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Review

Defense Mechanisms against Influenza Virus Infection in the Respiratory Tract Mucosa

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SUMMARY: 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, but if the viruses escape the early defense mechanisms, they are detected and eliminated specifically by adaptive immune mechanisms. The major adaptive immune mechanisms are as follows. (i) Specific secretory-IgA (S-IgA) antibodies (Abs) and CTLs ($CD8^+$ cytotoxic T lymphocytes) are involved in the recovery from influenza following viral infection of naïve mice. (ii) Preexisting specific S-IgA and IgG Abs in the immunized animals are involved in viral elimination by forming virus–Ig complexes shortly after re-infection. By their polymeric nature, the S-IgA Abs, which are carried to the mucus by transepithelial transport used for dimeric IgA (dIgA) Abs, provide not only protection against homologous virus infection but also cross-protection against drift virus infection. The IgG Abs, which transude from the serum to the mucus by diffusion, provide protection against homologous virus infection. They are largely distributed on the alveolar epithelia to prevent influenza pneumonia. (iii) In the absence of Abs in the pre-immunized animals, the production of specific IgA and IgG Abs by B memory cells is accelerated after re-infection, and these antibodies play a role in viral elimination from day 3 onwards after re-infection. (iv) In epithelial cells of infected animals, specific dIgA Abs being trafficked through the epithelial cells may be involved in the prevention of viral assembly by binding to newly synthesized viral proteins. (v) In the pre-immunized animals, CTL production by memory T cells is also accelerated and these cells appear to participate in the killing of the host cells infected with different subtype viruses (within the same type) from day 3 onwards after re-infection. (vi) Similarly, memory Th1 cells that mediate an accelerated delayed-type hypersensitivity response are involved in blockade of virus replication by secreting IFN-γ in mice challenged with different subtype viruses. These defense mechanisms suggest that the development of a mucosal vaccine, capable of inducing S-IgA Abs, which provide cross-protection against variant viruses within the same subtype, serum IgG Abs to prevent lethal influenza pneumonia and CTLs, which provide broad cross-protection against different subtype viruses, is strategically important to control influenza.

1. Introduction

Influenza is a contagious, acute respiratory disease caused by an influenza virus infection, which attacks the host respiratory tract mucosa (1,2). Influenza viruses infect host epithelial cells by binding to receptors (sialic acid) on the cell surface via one of the major viral surface glycoproteins, hemagglutinin (HA). The viruses 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, neuraminidase (NA). Influenza viruses are divided into types A, B and C, based on the antigenic differences of the core proteins. Influenza A viruses are further subdivided into subtype viruses (H1N1, H3N2, etc.), according to marked antigenic changes in the HA and NA molecules. These subtype viruses arose from an exchange of gene segments between the avian influenza gene

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pool and human influenza genes (antigenic shift). The A subtype viruses and B viruses cause an antigenic drift every year, resulting in an annual epidemic or local outbreak of influenza. To recover from influenza or to prevent influenza, both innate and adaptive immune responses must be induced in the respiratory mucosa following viral infection. Thus, the respiratory tract mucosa is not only the site of infection by influenza viruses but also the site of defense against viral infection in the host.

Most influenza viruses are detected and destroyed within a few hours by the innate immune mechanisms, which are not antigen-specific and do not require a prolonged period of induction (2,3). Several components such as mucus, macrophages, interferon (IFN) α, β and other cytokines, fever, natural killer (NK) cells and complement are involved in the innate immune system. If influenza viruses can escape these early defense mechanisms, they are detected and eliminated by adaptive immune mechanisms, where T and B cells and their products function as antigen-specific effectors (cytotoxic T lymphocytes [CTLs] and antibodies [Abs]) to target the virus. Also, antigen-specific memory cells (T and B cells) are involved in the prevention of the subsequent viral infection. The effector cells and molecules involved in the defense mechanisms following influenza virus infection are shown in Fig. 1. This review summarizes recent findings on these defense mechanisms induced following influenza virus infection in the respiratory tract mucosa. Furthermore, this review discusses that the development of a mucosal vaccine which is capable of inducing both secretory-IgA (S-IgA) and serum IgG Abs will be strategically important to control influenza. Such a vaccine can provide broad cross-protection against variant viruses including viruses with pandemic potential by the S-IgA Abs and prevent lethal influenza pneumonia by the serum IgG Abs.

2. Innate Immunity against influenza

2-1. Effectors involved in innate immunity

Influenza virus infection triggers the innate immune responses where the following effector cells, molecules and factors are implicated in the restriction of viral spread. Some of these effectors work within a few days following infection, not only as helpers to keep infection under control, but as communicators (antigen-presenting cells [APCs]) or activators (cytokines) in the subsequent adaptive immune response. Examples of the action of these effectors are listed in the following text. i) Inhibitory factors in the mucus of the respiratory tract, that are similar to or identical to N-acetylneuraminic acid-containing receptors for HA molecules reduce markedly the ability of the viruses to infect host cells (4-6). ii) Nasal and pulmonary levels of IFN-α and -β rise rapidly after infection and correlate directly with the degree of viral replication in ferrets, mice and humans (2,7,8). Alveolar macrophages or lymphocytes from infected lungs have been shown to release interferons in vitro (8). iii) Macrophages secrete IL-1, IL-6, TNF-α and IL-12 which activates NK cells (9). Macrophages recovered from infected lungs mediate lysis of infected cells, probably through apoptosis-dependent phagocytosis (10,11). In addition, cytokines (IL-1, TNF-α and IL-6) produced by macrophages induce fever, and the magnitude of the febrile response correlates strongly with the level of virus shedding in humans and animals (2,12). iv) The NK cells that are detected in pulmonary lymphocytes 48 h after influenza virus infection produce IFN-γ and limit viral spread by virus-infected cell lysis, which is mediated probably by pore formation in the infected cells involving perforin (13-16). v) Complement mediates protection; this is highlighted by the increase in mortality in C5-deficient mice infected with a lethal dose of influenza virus (17,18).

