

# Development of mucosal adjuvants for intranasal vaccine for H5N1 influenza viruses

Hideki Hasegawa  
Takeshi Ichinohe  
Akira Ainai  
Shin-ichi Tamura  
Takeshi Kurata

Laboratory of Infectious Disease  
Pathology, Department of Pathology,  
National Institute of Infectious  
Diseases, Tokyo, Japan

**Abstract:** An increasing number of infections of highly pathogenic avian influenza virus (H5N1) in humans has been reported in South-East Asia and other areas of the world. High mortality (>60%) of this viral infection and its pathosis of systemic infection are features of this new human disease. Moreover, there is great concern that this avian H5N1 virus could cause a pandemic of new influenza in humans, once it acquires the ability for human to human transmission. To prevent such highly contagious infectious diseases as influenza, it is essential to prepare effective vaccines. Especially in the case of new influenza virus, we cannot predict the strain which will cause the pandemic. In such a situation, a vaccine that induces cross-protective immunity against variant viruses is extremely important. However currently used parenteral seasonal influenza vaccine is strain-specific, and is less effective against variant viruses. In order to overcome the weakness of current vaccines we need to learn from the immune responses induced by natural infection with influenza viruses. In the case of mucosally acquired acute respiratory infection such as influenza, mucosal immunity induced by natural infection plays important role in protection against the infection, as mucosal secretory IgA antibody plays an important role in cross-protection. In this review we describe the advantages and development of mucosal vaccine against highly pathogenic H5N1 influenza viruses.

**Keywords:** influenza virus, mucosal immunity, secretory IgA antibody, adjuvant

## Influenza virus and its infection signal

Influenza is a contagious acute respiratory disease of birds and mammals caused by infection of the upper respiratory tract by viruses of the family Orthomyxoviridae. Types A and type B infect humans and cause respiratory symptoms and also encephalopathy in infants. Recently it has been reported that infection by highly pathogenic influenza viruses (HPIV) and the avian influenza virus (H5N1) in humans can be fatal. In cases where infection sites were not restricted to the respiratory system, it spread systemically including the gastrointestinal (GI) tract.<sup>1</sup> Although most human H5N1 infections have been caused by the direct transmission of virus from infected poultry, there is fear that a pandemic could result if subsequent transmission of H5N1 virus occurred between infected humans.<sup>2</sup> Therefore, there is an urgent and important public health need to develop effective vaccines against this highly pathogenic strain of avian influenza virus.

Annual epidemics of influenza are caused when the antigenic properties of the viral surface proteins hemagglutinin (HA) and neuraminidase (NA) are altered. HA is involved in binding of the virus to sialic acids on the surface of susceptible cells.<sup>3</sup> NA cleaves terminal sialic acid residues from carbohydrate moieties on the surfaces of infected cells, promoting the release of progeny viruses from infected cells. It has been shown that both HA and NA are among the most protective of the various viral proteins against influenza when immunized with plasmid DNAs encoding HA and NA.<sup>4</sup>

Correspondence: Hideki Hasegawa  
Department of Pathology, National  
Institute of Infectious Diseases  
4-7-1 Gakuen, Musashimurayama-shi,  
Tokyo 208-0011, Japan  
Tel +81-42-561-0771  
Fax +81-42-561-6572  
Email hasegawa@nih.go.jp

Influenza virus has single-stranded RNA as its genome, and this single-stranded RNA is recognized as an infection signal by host cells through Toll-like receptor 7 (TLR-7).<sup>5</sup> In the course of viral replication, double-stranded RNA is produced, which is recognized by TLR-3 as an infection signal. Thus, influenza virus is recognized by host immune cells at the very early stage of infection by the host through pathogen signals, and these receptors and the host immune system initiate the mucosal and systemic immune system against present and future viral infection. By verifying a series of events occurring at the infection site, we use our increased understanding of the immune response to develop and apply strategies to combat influenza viral infection.

## Innate immunity and adjuvant effect

Innate immunity is a set of nonspecific mechanisms that constitute the body's naturally occurring immune response to infection by microbes at any site. In influenza virus infection, the upper respiratory mucosal surface is the effector site of the innate immune system. The mechanical barrier of the mucosal epithelium, surface mucus, secretion of antimicrobial peptides such as defensins, secretion of type I and II interferons (IFNs), natural killer cells, and complement

factor all play important roles in innate immunity at the respiratory mucosa (Figure 1). Among these, the IFN response is required to signal viral infection. During influenza virus infection, genomic single-stranded RNA, and double-stranded RNA produced during viral replication, have been implicated as the molecular signals of infection that trigger IFN production.

