aluminium hydroxide and aluminium phosphate have also been effective in a human trial when tested as an adjuvant for inactivated whole-virus vaccine (63).

4-1-2. Non-parenteral vaccine

Inactivated vaccines are effective at protecting hosts against an epidemic of homologous viruses by inducing serum anti-HA IgG Abs, but they are relatively ineffective against an epidemic of heterologous viruses (45). On the other hand, the live-virus vaccine, which can induce IgA Abs, IgG Abs and CTLs (36), is only approved for the age group of 5 - 49 in the USA. In addition, the live vaccine appears to cause coryza, sore throat, and febrile reactions. Because of these problems, there is still room for improvement of the current vaccines.

4-1-2-1. Nasal split-product or whole-virion vaccine

Concentrated split-product vaccines (i.e., threefold concentrated vaccines), which are administered intranasally as an aerosol, are effective at inducing local HA-specific IgA Abs and at preventing infection by the live virus, according to results of human trials (64). Whole inactivated virus particles are also administered intranasally and result in an enhanced production of both local HA-specific IgA Abs and serum HI Abs in clinical trials (65-69). In addition, intranasal immunization with formalin-inactivated whole viruses, but not ether-split vaccines, induces a broad spectrum of heterosubtypic protective immunity in mice (70). The superior immunogenicity of whole-virion vaccines to ether-split vaccines might be accounted for by the adjuvant action of single-stranded RNA contained in the virus particles via TLR7 (17-19,71). Thus, intranasal immunization with inactivated whole-virus vaccines could provide broad cross-protection against drift virus infection in humans.

4-1-2-2. Adjuvant-combined nasal inactivated vaccine

Intranasal immunization with inactivated (ether-split) vaccines, used in conjunction with cholera toxin B subunit (CTB) containing a trace amount of cholera toxin (0.1%) (CTB*) [or the *Escherichia coli* heat-labile toxin B subunit (LTB) containing a trace amount of the heat-labile toxin (0.5%) (LTB*)], provides effective cross-protection in the upper RT against variants (drift viruses) within the subtype of influenza A viruses or variants of the B-type viruses (72-76). The strong cross-protection in the upper RT is provided mainly by S-IgA Abs, whereas the weak cross-protection in the lower RT is provided by IgG Abs (35,73). However, the use of LT or CT as an adjuvant with the nasal influenza vaccine may not be clinically safe, because an intranasal virosomal vaccine adjuvanted with LT (NasalFlu, Berna Biotech), following licensing in 2001, has been linked to several cases of transient Bell's palsy (facial paralysis) (77,78). Thus, a clinically safer and more effective adjuvant is still needed for the intranasal administration of inactivated split-product or subunit vaccines.

The mechanisms by which CT or LT enhances mucosal immune responses against influenza viral antigens involve the stimulation of the innate immune system (15,16,79). Thus, CT or LT alone can reduce the replication of viruses non-specifically in the upper RT when administered intranasally to mice together with infectious viruses (79). In addition, the reduction of viral replication correlates with the activation of APCs (macrophages, DCs, etc.). Therefore, new and effective adjuvants may arise from the screening of materials that stimulate the function of APCs, including ganglioside GM1 and ligands of several TLRs (16-19,79-84). One attempt involved development of a nontoxic form of an adjuvant based on LT and CT together (85,86). Another is the use of ligands for the TLR family of receptors on APCs, e.g., CpG DNA and double-stranded RNA (87,88). Other mechanisms to stimulate the innate immune system, such as some cytokines and complements, are also involved in the induction of mucosal immune responses against influenza viral antigens (89-92) It is therefore clear that the development of a new and promising adjuvant will help to realize a safer and more effective adjuvant-combined nasal influenza vaccine in humans.

4-1-2-3. Epidermal immunization vaccine

Epidermal powder immunization (EPI) using special delivery systems (Powder Ject ND5.2) can efficiently deliver the powdered trivalent influenza vaccine to the epidermis in humans (93-95). When subjects receive either intramuscular injection (vaccine containing 15 μg HA of each influenza viral strain), a single EPI vaccination or two adjacent EPI, seroconversions, the titer increases and the geometric mean titers to all strains are equivalent or higher in the EPI-immunized groups than in the intramuscular-immunized group. EPI may facilitate antigen-recognition and uptake by Langerhans cells and DCs in the dermis so as to elicit both high serum IgG levels and low mucosal IgA Ab responses to an inactivated virus vaccine.