2-2. Enhancement of innate immunity

Efforts to activate the effectors involved in innate immune responses would lead to the enhancement in the adoptive immune responses, because some of the effectors act in both systems as a bridge. For example, the intranasal administration of cholera toxin (CT) prior to infection with influenza viruses activates macrophages and NK cells via the binding of CT to the receptor (ganglioside GM1) on the cells and results in non-specific replication reduction of the infected viruses in the respiratory tract (19) (Fig. 2). The non-specific activation of macrophages (APCs) and the enhancement of cytokine production by CT cause the subsequent enhance-

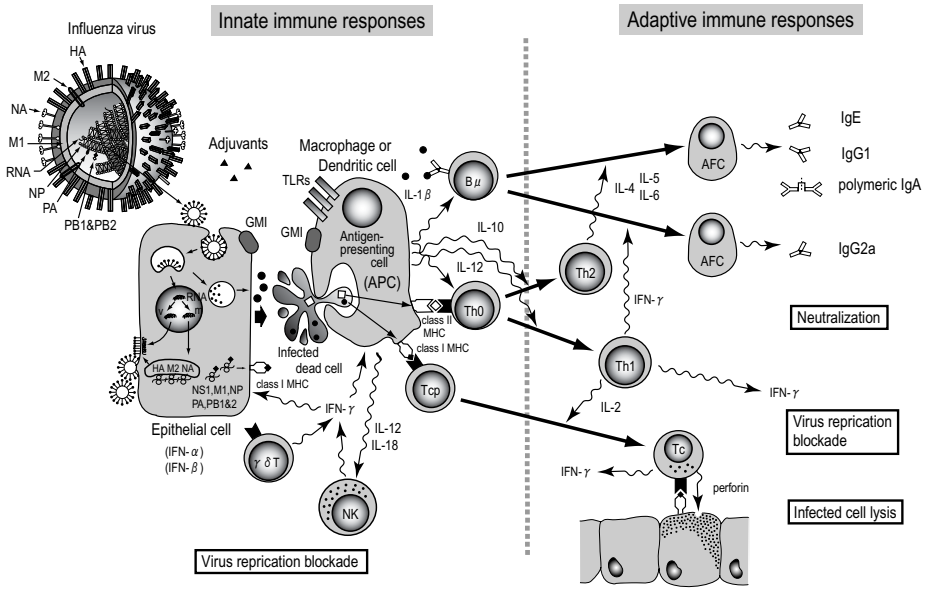


Fig. 1. Defense mechanisms induced by influenza virus infection.

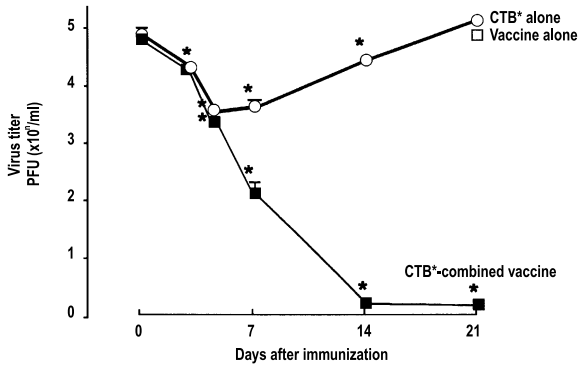


Fig. 2. Non-specific reduction of influenza viral replication by pre-treatment with cholera toxin adjuvant (CTB*). Mice were immunized intranasally with CTB* (1 μg) alone (○), A/PR8 vaccine alone (1 μg) (□) or, CTB*-combined vaccine (■), and then challenged 3, 5, 7, 14 and 21 days later with A/PR8 viruses. Three days after challenge, the virus titer in the nasal wash (PFU/ml) was measured. Each value represents the mean ± SD of the virus titer/ml in the nasal washes of the mice in each group of six mice. The asterisk represents a statistically significant difference between non-immunized and infected mice.

ment of the adaptive immune responses against influenza viruses, which results in complete protection against infection. Similarly, pretreatment of mice with *Propionibacterium acnes* (*Corynebacterium parvum*) or baculovirus before a lethal viral challenge results in lower lung-viral titers and lower mortality (20-22). In these cases, various constituents of the pathogens used for the pretreatment can activate macrophages and dendritic cells (DCs) via members of the Toll-like receptor (TLR) family on these cells to induce protection against infection (23-25). For example, bacterial DNA has stimulatory effects on mammalian immune cells by the presence of unmethylated CpG dinucreotides, which are far more common in bacterial DNA than in vertebrate DNA (26,27). Recognition of CpG DNA by mammalian immune cells is mediated by TLR9, which can distinguish bacterial DNA from self-DNA (23). Intranasal administration of CpG DNA can enhance mucosal Ab responses to co-administered inactivated influenza vaccines (28). Thus, various constituents of the pathogens can be used to enhance innate immune responses via various receptors, such as TLRs and GM1 gangliocide, on macrophages and DCs, so as to induce augmented mucosal immune responses in the respiratory tract (29).

3. Adaptive immunity against influenza

3-1. Roles of APCs in adaptive immunity

APCs (macrophages and DCs) are essential in the induction of the adaptive immune responses (25). Exogenous viral antigens, which comprise inactive viral particles, intact viruses and apoptotic, infected cells, are taken up by APCs through endocytosis and provide a potential source of peptides that could bind to MHC class I or II molecules in the APCs (30-34). In addition, influenza-infected macrophages can also act as APCs (35), and APCs secrete IL-12, which contributes to Th1-type helper cell development (9). APCs also secrete IL-1β, one of the most important cytokines in bridging between the innate and adaptive immune systems (19).