The innate immune system senses viral infection by recognizing a variety of viral components, including double-stranded (ds) RNA, and triggering antiviral responses. The cytoplasmic helicase protein retinoic-acid-inducible protein I (RIG-I, also known as Ddx58) and melanoma differentiation-associated gene 5 (MDA5, also known as Ifih1 or Helicard) have been implicated in recognition of viral dsRNA. Viral dsRNA binds to RIG-I and MDA5 in the cytoplasm, which leads to activation of IFN regulatory factors.<sup>6</sup> In vitro studies suggest that RIG-I and MDA5 recognize both RNA viruses, and polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analog. RIG-I is essential for the production of IFNs in response to RNA viruses, including paramyxovirus, influenza virus, and Japanese encephalitis virus, whereas MDA5 is critical for the detection of picornavirus.<sup>6</sup> The recognition of viral infection by the innate immune system

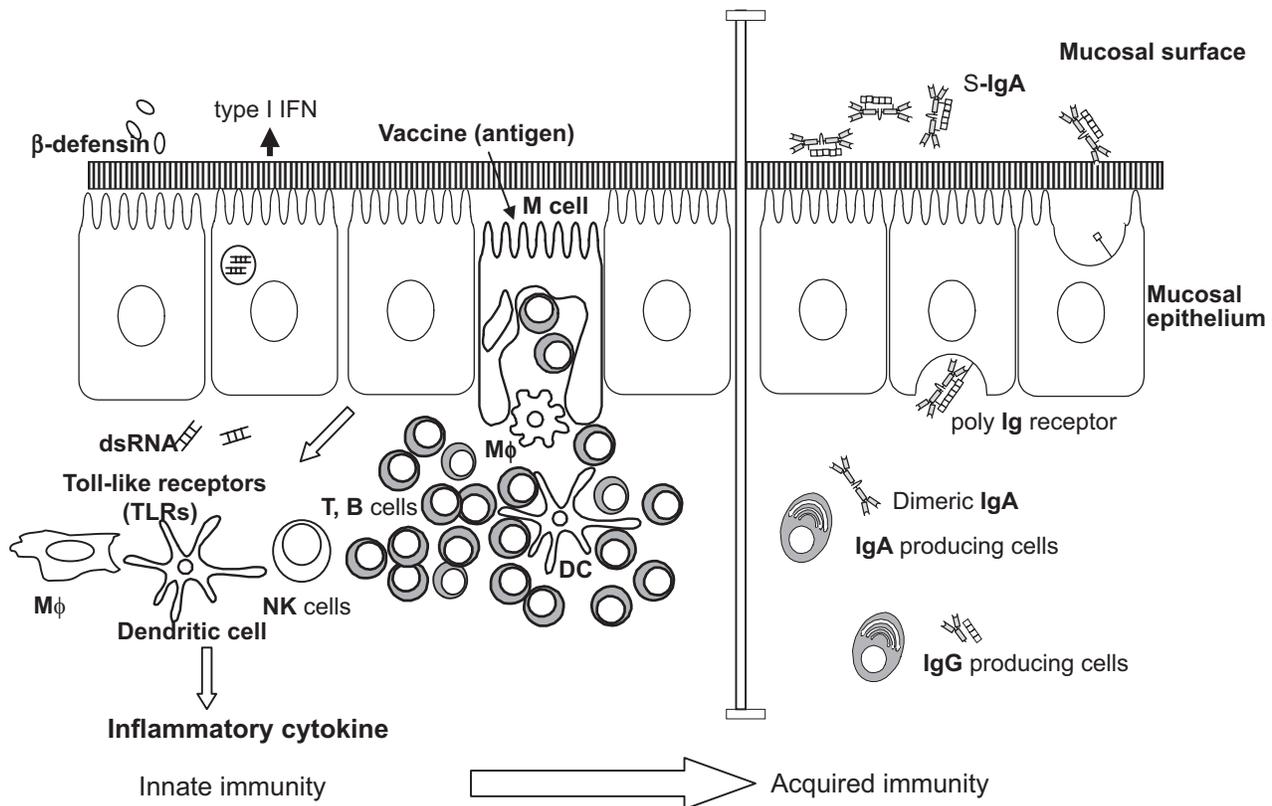


Figure 1 Defence mechanism at mucosal site, innate and adaptive immunity.

bridges the transition between innate and adaptive immune responses. This is a particularly important facet of innate immunity involved in mucosal immune responses. We can take advantage of the mucosal innate immune response to enhance vaccine efficacy, which we will discuss later in this review.

Among the several innate immune receptors, the Toll-like receptor family plays a central role in the recognition of viral nucleic acid. This recognition leads to the induction of type I IFN. We previously demonstrated that the synthetic double-stranded RNA (dsRNA) poly(I:C), a TLR-3 agonist, has mucosal adjuvant activity when co-administered intranasally with an influenza HA vaccine, and increases both the mucosal and systemic humoral response, resulting in complete protection against challenge by homologous avirulent (H1N1) and highly pathogenic (H5N1) influenza viruses in mice.<sup>7,8</sup> Sloat and Cui<sup>9</sup> also reported that mice immunized intranasally with recombinant anthrax protective antigen adjuvanted with poly(I:C) developed strong systemic and mucosal anti-anthrax antigen responses with lethal toxin neutralization activity. Thus, the signals conducted by innate immune receptors work as adjuvants which act as a bridge between innate immunity and acquired immunity.