4-1-3. Improved vaccine constituents

4-1-3-1. NA

There is a substantial amount of evidence that NA molecules, as well as HA molecules, contribute to protection against influenza virus infection (10-12,96-103). Naturally or experimentally acquired Abs to NA confer resistance to experimental or natural viral infections in both humans and mice (96-101). Both HA and NA are the most important protective viral components among the various proteins in mice immunized with plasmid DNA encoding various proteins from influenza A and B viruses (10-12). The addition of purified NA molecules to conventional vaccines provides more effective prophylaxis of influenza (102,103). Moreover, the NA protein of the influenza A (H1N1 subtype) virus vaccine is expected to improve the efficacy of protection against influenza among low-responders to the HA protein (104). Thus, increasing the dose of the NA component can provide better cross-protection; however at the present time, only the HA content is standardized, i.e., at 15 μg per dose for each viral strain.

4-1-3-2. Conserved HA epitope

The HA molecule contains an amino acid sequence that is conserved not only within specific subtypes but also between subtypes (105-107). Unmasking of the cross-reactive antigenic determinants in the stem region of the HA molecule

can induce neutralizing Abs to H1 and H2 subtype viruses, although neutralizing Abs that recognize the stem region of HA are not induced by virus infection or by current influenza vaccines. Mice immunized with cells transfected with cDNA coding for a deletion mutant of HA lacking the globular region from A/Okuda/57 (H2N2) virus provide partial cross-protection against a challenge with a lethal dose of A/FM/1/47 (H1N1) virus. Thus, such headless HA molecules, which provide cross-protection by eliciting neutralizing Abs, may be useful in a vaccine against emerging influenza viruses.

4-1-3-3. M2 protein

The extracellular domain of the M2 protein, which is an integral membrane protein of the influenza A virus, is nearly invariant in all A virus strains (108,109). Vaccination with the M2 protein provides enhanced viral clearance in mice challenged by homologous and heterologous influenza A viruses. This protection is mediated by M2-specific serum Abs, which provide partial protection against challenge infection when transferred into naïve mice. Thus, the M2 protein may be useful to provide cross-protection as a vaccine against emerging influenza viruses, although the ability of M2 protein to provide protection still appears to be low, compared with that of HA or NA proteins.

4-1-4. DNA vaccine

The injection of plasmid DNA encoding influenza A virus (PR8, H1N1) NP into the quadriceps of BALB/c mice results in the generation of NP-specific CTLs and protection against challenge infection by the A/HK/68 (H3N2) virus (13). Thus, cross-protection between different subtypes of influenza is provided by the injection of NP-expressing DNA. However, the efficacy of protection by NP-expressing DNAs is not as great as that obtained with HA and NA-expressing DNAs (10-12). The injection of DNA expressing the HA fused to three copies of murine C3d of complement (HA-C3d3) more efficiently elicits hetrosubtypically cross-reactive Abs than did non-fused forms of HA and protects mice from lethal challenges by influenza of different subtypes (110). Thus, the HA-C3d_3 DNA vaccine provides broader protection than does the HA DNA vaccine by inducing cross-reactive Abs.

4-2. Live virus vaccine

4-2-1. Recombinant live virus vaccine

The advantage of live viral vaccines are inducing not only mucosal IgA and serum IgG Ab responses, but also CTL responses and providing cross-protection between different subtypes of influenza. Current cold-adapted live-attenuated influenza virus vaccines are growth-restricted to the upper RT. These *ca*-master strains have several genetic changes, the background of attenuation of which has not yet been elucidated. Alternative rational approaches to the design of live virus vaccines have been proposed (111-115). One such approach has been the development of live attenuated viruses encoding altered NS1 proteins, which are attenuated for the function of an antiviral state of type I IFNs (111,113). In addition, the development of a reverse-genetics system that allows one to generate influenza A and B viruses entirely from cloned cDNAs will make it easy to generate new attenuated virus vaccines (112,114,115). The eight-plasmid system also enables the rapid and reproducible generation of reassortant influenza A and B viruses for use in the manufacture of vaccines.

4-2-2. Virus-vectored vaccine

A vectored influenza vaccine, which is a replication-defective human adenovirus-vector encoding A/PR8 HA, has been determined as safe and immunologically effective in humans when administered as a nasal spray or skin patch (116). The adenovirus-vectored vaccine may be useful for some epidemic strains of influenza virus in cases when there is difficulty growing them in embryonated eggs for vaccine production.