3-2. Roles of T and B cells in adaptive immunity

Virus-specific $CD4^+$ helper T cell precursors, from which Th1-type and Th2-type cells are produced, and $CD8^+$ precursor T cells for CTLs recognize MHC class II- and MHC class I-antigenic peptide complexes on APCs, respectively. These cells are subsequently activated by cytokines produced by APCs (36). Th1 cells secrete IFN-γ and IL-2, and help IgG2a Ab production by Ab-forming cells (AFCs) in mice, while Th2 cells secrete IL-4 and IL-5, and help IgA, IgG1 and IgE Ab production by AFCs (3). Th1 cells also enhance the proliferation of $CD8^+$ CTLs by secreting IL-2 (37,38). The Abs produced during these responses contribute to viral neutralization (NT) by binding with viral antigens (4,30). Th1 cells mediate delayed-type hypersensitivity (DTH) reaction by secreting IFN-γ, which results in the inhibition of virus replication (40-42). In turn, CTLs recognize MHC class I-antigenic peptide complexes on virus-infected epithelial cells and destruct the virus-infected cells mainly by exocytosis of granules containing perforin and granzymes (43-45). IFN-γ secreted by NK and $CD8^+$ T cells seems not to be essential for the target cell lysis (46,47).

4. Mucosal immune system in the respiratory tract

The mucosal immune system can be divided into two sites, inductive sites and effector sites. The inductive sites are mucosal-associated lymphoid tissues (MALT), where initial responses including antigen uptake by APCs and priming of T and B cells for IgA Ab production are induced and the effector sites are the mucosa that covers the internal surface of the whole body, where IgA AFCs are found and where S-IgA Abs play a protective role. Thus, specific IgA AFC precursor populations, induced by antigenic stimulation at one inductive site, migrate not only to the effector site near the original inductive site, but also to other mucosal sites via the homing pathways. This system is collectively referred to as the common mucosal immune system (48-54), and is responsible for the recovery from influenza and for influenza prevention in the respiratory tract.

4-1. Common mucosal immune system

The mucosal immune response in the upper respiratory tract is induced in the nasopharyngeal-associated lymphoid tissues (NALT) in rodents (52,53). The NALT seems to be functionally and anatomically different from Waldeyer's ring, which comprises the nasopharyngeal tonsil (adenoid), the paired tubal tonsils, the paired palantine tonsils and the linguial tonsil in humans (55). Since AFC responses are induced in NALT cell cultures from naïve mice after in vitro culture with influenza virus, it is evident that the NALT comprises inductive tissues from which mucosal AFC precursors originate (56). NALT is also a mucosal inductive site for virus-specific cellular immune responses (57). Exogenous antigens penetrate through highly pinocytotic and phagocytotic M cells present on the NALT and interact with resident T and B cells, resulting in a large number of IgA AFC precursors (47,48,51). The primed T and B cells then leave the NALT and enter the cervical lymph nodes and eventually the general circulation via the thoracic duct. The primed T and B cells then migrate to the lamina propria mucosae of the respiratory tract, intestinal tract, and other sites where IgA AFC precursors differentiate into specific IgA AFCs. Of relevance to this, intranasal immunization is superior to oral immunization in inducing S-IgA Abs in not only the respiratory tract but also the gastrointestinal tract (58). Thus, the NALT appear to be the inductive site that most effectively provides S-IgA Ab in the respiratory tract that is required for protection against

influenza virus infection. In addition, the intranasal route may be the most practical for other vaccines whose protective sites are mucosa, other than the respiratory tract, and that are sensitive to gastrointestinal conditions such as low pH and the presence of proteolytic enzymes (59).

The mucosal immune responses in the lower respiratory tract are induced in the bronchus-associated lymphoid tissue (BALT), which has been well characterized in rats and rabbits, but not in humans and mice (54). Since AFC responses are induced in lung cultures from naïve mice after in vitro culture with influenza viruses, it seems probable that the BALT or its equivalent tissue in mice works as the inductive site in the lung from which mucosal AFC precursors originate (60).

4-2. NALT

The NALT, which is composed of paired lymphoid cell aggregates in the noses of rodents, is the only well-organized MALT in the upper respiratory tract (52,53). It is situated in the mucosa of both lateral walls of the nasal cavity, near the nasal floor on the posterior side of the palate (Fig. 3). It can be isolated easily by peeling away the palate from the upper jaw. It consists of a reticular network filled with various types of lymphoid and non-lymphoid cells. The NALT is not only an inductive tissue from which precursors of mucosal AFCs and CTLs originate (56,57), but also an important site in lymphocyte re-circulation, since NALT lymphocytes migrate back to the NALT and cervical lymph nodes (CLNs) in far greater numbers than cells from Peyer's patches, and, reciprocally, cells from the CLNs migrate back more frequently to the NALT than to Peyer's patches (52).

4-3. Secretion of S-IgA Abs

AFCs that disseminate to the lamina propria underneath the mucosal epithelium via the general circulation cause S-IgA Ab production in the mucosal secretions. The S-IgA Abs are the J-chain-containing dimeric IgA (dIgA) Abs that are produced by IgA AFCs. The dIgA Abs bind to a polymeric Ig receptor (pIgR) on the basolateral surface of the epithelial cells and are carried to the apical surface. They are released as S-IgA Abs by combining with the secretory component (SC), which is the extracellular region of pIgR cleaved by a specific protease (50). Thus, anti-influenza HA-specific polymeric IgA (pIgA) Abs injected intravenously could be transported more efficiently into nasal secretions than monomeric IgA (mIgA) or IgG1 Abs (61). Blocking of the transcytosis of dIgA in pIgR-knockout mice immunized intranasally with an adjuvant-combined influenza vaccine resulted in a marked increase in serum IgA concentration and a decrease in the nasal IgA concentration (62).