## Mucosal vaccine

Seasonal influenza vaccines are prepared based on the prediction of the expected strain of epidemic of the next season. These are parenterally injected vaccines which does not prevent the infection itself, which reduce the severity and complications after the infection. Parenteral vaccines can induce the neutralizing IgG antibody in the serum but they cannot induce the secretory IgA antibody which acts on the mucosal surface. Secretory IgA antibodies on the mucosal membrane surface are highly effective for preventing infection because they react on the surface of the mucosal membrane before the pathogens attach to the epithelial cell surface, which is the first target of influenza viral infection. Moreover, serum IgG antibodies are less effective against drifted viral strains because they act more specifically than secretory IgA antibodies. Secretory IgA antibodies have cross-protective effects against variant strains of the influenza virus. The exact mechanism of the cross-reactive effects of IgA is still unknown, but this phenomenon is a great advantage in preventing infection. In fact, natural influenza virus infection was shown to be superior to vaccination with inactivated virus in inducing cross-protection against infection by mutated

viruses within a particular subtype of the A-type virus in humans.<sup>10–12</sup> Another reason why the mucosal immune system is adept at preventing infection is that the effector sites are not restricted to the originally sensitized mucosa. IgA-specific antibody forming cell (AFC) precursors migrate from mucosal sites throughout the entire body via site-specific homing pathways. This system is referred to as the common mucosal immune system.<sup>13–17</sup> Because of the advantages of induction of mucosal immunity for preventing influenza, several strategies have been used to attempt to development a mucosal vaccine. For effective induction of secretory IgA by inactivated vaccine, mucosal co-administration of vaccine with adjuvant is necessary. As a mucosal adjuvant, a bacterial toxin such as cholera toxin (CT) or *Escherichia coli* heat-labile toxin (LT) have been used experimentally.<sup>18,19</sup> Although LT is an effective adjuvant for the production of mucosal IgA, it has adverse clinical side effects, such as facial paralysis (Bell's palsy).<sup>20</sup> New, clinically safe and effective adjuvants are necessary for the administration of intranasal influenza vaccines to humans. The most promising candidate for mucosal adjuvant is PolyI:PolyC<sub>12</sub>U (Ampligen<sup>®</sup>), which is synthetic dsRNA and has a good safety profile based on clinical trials, including a recent double-blind, placebo-controlled Phase III clinical trial for chronic fatigue syndrome (CFS).<sup>21–23</sup> To date, >75,000 doses of Ampligen<sup>®</sup> have been administered to humans, at an average dose of 400 mg, and it has been generally well tolerated. Recently, it was shown that PolyI:PolyC<sub>12</sub>U was as effective as poly(I:C) in inducing maturation of human monocyte-derived dendritic cells in vitro.<sup>24</sup> So PolyI:PolyC<sub>12</sub>U (Ampligen<sup>®</sup>) was examined as an adjuvant for mucosal influenza H5N1 vaccine administered intranasally in mice together with synthetic dsRNAs (poly(I:C) and Ampligen<sup>®</sup>) as powerful TLR-3 agonists.

## Highly pathogenic avian influenza virus H5N1

The first outbreak of the highly pathogenic avian influenza virus H5N1 was reported in humans and birds in Hong Kong in 1997, during which six out of 18 infected people died.<sup>25</sup> Subsequently, re-emergence of the H5N1 virus associated with a high fatality rate (greater than 60%) has been reported in southern China, Vietnam, Thailand, Cambodia, Indonesia, Turkey, and Iraq. From January 2003 to September 2008, 387 laboratory-confirmed human cases of H5N1 were reported to the World Health Organization (WHO). Although most human H5N1

infections have been caused by the direct transmission of virus from infected poultry, there is fear that a pandemic could result if subsequent transmission of H5N1 virus occurred between infected humans.<sup>2</sup> Because the ability to be transmitted from human to human represents the final barrier to a new pandemic of H5N1, there is an urgent and important public health need to develop effective vaccines in preparation for such a pandemic. However developing a vaccine against the H5N1 virus poses a number of problems. A highly contained facility is required, and the virus grows very poorly in embryonated eggs because it kills chickens. Attenuation of the vaccine strain is necessary to eliminate these problems. Currently licensed human vaccines are strain-specific and do not protect against heterotypic influenza viruses. This is problematic, because influenza A (H5N1) continues to evolve into antigenically distinct clades. The question remains of how an effective vaccine can be prepared for an impending pandemic of a new influenza, which might be caused by a highly pathogenic strain of avian influenza virus. Influenza virus A (H5N1) is not the only strain that could cause a new pandemic in humans.