5. Perspectives for the development of new influenza vaccines using mice

The development of a new and promising adjuvant is required to realize a safer and more effective adjuvant-combined influenza vaccine in humans. Under the present circumstances, it appears to be very useful to examine whether or not a mouse influenza model can be used to directly assess the effectiveness of a new vaccine in humans. The critical parameters affecting the outcome of an influenza vaccine are the dose, volume, concentration, and formulation of the vaccine. Generally speaking, the higher the dose of vaccine used for immunization, the better the immune response induced in mice; greater immune responses usually provide more cross-protective immune responses. However, the amount of vaccine used in humans would need to be restricted from the standpoint of safety. To distinguish between the critical parameters for the vaccine in humans and those used for mouse model experiments, differences between humans and mice in body weight, surface area, and other factors need to be considered (117,118). In addition, the parameters regarding the responsiveness of each mouse strain to the vaccine must be considered, because dose-dependent responsiveness, which is controlled by the MHC gene, changes greatly from strain to strain (104). Therefore, in order to determine the vaccine doses, calculations allowing extrapolation from mice to humans would need to be altered with the strain of mouse used in vaccine experiments.

There appear to be good mouse models available, thus rendering it possible to assess the effectiveness of new influenza vaccines in humans. Experiments involving the intranasal administration of split-product vaccine together with an adjuvant have been conducted on both mice and humans (72-76,85,119,120). Reports regarding such experiments have provided important information for the calculation of vaccine doses extrapolated from mice to humans. Thus, the dose of the CTB* (0.1 μg)-combined split vaccine, which was administered intranasally to BALB/c mice twice in a 4-week interval, was 0.1 μg monovalent vaccine/mouse (1 μl/nostril $\times$ 2). In these immunized mice, cross-reactive nasal IgA Abs were induced, as were less cross-reactive serum IgG Abs, together with complete protection or partial cross-protection against a lethal dose of homologous or heterologous viral infection (85). On the other hand, the dose of LTB* (100 μg)-combined split vaccine, which was administered intranasally to volunteers twice in a 4-week interval, was 140 μg split-product trivalent vaccine/person (125 μl/nostril $\times$ 2), which roughly corresponded to the vaccine dose used in the subcutaneous injection. In the immunized volunteers, significantly high levels of saliva IgA and serum IgG Abs were induced, compared with the levels of volunteers who received the intranasal vaccine alone, and the effectiveness of the vaccine was estimated to be approximately 60% (119,120). Thus, the vaccine dose in mice (0.1 μg monovalent vaccine/20-g mouse) corresponded to approximately 5 μg/kg, while the vaccine dose in humans (approximetely 45 μg monovalent vaccine/45-kg person) corresponded to approximately 1 μg/kg. These data suggest that the vaccine

dose in BALB/c mice corresponded to approximately five times the dose used in humans (dose/kg). Therefore, the effectiveness of new vaccines in humans could be roughly assessed by the ability of approximately 0.1 μg vaccine to induce an immune response and to provide protection against infection in BALB/c mice immunized intranasally according to a two-dose regimen.

The calculation that would allow extrapolation from mice to humans to determine the appropriate vaccine dosage was also confirmed by the use of another experimental model. Thus, the results of clinical trials, in which whole inactivated virus particles alone were administered intranasally and resulted in the enhanced production of both local HA-specific IgA Abs and serum HI Abs (65-69), were also roughly repeated in BALB/c mice immunized intranasally with 0.1 μg whole inactivated virus particles without adjuvant (0.1 μg/mouse) (unpublished data). The high immunogenicity of the inactivated whole-virion vaccine, which is almost equivalent to that of the adjuvant-combined split-product vaccine, could be explained by the adjuvant action of single-stranded RNAs via TLR7 (17-19,71). Thus, the inactivated virion vaccine appears to be one of the promising vaccines due to its low production costs. Although this calculation that would allow extrapolation from BALB/c mice to humans would be useful for assessment of the effectiveness of new influenza vaccines in humans, such a calculation cannot be applied to trials involving other strains of mice, which exhibit less of a response to the vaccine (70).

As described above, the mouse influenza model appears to be very useful for the development of new vaccines for humans. It is hoped that the use of mouse models will lead to the development of a safer and more effective vaccine in the near future.

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