5. Recovery from influenza following primary viral infection by adaptive immune responses

5-1. Primary Ab responses

5-1-1. Ab responses in humans experimentally infected with influenza virus

In naïve children experimentally infected with influenza A virus, infection with live, attenuated A viruses induces a high-level anti-HA IgA Ab response with lower levels of IgM and IgG Abs observed in the nasal wash within 2 weeks after infection (63). The nasal wash IgA and IgG Ab titers persist for 1 year in about half of the vaccinated subjects. Approximately 85% of seronegative adult volunteers who are infected with a live attenuated virus developed an IgA Ab response. In addition, there is evidence for active secretion of IgA Abs. In individuals infected with wild-type or attenuated viruses, the nasal wash IgA Abs are mainly polymeric and almost

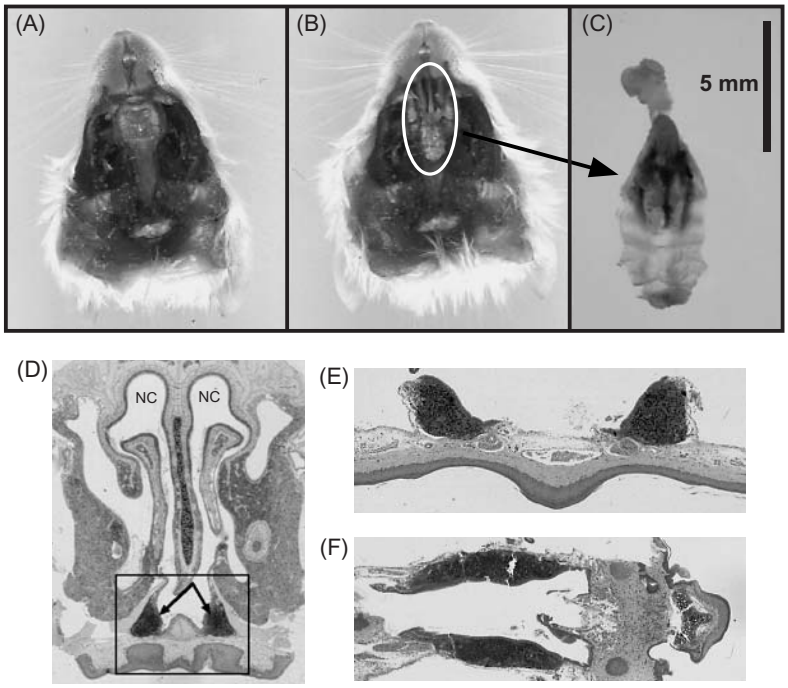


Fig. 3. Isolation method and histology of NALT of BALB/c mice. NALT was isolated from the rest of the nasal tissue by peeling away the palate from the upper jaw (A and B). A palate fragment isolated from the upper jaw, including a pair of NALT on the posterior surface (C). A frontal section of the upper jaw stained by hematoxylin and eosin showing that the NALT are situated in the mucosa of both lateral walls of the nasal cavity, near the nasal floor (D). A cross section of an isolated palate showing that the NALT is found beneath the columnar epithelium of the nasal cavity (E). A horizontal section of an isolated palate showing the longitudinal presence of NALT (F).

completely IgA1. IgA1 is one of two subclasses of human IgA and possesses an elongated proline-rich hinge region (64). It is predominant subclass in secretions (70 - 95% of total IgA) and in serum (about 90% of total IgA), although IgA2 predominates in the colon (about 60% of total IgA).

5-1-2. Ab responses in the upper respiratory tract of naïve mice infected with mouse-adapted virus

Infection with mouse-adapted viral strains results in either non-lethal respiratory disease (influenza model) or lethal viral pneumonia (viral pneumonia model), depending on the volume of virus suspension used for the intranasal administration under anaesthesia (65,66). Nasal virus titers in the influenza model, in which 2 μl of a virus suspension (1 μl to each nostril) is administered by intranasal dropping, peak within 3 - 5 days and decline to undetectable levels by 10 days (56) (Fig. 4A). The expression of nucleoprotein (NP) mRNA in the epithelial cells adjacent to the NALT, which shows the presence of the infected cells, changes in parallel with the viral titer (Fig. 4B). This infection also induces a significant accumulation of lymphoid cells (T and B cells) in the NALT, which peaks at approximately day 7 postinfection (Fig. 4C). In parallel with this change, all of virus-specific IgA, IgG and IgM AFC responses, developing on day 5 and peaking on day 7, are found in the lamina propria mucosae adjacent to the NALT (nasal mucosa); IgA and IgG Ab production predominates, followed by IgM Abs (Fig. 4D). On day 7 postinfection, the nasal mucosa adjacent to the NALT contains the greatest number of IgA AFCs per total cells in the CLNs, nasal mucosa adjacent to the NALT and the other nasal mucosa. The appearance of AFCs is accompanied by the appearance of virus-specific IgA Abs in the nasal wash (Fig. 4D). The appearance of the Abs correlates inversely with decrease of virus titers in the nasal area (Fig. 4A) and implies their involvement in the recovery from primary infection. Thus, the diffuse lining (the lamina propria mucosae) of the nasal passage is the site of virus-specific Ab production in response to influenza virus infection. In addition, it is the site of long-term virus-specific IgA Ab production, persisting for the life of the animal (67, 68).