H5 vaccine candidates must be continually updated to match the antigenicity of circulating viruses because of the differences in HA antigenicity among 1997, 2003, and 2004 H5 viruses.<sup>26</sup> In addition, it is difficult to predict which strain of virus (H5 or other avian-associated HA) will be responsible for a pandemic. In such circumstances, the ideal approach is to prepare a vaccine that confers strong cross-protective immunity against variants of a particular virus strain. Mucosal immunity induced through natural infection by influenza virus has potent cross-protective activity, compared with subcutaneous vaccination-induced systemic immunity. Cross-protective activity is correlated with mucosal secretory IgA, which is not induced after subcutaneous vaccination.<sup>27</sup> In order to induce cross-protective mucosal immunity through influenza vaccination, we have examined the effect of intranasal administration of an inactivated viral vaccine with various adjuvants, and found that mucosal IgA plays an important role in cross-protection against variant influenza A and B virus infection.<sup>7,28–30</sup> Nicholson and colleagues reported that the H5N1 vaccine is poorly antigenic in humans, and requires adjuvant to elicit a detectable antibody response.<sup>31</sup> Several groups looking at avian influenza H5N1 vaccines have reported that intranasal administration of a formalin-inactivated whole virus vaccine with or without mutant *E. coli* LT adjuvant (R192G), or an adenoviral vector-based influenza vaccine,

protected mice from lethal challenge by a heterologous H5N1 virus.<sup>32–34</sup>

## Development of adjuvant-combined inactivated nasal vaccines

Subcutaneous injection of inactivated vaccines would be an effective strategy in an epidemic caused by a homologous virus, as it induces specific serum IgG, but would be less effective in an epidemic caused by a heterologous virus. On the other hand, live attenuated vaccines effectively protect against heterologous virus infection by inducing secretory IgA, IgG, and cytotoxic lymphocyte (CTL) responses. However, because their safety has been proven only in healthy people between the age of 5 and 49, their use is approved only for this group of people in the US. Intranasal administration of inactivated vaccines represents a potential solution to overcoming these problems.

In clinical trials, inactivated whole virus particles and split-product vaccines have been shown to be effective in preventing live virus infection when administered intranasally.<sup>35–39</sup> Moreover, intranasal administration of an inactivated whole virion vaccine induced a broad spectrum of heterosubtypic immunity in mice, which was not observed using an ether-split vaccine.<sup>33</sup> The stronger immunogenicity of the inactivated whole virion vaccine was likely due to the stimulation of innate immunity by genomic single-stranded RNA, via TL-R7.<sup>5,40</sup>

Intranasal administration of an inactivated ether-split vaccine and the synthetic dsRNA poly(I:C) conferred effective cross-protection in the upper respiratory tract (RT) against viral variants (drift viruses) of influenza A, or B-type viruses.<sup>7</sup> Because most viruses produce dsRNA during replication,<sup>41</sup> synthetic dsRNA likely acts as a molecular mimic of viral infection. The mammalian TLR-3 receptor recognizes dsRNA, and activates the NF- $\kappa$ B<sup>42</sup> pathway, resulting in activation of type I IFN, which in turn enhances the primary antibody response to subcutaneous immunization of soluble materials.<sup>43</sup> This adjuvant activity of type I IFN appears to play an important role in bridging the gap between innate and adaptive immunity.<sup>43</sup>

In mice, intranasal administration of an ether-split vaccine from PR8 (influenza strain H1N1) and poly(I:C) adjuvant induced a strong anti-HA IgA and IgG response in nasal washes and serum, respectively, while vaccination without poly(I:C) induced very little response. In addition, administration of either an A/Beijing (H1N1) or A/Yamagata (H1N1) vaccine and poly(I:C) conferred complete protection against PR8 virus challenge in a mouse model of nasal infection,

suggesting that intranasal vaccination with poly(I:C) adjuvant confers cross-protection against variant viruses. Although the systemic antigen-specific T-cell responses were induced by intranasal vaccination with poly(I:C) adjuvant, T-cell responses against heterologous influenza viruses were weak. Moreover, TLR3, which is a receptor for dsRNA in nasal-associated lymphoid tissue (NALT), was upregulated at the level of mRNA expression upon intranasal administration of a split vaccine and poly(I:C).<sup>7</sup> Recently, a clinically safe dsRNA, PolyI:PolyC<sub>12</sub>U (Ampligen<sup>®</sup>), was investigated as a dsRNA adjuvant for intranasal avian flu vaccines.

To evaluate the adjuvant effect of Ampligen<sup>®</sup>, the protective effect of intranasal administration of vaccine and Ampligen<sup>®</sup> adjuvant against homologous (A/Vietnam) and heterologous (A/Hong Kong and A/Indonesia) H5N1 influenza virus challenge was examined<sup>44</sup> (Figure 2). Two groups of mice were immunized either intranasally or subcutaneously with 1 µg of vaccine from Vietnam strain and 10 µg of Ampligen<sup>®</sup>, then challenged by intranasal administration of 1000 PFU of H5N1 influenza virus at 2 weeks after the final immunization. A third group of control mice was immunized intranasally with 10 µg of Ampligen<sup>®</sup> alone. In response to homologous viral challenge, all the mice immunized intranasally with vaccine and Ampligen<sup>®</sup> completely cleared viruses in their nasal cavity. By contrast, significantly higher levels of virus in nasal wash were detected in mice immunized subcutaneously with vaccine and Ampligen<sup>®</sup>. All mice of both groups survived after homologous A/Vietnam/1194/2004 viral challenge. In the heterologous virus challenge group, virus titers in nasal wash of intranasal vaccination group were significantly lower than in the subcutaneous vaccination group after A/Hong Kong or A/Indonesia viral challenge. Consequently, although intranasally immunized mice survived a potentially lethal infection with A/Hong Kong or A/Indonesia viruses, most influenza-challenged mice died (Figure 2). These results clearly indicated that intranasal administration of H5N1 vaccine and Ampligen<sup>®</sup> adjuvant is more effective than subcutaneous vaccination against homologous and heterologous H5N1 influenza virus challenge.

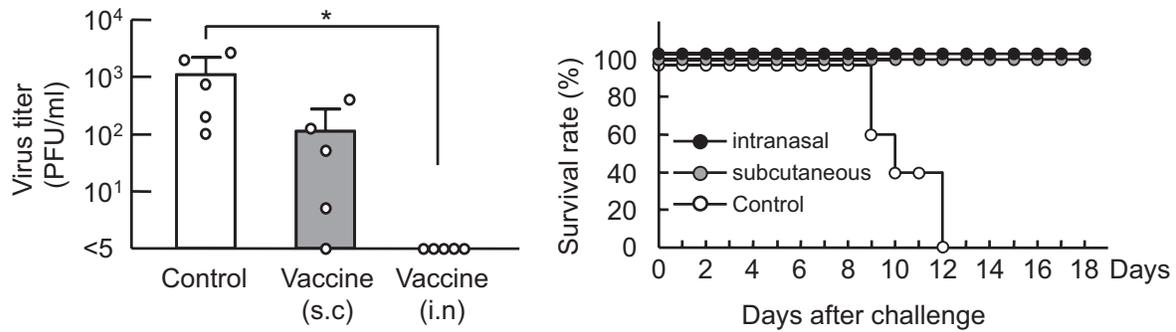
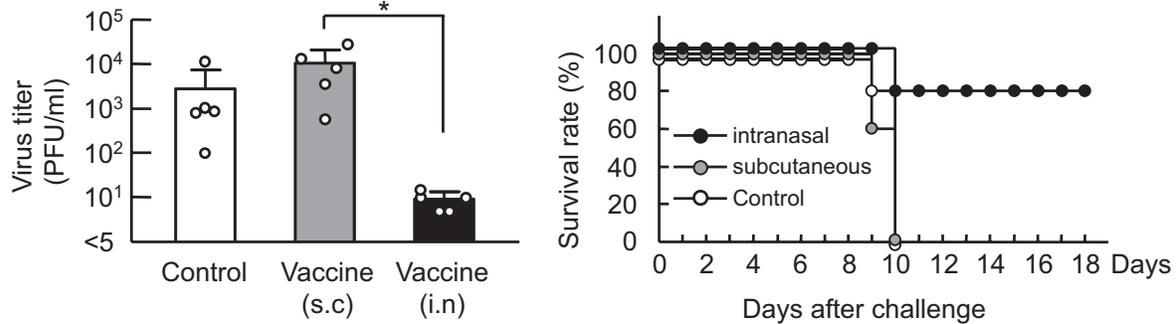
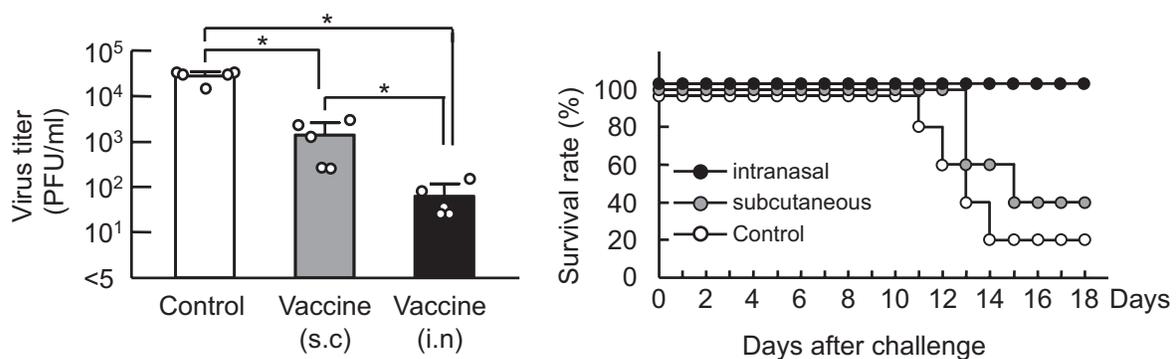
BALB/c mice were immunized three times intranasally or subcutaneously with trivalent inactivated influenza vaccine licensed in Japan for the 2005–2006 season.<sup>45</sup> The vaccine included A/New Caledonia/20/99 (H1N1), A/New York/55/2004 (H3N2), and B/Shanghai/361/2002 viral strains and was administered with PolyI:PolyC<sub>12</sub>U (Ampligen<sup>®</sup>) as an adjuvant. The immunized mice were challenged with A/Hong Kong, A/Vietnam, or A/Indonesia

H5N1 influenza viruses 2 weeks after the final immunization. Mice immunized intranasally manifested cross-reactivity of mucosal IgA and serum IgG with H5N1 virus as well as a reduced H5N1 viral titer in nasal wash, and their survival was higher after H5N1 virus challenge compared with nonimmunized animals. Subcutaneous immunization did not induce a cross-reactive IgA response and did not afford protection against H5N1 viral infection. These results suggest that intranasal immunization with annual influenza vaccine may overcome the problem of a limited supply of H5N1 virus vaccine by providing cross-protective mucosal immunity in humans against H5N1 viruses with pandemic potential.