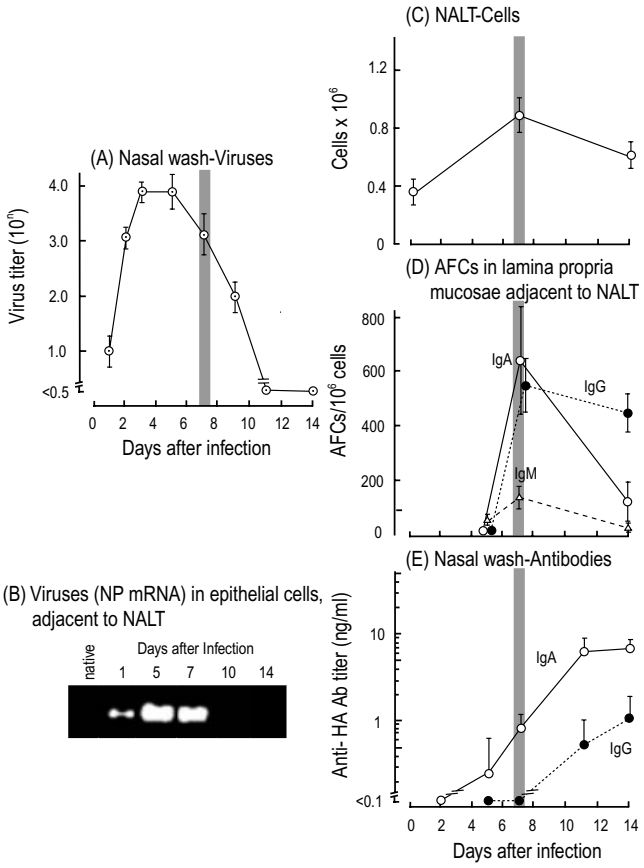


Fig. 4. Kinetics of virus titers in the nasal wash (A), expression of NP mRNA in the epithelial cells (B), number of lymphoid cells in the isolated NALT (C), AFC responses in the isolated NALT, including the mucosa (D), and Ab responses in the nasal wash in mice infected with a small volume of A/PR8 virus suspension (E). The shaded column in each figure indicates day 7 post-infection.

5-1-3. Ab responses in the lung of naïve mice infected with influenza A virus

A study of AFC responses within lung tissue following primary intranasal infection showed that IgM AFCs are first detected at day 5 and then peaked at day 10, whereas IgG and IgA AFCs are detected at day 10 and peaked around day 18 with slightly more IgG than IgA AFCs (60). The AFCs appear earlier in the spleen than in the lung and disappeared more rapidly from splenic tissue. A possible causal relationship between the AFC responses and recovery from influenza or viral pneumonia remains to be examined.

5-1-4. Intraepithelial cell prevention of viral assembly by S-IgA Abs in naïve mice infected with mouse-adapted influenza A virus

It has been postulated that the dIgA Abs, which are actively transcytosed across epithelial cells via pIgR, can bind to newly synthesized viral proteins within the epithelial cells to prevent viral assembly (69). This defense mechanism may be involved in either recovery from influenza after primary viral infection or prevention of influenza by re-infection.

5-2. T cell-mediated immune responses following primary viral infection

5-2-1. Involvement of CTLs in the recovery from influenza in mice

Mice infected with a sublethal dose of virus usually clear the infective particles from the respiratory tract within 10 days after primary infection (Fig. 4A). Influenza virus-specific $CD8^+$ T cells appear from day 5 and accumulate in the nasal mucosa, peaking on day 7 after the primary infection (70). The T cell accumulation is detected marginally in the NALT, moderately in the CLNs with a peak on day 7, and most abundantly in the spleen with a peak around day 13. In addition, they are found in the lamina propria and the intraepithelial lymphocyte compartment of the respiratory epithelium in the nasal mucosa. The recruitment of the virus-specific $CD8^+$ T cells into the nasal mucosa following a primary intranasal infection is analogous to the recruitment of the same effector cells into the lung following a pulmonary infection (71,72). These evidences suggest that influenza virus-specific CTLs, as well as the S-IgA Abs, which are induced in the NALT and recruited into the nasal mucosa, are involved in the recovery from influenza in the upper respiratory tract (56,57). CLNs may serve to amplify mucosal immune responses initiated in NALT.

5-2-2. Involvement of Th1 cells in the recovery from influenza in mice

Low-level DTH responses are induced by Th1 cells on day 7 after infection with live virus in mice (40-42). The involvement of Th1 cells in the recovery from influenza remains to be investigated, although Th1 cells can prevent viral replication by producing IFN-γ (42,73).

5-2-3. Cytokine responses in naïve mice infected with influenza A virus

Strong IL-2, weak IL-4, strong IL-6 and strong IFN-γ mRNA expressions are induced in the NALT of infected mice during the early days of infection (74). IFN-γ mRNA is expressed by both $CD4^+$ and $CD8^+$ T cells at around 7 days postinfection. Both anti-viral IgA Abs in the nasal wash and IgG2a-rich Abs in the serum are also detected at 11 days after the infection. In addition, persistent expression of IL-5 and IL-10 (Th2-type cytokines), together with IFN-γ, was detected in the T cell fraction isolated from lung tissue and airways of infected mice (75,76). Two cytokines are involved in IgA responses; TGF-β induces switching to IgA production and IL-5 or IL-6 acts on B cells committed to IgA production to differentiate into IgA-secreting cells (77,78). On the other hand, IFN-γ released from the Th1 cells participate in isotype switching to IgG2a in the mouse (36,79). Based on these collective results, the cytokine profile in the respiratory tract of infected mice can be classified as a mixed type of Th1 and Th2.