## Cross-protection by other vaccines

Parenteral inactivated vaccines, including split-product, subunit vaccines and whole virion vaccines, induce mainly serum IgG antibodies that are weakly cross-protective against drift viruses within a subtype. These IgG antibodies would be effective against an epidemic of homologous virus, but would rarely be effective against an epidemic caused by a heterologous virus. Thus, an inactivated parenteral vaccine can effectively protect against an epidemic caused by a homologous virus, but would be relatively ineffective against an epidemic caused by a heterologous virus.

On the other hand, a cold-adapted, live-attenuated virus vaccine licensed in Russia and in the USA<sup>46–48</sup> appeared to mimic the natural course of infection, and provided cross-protective immunity against different subtypes of viruses by inducing secretory (S)-IgA antibodies, serum IgG antibodies, and a CTL response.<sup>49,50</sup> The advantage of live viral vaccines is that they induce not only mucosal IgA and serum IgG antibody responses, but also CTL responses, and confer cross-protection against different subtypes of influenza virus. Current cold-adapted (ca) live-attenuated influenza virus vaccines are growth-restricted to the upper RT. Using reverse genetics, a live attenuated vaccine was generated that encodes a modified form of H5 HA and wild-type-type N1 neuraminidase from influenza A virus strain H5N1, with the remaining gene segments derived from the cold-adapted (CA) influenza A vaccine donor strain. This vaccine was immunogenic in mice.<sup>51</sup> Four weeks after receiving a single intranasally administered dose of CA vaccine, mice were fully protected from lethal challenge with homologous and antigenically distinct, heterologous wt strain H5N1 viruses from different genetic sublineages.<sup>51</sup> Because live attenuated vaccine can induce immune responses equivalent to those induced by natural infection, a live vaccine that has no side

**A A/Vietnam/1194/2004****B A/Hong Kong/483/97****C A/Indonesia/6/2005**

**Figure 2** H5N1 virus titers in nasal washes and survival rates after lethal challenge with homologous A/Vietnam, heterologous A/Hong Kong, or heterologous A/Indonesia viruses. Mice were immunized intranasally (solid bar) or subcutaneously (gray bar) with vaccine and Ampligen<sup>®</sup>, then challenged by intranasal administration of 1000 PFU of A/Vietnam (A), A/Hong Kong (B), or A/Indonesia (C) virus 14 days after the final immunization. Nasal washes were collected three days post infection (d.p.i), and virus titers were measured by plaque assay. Each bar represents the mean  $\pm$  SD of five mice and open circles indicate individual animals. For statistical analysis, virus titers were compared to those from control mice (open bar) that received intranasal administration of 10  $\mu$ g of Ampligen<sup>®</sup> alone. Survival rates were monitored for 18 days. Copyright © 2007. Reproduced with permission from Ichinohe T, Kawaguchi A, Tamura S, et al. Intranasal immunization with H5N1 vaccine plus Poly I:Poly C12U, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge. *Microbes Infect.* 2007; 9:1333–1340.

**Note:** \* $p < 0.05$ .

effects would be good candidate of pandemic vaccine, if it could be produced.

## Conclusion

Now that a pandemic of new influenza virus seems possible, and because it will be difficult to know when a pandemic will occur or which strain of virus will be the cause, it is in our best interests to develop broadly effective and safe vaccines against the influenza virus. For the development of

a broadly effective vaccine, induction of mucosal immunity is an inevitable requirement, as mucosal secretory IgA plays an important role in cross-protection. Vaccines designed to induce mucosal immunity are necessary to combat a new influenza pandemic. As stated above, one of the requirements for inducing mucosal immunity is administration of the vaccine at mucosal sites, such as the nasal mucosa. For this reason, intranasal administration of inactivated vaccine plus adjuvant, or live attenuated vaccines, are promising candidates for

inducing cross-protective immunity against variant influenza viruses. However, for safety reasons, the ideal vaccine for induction of cross-protective mucosal immunity may be an inactivated vaccine. Recently, several candidate adjuvants that are effective in mucosal vaccine administration have emerged, including dsRNA (Ampligen®),<sup>7,8</sup> CMPs, SMPs,<sup>30</sup> and mutant CT.<sup>52</sup> These mucosal adjuvants represent promising approaches to the development of safe and effective vaccines for a potential influenza pandemic.

## Acknowledgments

The authors wish to express appreciation to all participants in their work cited in this review. We thank Dr. Wilina Lim at Department of Health, The government of Hong Kong for supplying A/Hong Kong/483/97 virus and A/Vietnam/1194/2004 virus, and Dr. Le Mai Thi Quynh at National Institute of Hygiene and Epidemiology, Vietnam for supplying A/Vietnam/1194/2004 virus, Dr. Triono Soendoro at National Institute of Health Research and Development, Ministry of Health Republic of Indonesia for supplying A/Indonesia 6/2005 virus, and Dr. John Wood at NIBSC for providing NIBRG-14 virus. We thank Dr. Tashiro M, and Dr. Odagiri T for helpful discussion. We are grateful to Dr. W Carter and D Strayer (Hemisphere, Biopharma, Philadelphia, PA) for supplying Ampligen®. This work was supported by grants from the Ministry of Health, Labor, and Welfare.

## References

- Uiprasertkul M, Puthavathana P, Sangsiriwut K, et al. Influenza A H5N1 replication sites in humans. *Emerg Infect Dis.* 2005;11(7):1036–1041.
- Ungchusak K, Auewarakul P, Dowell SF, et al. Probable person-to-person transmission of avian influenza A (H5N1). *N Engl J Med.* 2005;352(4):333–340.
- Renger KB, Small PA Jr. Passive immunization: systemic and mucosal. In: Ogra LL, Lamm ME, McGhee JR, Mestecky J, Strober W, Bienenstock J, editors. *Handbook of Mucosal Immunology*. San Diego, CA: Academic Press; 1994:347–356.
- Chen Z, Sahashi Y, Matsuo K, et al. Comparison of the ability of viral protein-expressing plasmid DNAs to protect against influenza. *Vaccine.* 1998;16(16):1544–1549.
- Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science.* 2004;303(5663):1529–1531.
- Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature.* 2006;441(7089):101–105.
- Ichinohe T, Watanabe I, Ito S, et al. Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. *J Virol.* 2005;79(5):2910–2919.
- Asahi-Ozaki Y, Itamura S, Ichinohe T, et al. Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. *Microbes Infect.* 2006;8(12–13):2706–2714.
- Sloat BR, Cui Z. Nasal immunization with a dual antigen anthrax vaccine induced strong mucosal and systemic immune responses against toxins and bacilli. *Vaccine.* 2006;24(40–41):6405–6413.
- Couch RB, Kasel JA. Immunity to influenza in man. *Annu Rev Microbiol.* 1983;37:529–549.
- Hoskins TW, Davies JR, Smith AJ, Allchin A, Miller CL, Pollock TM. Influenza at Christ's Hospital: March, 1974. *Lancet.* 1976;1(7951):105–108.
- Hoskins TW, Davies JR, Smith AJ, Miller CL, Allchin A. Assessment of inactivated influenza-A vaccine after three outbreaks of influenza A at Christ's Hospital. *Lancet.* 1979;1(8106):33–35.
- Asanuma H, Thompson AH, Iwasaki T, et al. Isolation and characterization of mouse nasal-associated lymphoid tissue. *J Immunol Methods.* 1997;202(2):123–131.
- Brandtzaeg P. Overview of the mucosal immune system. *Curr Top Microbiol Immunol.* 1989;146:13–25.
- Brandtzaeg P, Baekkevold ES, Farstad IN, et al. Regional specialization in the mucosal immune system: what happens in the microcompartments? *Immunol Today.* 1999;20(3):141–151.
- Kuper CF, Koornstra PJ, Hameleers DM, et al. The role of nasopharyngeal lymphoid tissue. *Immunol Today.* 1992;13(6):219–224.
- McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H. The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine.* 1992;10(2):75–88.
- Tamura SI, Asanuma H, Ito Y, et al. Superior cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza hemagglutinin vaccine. *Eur J Immunol.* 1992;22(2):477–481.
- Tamura S, Ito Y, Asanuma H, et al. Cross-protection against influenza virus infection afforded by trivalent inactivated vaccines inoculated intranasally with cholera toxin B subunit. *J Immunol.* 1992;149(3):981–988.
- Mutsch M, Zhou W, Rhodes P, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N Engl J Med.* 2004;350(9):896–903.
- Suhadolnik RJ, Reichenbach NL, Hitzges P, et al. Changes in the 2–5A synthetase/RNase L antiviral pathway in a controlled clinical trial with poly(I)-poly(C12U) in chronic fatigue syndrome. *In Vivo.* 1994;8(4):599–604.
- Suhadolnik RJ, Reichenbach NL, Hitzges P, et al. Upregulation of the 2–5A synthetase/RNase L antiviral pathway associated with chronic fatigue syndrome. *Clin Infect Dis.* 1994;18(Suppl 1):S96–104.
- Thompson KA, Strayer DR, Salvato PD, et al. Results of a double-blind placebo-controlled study of the double-stranded RNA drug poly(I):poly(C12U) in the treatment of HIV infection. *Eur J Clin Microbiol Infect Dis.* 1996;15(7):580–587.
- Adams M, Navabi H, Jasani B, et al. Dendritic cell (DC) based therapy for cervical cancer: use of DC pulsed with tumour lysate and matured with a novel synthetic clinically non-toxic double stranded RNA analogue poly [I]:poly [C(12U)] (Ampligen R). *Vaccine.* 2003;21(7–8):787–790.
- Subbarao K, Klimov A, Katz J, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science.* 1998;279(5349):393–396.
- Horimoto T, Fukuda N, Iwatsuki-Horimoto K, et al. Antigenic differences between H5N1 human influenza viruses isolated in 1997 and 2003. *J Vet Med Sci.* 2004;66(3):303–305.
- Liew FY, Russell SM, Appleyard G, Brand CM, Beale J. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. *Eur J Immunol.* 1984;14(4):350–356.
- Asahi Y, Yoshikawa T, Watanabe I, et al. Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. *J Immunol.* 2002;168(6):2930–2938.
- Hasegawa H, Ichinohe T, Strong P, et al. Protection against influenza virus infection by intranasal administration of hemagglutinin vaccine with chitin microparticles as an adjuvant. *J Med Virol.* 2005;75(1):130–136.