5-3. Involvement of both Abs and CTLs in the recovery from influenza

For many years, MHC class I-restricted CTLs that recognize NP and other conserved gene products were thought to be the principal effectors in the recovery from primary influenza virus infection (80,81). However, $CD4^+$ T-cell-dependent anti-viral Ab responses, as well as $CD8^+$ CTLs, seem to be indispensable for the recovery of mice from primary influenza virus infection (73,82-84). For example, virus-neutralizing Abs of IgG but not IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice (85). In addition, the $CD4^+$ T cells and MHC class II+/+ bone marrow cells in the short term radiation chimeras made with MHC class II–/– recipients are able to clear influenza virus from MHC class II–/– lung cells (86). This implies that immune $CD4^+$ T cells in these chimeras function to help the Ig-producing B cells. The recovery process involves two phases: an early phase (days 5 - 7), characterized by a rapid decrease in virus titer, is T-cell-dependent, while a late phase (day 7 onwards), characterized by a more protracted decrease that ultimately results in clearance, is B-cell-dependent (87, 88). These results suggest that influenza viruses after primary infection are eliminated initially via killing of the virus-infected epithelial cells by MHC class I-restricted $CD8^+$ CTLs, which appear transiently in the respiratory mucosa with a peak on day 7 postinfection. The viruses, which still survive, are then eliminated via NT by mucosal IgA Abs, which are detected on day 5 and reach a plateau at around day 11, and IgG Abs, which diffuse from serum across the mucosa.

6. Prevention of influenza following secondary viral infection by adaptive immune responses

6-1. Prevention of influenza by preexisting Abs

6-1-1. Direct role of S-IgA Abs in protection against virus infection

Anti-influenza S-IgA Abs purified from the respiratory tracts of mice immunized with influenza viral HA molecules, when administered intranasally, protect non-immune mice from influenza virus infection (89,90). Treatment with anti-IgA Abs, but not with anti-IgG or anti-IgM Abs, of mice immunized with live influenza virus abrogates the protection (91,92). Thus, IgA Abs play a direct role in protection against influenza. In addition, pIgA and S-IgA have several-fold higher activities than monomeric IgA (mIgA) in hemagglutination inhibition (HI) and virus NT, which are derived from their polymeric nature (93). Thus, S-IgA Abs in the respiratory tract play a causal role in providing cross-protection against infection with variant (drift) viruses within a subtype and different subtype viruses within the A virus .

6-1-2. Cross-protection by S-IgA induced by primary viral infection

Mice previously infected with A/Rec 31 (H3N1) virus are strongly protected against challenge with A/Vic (H3N2) virus in parallel with the presence of cross-reactive S-IgA Abs in the lung (94). Mice previously infected with A/Yamagata (H1N1) viruses are also protected against challenge with PR8 (H1N1) virus in proportion to the amount of cross-reactive S-IgA Abs in the nasal wash (4). Thus, S-IgA Abs in the respiratory tract are directly involved in cross-protection against infection with variant (drift) viruses within a subtype of influenza A viruses.

6-1-3. Virus-Ig complex formation in the upper respiratory tract

Elimination of challenge viruses (A/PR8 virus) from the nasal area occurs earlier in mice immunized 4 weeks previously with A/PR8 (H1N1), A/Yamagata (H1N1) and A/Guizhou-X (H3N2) viruses, in that order, compared to naïve mice (4). The early viral elimination, as assessed by the PFU (infectious virus) reduction, correlates with the level of A/PR8 virus-reactive Abs in the immunized mice and the appearance of viral-Ig complexes shortly after challenge infection. Thus, local Abs present at the time of challenge virus infection are involved in the prevention of influenza by forming virus-Ig complexes shortly after infection.

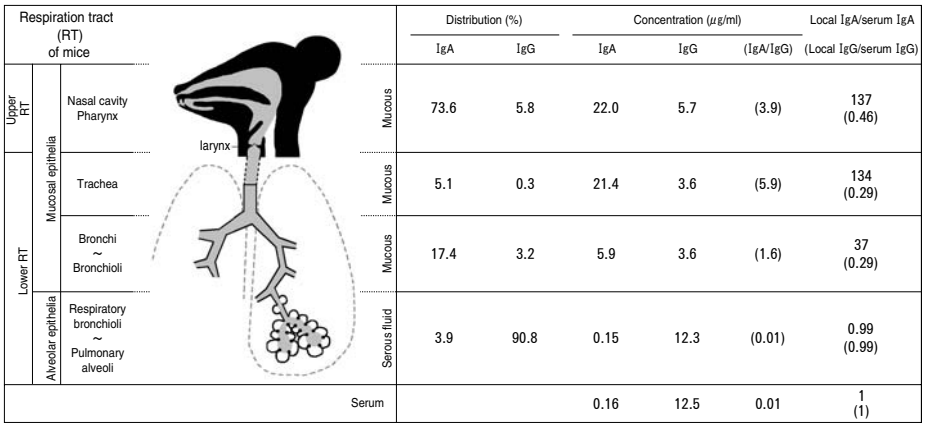
6-2. Prevention of influenza by secondary Ab responses

6-2-1. Prevention of influenza by secondary Ab responses in humans

Children who previously experienced natural infection or who received a live virus vaccine exhibit a marked reduction in both the amount and duration of virus shedding when compared to subjects without prior exposure to influenza A virus infection (39, 63). The nasal wash IgA Ab response to the influenza HA correlates with this resistance to challenge infection. Thus, re-infection results in a secondary IgA Ab response, which is provided by memory Th and B cells and characterized by a rapid rise in IgA Ab titer, a higher peak titer and maintenance of detectable levels of Ab over a longer period of time.