30. Ichinohe T, Watanabe I, Tao E, et al. Protection against influenza virus infection by intranasal vaccine with surf clam microparticles (SMP) as an adjuvant. *J Med Virol.* 2006;78(7):954–963.
31. Nicholson KG, Colegate AE, Podda A, et al. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. *Lancet.* 2001;357(9272):1937–1943.
32. Hoelscher MA, Garg S, Bangari DS, et al. Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. *Lancet.* 2006;367(9509):475–481.
33. Takada A, Kuboki N, Okazaki K, et al. Avirulent Avian influenza virus as a vaccine strain against a potential human pandemic. *J Virol.* 1999;73(10):8303–8307.
34. Tumpey TM, Renshaw M, Clements JD, Katz JM. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. *J Virol.* 2001;75(11):5141–5150.
35. Greenbaum E, Engelhard D, Levy R, Schlezinger M, Morag A, Zakay-Rones Z. Mucosal (SIgA) and serum (IgG) immunologic responses in young adults following intranasal administration of one or two doses of inactivated, trivalent anti-influenza vaccine. *Vaccine.* 2004;22(20):2566–2577.
36. Greenbaum E, Furst A, Kiderman A, et al. Serum and mucosal immunologic responses in children following the administration of a new inactivated intranasal anti-influenza vaccine. *J Med Virol.* 2001;65(1):178–184.
37. Kuno-Sakai H, Kimura M, Ohta K, Shimojima R, Oh Y, Fukumi H. Developments in mucosal influenza virus vaccines. *Vaccine.* 1994;12(14):1303–1310.
38. Muszkat M, Greenbaum E, Ben-Yehuda A, et al. Local and systemic immune response in nursing-home elderly following intranasal or intramuscular immunization with inactivated influenza vaccine. *Vaccine.* 2003;21(11–12):1180–1186.
39. Muszkat M, Yehuda AB, Schein MH, et al. Local and systemic immune response in community-dwelling elderly after intranasal or intramuscular immunization with inactivated influenza vaccine. *J Med Virol.* 2000;61(1):100–106.
40. Lund JM, Alexopoulou L, Sato A, et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A.* 2004;101(15):5598–5603.
41. Jacobs BL, Langland JO. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. *Virology.* 1996;219(2):339–349.
42. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature.* 2001;413(6857):732–738.
43. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells *in vivo*. *Immunity.* 2001;14(4):461–470.
44. Ichinohe T, Kawaguchi A, Tamura S, et al. Intranasal immunization with H5N1 vaccine plus Poly I:Poly C12U, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge. *Microbes Infect.* 2007;9(11):1333–1340.
45. Ichinohe T, Tamura S, Kawaguchi A, et al. Cross-protection against H5N1 influenza virus infection is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine. *J Infect Dis.* 2007;196(9):1313–1320.
46. Ghendon Y. Cold-adapted, live influenza vaccines developed in Russia. In: Nicholson KG, Webster RG, Hay AJ, editors. *Textbook of Influenza*. Boston, MA: Blackwell Science; 1998:391–399.
47. Keitel WA, Piedra PA. Live cold-adapted, reassortant influenza vaccines (USA). In: Nicholson KG, Webster RG, Hay AJ, editor. *Textbook of Influenza*. Boston, MA: Blackwell Science; 1998:373–390.
48. Maassab HF. Adaptation and growth characteristics of influenza virus at 25 degrees C. *Nature.* 1967;213(76):612–614.
49. Murphy BR. Mucosal immunity to viruses. In: Ogra LL, Lamm ME, McGhee JR, Mestecky J, Strober W, Bienenstock J, editors. *Handbook of Mucosal Immunology*. San Diego, CA: Academic Press; 1994.
50. Murphy BR, Clements ML. The systemic and mucosal immune response of humans to influenza A virus. *Curr Top Microbiol Immunol.* 1989;146:107–116.
51. Suguitan AL, McAuliffe J, Mills KL, et al. Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. *PLoS Med.* 2006;3(9):e360.
52. Yoshino N, Lu FX, Fujihashi K, et al. A novel adjuvant for mucosal immunity to HIV-1 gp120 in nonhuman primates. *J Immunol.* 2004;173(11):6850–6857.