6-2-2. Protective roles of local S-IgA Abs and systemic IgG Abs in the respiratory tract

Serum IgG Abs in the immunized mice seem to be important for preventing lethal influenza pneumonia (95). To confirm this hypothesis, the distribution and concentration of specific IgA and IgG Abs in the mucus or serous fluid from different sites of the respiratory tract were examined under conditions of complete protection against challenge infection with a lethal dose of influenza virus in mice immunized intranasally with the vaccine (62,96). The specific S-IgA Abs, which are secreted actively across the mucosal membrane, are present at high levels in the mucus of nose, trachea, bronchi and bronchioli, whereas the specific IgG Abs, which could access the mucosal surfaces by passive diffusion from serum, are found predominantly in the serous fluid of alveolar epithelia (Fig. 5). Thus, S-IgA Abs are involved primarily in the prevention of influenza in the upper respiratory tract, whereas serum IgG Abs predominate in the prevention of lethal influenza pneumonia.



Respiration tract (RT) of mice			Distribution (%)		Concentration (μg/ml)			Local IgA/serum IgA
			IgA	IgG	IgA	IgG	(IgA/IgG)	(Local IgG/serum IgG)
Upper RT	Mucosal epithelia	Nasal cavity Pharynx	73.6	5.8	22.0	5.7	(3.9)	137 (0.46)
Lower RT	Mucosal epithelia	Trachea	5.1	0.3	21.4	3.6	(5.9)	134 (0.29)
Lower RT	Mucosal epithelia	Bronchi ~ Bronchioli	17.4	3.2	5.9	3.6	(1.6)	37 (0.29)
Lower RT	Alveolar epithelia	Respiratory bronchioli ~ Pulmonary alveoli	3.9	90.8	0.15	12.3	(0.01)	0.99 (0.99)
Serum					0.16	12.5	0.01	1 (1)

Fig. 5. Distribution and concentration of A/PR8 HA-specific IgA and IgG Abs in different sites of the respiratory tract (RT) of immunized mice. Mice were immunized intranasally with an adjuvant-combined A/PR8 inactivated vaccine, which provided a minimal dose for complete protection against challenge infection with a lethal dose of the virus. In the immunized mice, anti-HA IgA and IgG Ab titers in nasal wash, tracheal wash, broncho-alveolar wash and serum were measured. The Ab titers were converted to the concentration of mucus (or serous fluid) in different sites of the RT, based on the mucus (or serous fluid) volume in each site, which was calculated from the surface area of each site and the estimated thickness of mucus (or serous fluid). The surface area of each site was estimated using serial tissue sections of nose, trachea and lungs. The distribution (%) shows the ratio of the Ab amount in each site of RT (mucus or serous fluid) to that in the total RT.

6-3. Prevention of influenza by secondary T cell-mediated immune responses

6-3-1. Prevention of influenza by secondary CTL responses in humans

Volunteers with a high CTL (class I MHC-restricted $CD8^+$ cytotoxic T lymphocyte) activity shed fewer viruses than those with a low CTL activity when experimentally administered with wild-type influenza A virus (97). Thus, the CTL activity of memory T cells correlates with resistance to influenza. However, the epidemiologic behavior of influenza viruses in humans suggests that the overall contribution of CTL to disease reduction during re-infection with influenza A virus is small, because repeated infection of humans with influenza A virus bearing internal viral antigens provides little resistance to disease caused by new influenza variants (63,98).

6-3-2. Prevention of influenza by secondary CTL responses in mice

CTL memory cells induced by primary infection are stimulated by re-infection, resulting in an accelerated appearance of CTL activity in mice challenged with different subtype viruses (70-72, 99). Thus, secondary influenza virus specific-CTL responses appear about 2 days earlier, and with higher activities, than primary CTL responses, and are involved in the clearance of different subtype viruses from the lung and nose.

6-3-3. Prevention of influenza by secondary Th responses in mice

The memory Th1 cells have antiviral activity in mice challenged with different subtype viruses (42,100). Specific T cells mediate protection and recovery of JHD−/− mice (B cell-deficient mice) immunized with live virus and challenged with a lethal dose of influenza virus (101). $CD4^+$ and $CD8^+$ T cells, but not Abs, are involved in cross-protection between viruses (H1N1, H2N2 and H3N2) for primary infection and viruses (H1N1 and H3N2) for challenge infection (heterosubtypic immunity) using mice lacking IgA, all Ig, NKT cells, or $\gamma\delta$ T cells (102). These results imply the involvement of memory Th cells and CTLs in heterosubtypic immunity. In contrast, β2-microglobulin-deficient mice, infected with the H3N2 influenza virus and challenged with the H1N1 influenza virus 3-4 weeks later, exhibit increased survival and enhanced clearance of virus relative to non-immune controls. This suggests that $CD4^+$ T cells and Abs are involved in heterosubtypic immunity (103). The defense mechanisms are redundant, raising the possibility that one of protective mechanisms that are the primary means of protection in the respiratory tract in wild-type mice may function preferentially in T cell subset, B cell or Ig-deficient mice. Thus, IgA Abs, as well as CTLs, seem to play a major role in heterosubtypic immunity.

7. Basis for the development of an effective mucosal influenza vaccine and perspectives

The major adaptive immune responses involved in defense against influenza in the respiratory mucosa are summarized in Fig. 6 and in the following text. (i) In naïve mice, specific S-IgA Abs and CTLs are the major effectors involved in the recovery from influenza following primary virus infection. The S-IgA Abs first appear at day 5, then increase and reach a plateau at around day 11 postinfection. The CTLs also appear transiently in the nasal mucosa with a peak around day 7 postinfection. Sublethal doses of influenza viruses are eliminated from the upper respiratory tract within 10 days after primary viral infection. (ii) In the pre-immunized animals, the preexisting S-IgA and IgG Abs encounter and inactivate the re-infected viruses shortly after infection by forming virus-Ig complexes. The local S-IgA Abs react not only to homologous viruses but also to variant viruses in the same subtype. The strong cross-reactivity of the S-IgA Abs appears to derive from its polymeric nature, resulting in an overall increase in avidity of the Ab for the influenza virus compared to the serum IgG Abs. IgM Abs, as well as IgA Abs, are actively secreted because of the pIgR-mediated active transport of J chain-containing polymeric immunoglobulin molecules, although active secretion was most frequent for IgA Abs in children immunized with live virus vaccines (63). The mechanism underlying the role of IgM Abs in protection against influenza remains to be investigated. IgG Abs, which transude from the serum to the mucus by diffusion, react mainly with homologous virus. (iii) In the pre-immunized animals, CTL memory cells induce an accel-

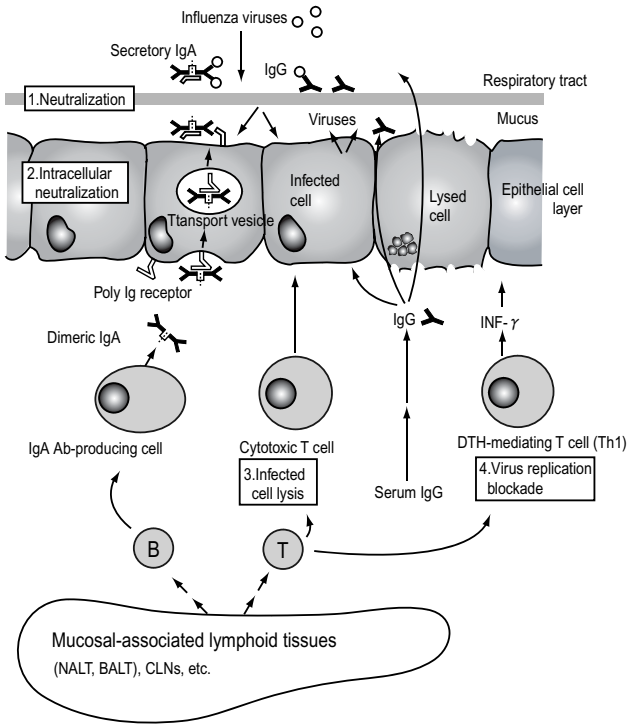


Fig. 6. Major adaptive immune responses involved in defense against influenza in the respiratory tract mucosa.

erated CTL production from day 3 onwards after re-infection and the produced CTLs are involved in the lysis of the epithelial cells infected with different subtype viruses (within the same type) to prevent the spread of infection. In the absence of the preexisting Abs, B memory cells induce an accelerated IgA and IgG Ab production from day 3 onwards after re-infection and the produced Abs are involved mainly in the elimination of both homologous and drift viruses within the same subtype by forming virus-Ig complexes. (iv) The memory Th1 cells that mediate DTH are involved in blocking viral replication by secreting IFN-γ in mice challenged with different subtype viruses, although the Th1 cell induction following live virus infection is lower than that induced by inactivated virus and viral antigens. (v) On the transport of dIgA Abs by pIgR through infected epithelial cells, the dIgA Abs bind to newly synthesized viral proteins within the infected cells and prevent viral assembly thereby preventing the spread of infection.

To control influenza, protective immunity must be induced in advance by the administration of a vaccine. Currently available, inactivated vaccines, which are composed of either entire virions ("whole virus" vaccines), virions subjected to treatment with ether ("split-product" vaccines) or purified glycoproteins ("subunit vaccines"), are injected parenterally (2). As discussed and shown in Fig. 6, the major protective immunity induced by influenza virus infection is provided by S-IgA Abs, IgG Abs and CTLs in the respiratory tract. However, inactivated vaccines induce mainly serum IgG Abs rather than mucosal IgA Abs, which are cross-reactive among drift viruses within a subtype, and CTLs that are cross-reactive among different subtypes. Thus, inactivated vaccines are effective in protecting against an epidemic of homologous viruses but relatively ineffective against an epidemic of heterologous viruses (2,63,98). Therefore, we advocate an intranasal administration of inactivated vaccine to elicit S-IgA Ab induction to improve the protective efficacy of the inactivated vaccines (104,105). Furthermore, the FDA recently approved a cold-adapted, live-attenuated vaccine (Flumist, Medlmmune Vaccines, Inc., USA) for intranasal administration. This vaccine can induce IgA Abs, IgG Abs and CTLs (3,106). However, the live-virus vaccine is only approved for the age group of 5 - 49 years, thus excluding two major high-risk groups, the infants and the elderly, in addition to immunodeficient patients and pregnant women. The live vaccine seems to cause coryza, sore throat and febrile reactions. Because of these problems, several trials are currently underway to test new mucosal vaccines using inactivated viruses or viral components (28,107-109).

We have demonstrated that intranasal immunization with inactivated vaccines, used in conjunction with CT B subunit (CTB) containing a trace amount of CT (0.1%) (CTB*) [or *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 respiratory tract against variants (drift viruses) within the subtype of the influenza A viruses or variants of the B viruses (109-112). 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 (111-113). However, the use of heat-labile enterotoxin (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, Switzerland), following licensing in 2001, has been linked to several cases of transient Bell's palsy (facial paralysis) (114). Thus, clinically safer and more effective adjuvants are required for the intranasal administration of inactivated influenza vaccine.

The mechanisms by which CT or LT enhances mucosal immune responses against influenza viral antigens involve stimulation of the innate immune system (17). Thus, CT or LT alone can reduce the replication of the viruses non-specifically in the upper respiratory tract when administered intranasally into mice together with infectious viruses. In addition, reduction of viral replication correlates with the activation of APCs (macrophages, DCs and others). Therefore, new and effective adjuvants may arise from a screen of materials that stimulate the function of APCs, including ganglioside GM1 and ligands of several TLRs (18,22-29). One attempt involved development of a nontoxic form of an adjuvant based on LT and CT together (115). Another is the use of ligands for TLR family of receptors on the APCs, such as CpG DNA (28). 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.

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