Influenza

Influenza is a contagious acute respiratory disease caused by infection of the upper respiratory tract by influenza virus. Influenza viruses belong to the Influenza A genus of the family *Orthomyxoviridae*. The genome of influenza virus consists of eight single-stranded RNA segments (*HA*, *NA*, *M*, *NP*, *NS*, *PA*, *PB1* and *PB2*) that encode 11 proteins. Annual epidemics of influenza occur when the antigenic properties of the viral surface protein hemagglutinin (HA) and neuraminidase (NA) are altered. HA is one of the major viral surface glycoproteins and is involved in the binding of the virus to sialic acid on the surface of susceptible cells.[22] NA is also a viral surface glycoprotein that cleaves terminal sialic acid residues from carbohydrate moieties on the surface of infected cells, promoting the release of progeny viruses. Influenza A viruses can be divided into subtypes on the basis of genetic and antigenic differences in their HA and NA proteins (HA, H1–H16; and NA, N1–N9, respectively). Two mechanisms underlie this altered antigenicity: antigenic shift, caused by genetic rearrangement between human and animal viruses after double infection of host cells (which can result in a pandemic strain); and antigenic drift, caused by small changes in HA and NA on the virus surface (which can generate epidemic strains).[9] The emergence of variant virus strains by these two mechanisms is the cause of influenza epidemics. By contrast, the genes that encode the internal viral proteins are highly conserved among influenza A viruses.

Among the 11 proteins encoded by influenza virus, HA and NA are the central targets of protective humoral immune responses. For example, studies have demonstrated that passive transfer of antibodies to the conserved M1 or NP proteins fails to confer protective immunity, while a DNA vaccine encoding NA and HA is protective.[23,24] Other studies have shown that the expression of HA and NA in vivo from plasmid-based vaccines is more protective than plasmid-based expression of internal viral proteins.[23] Thus, HA and NA are important target antigens in developing an effective influenza vaccine.

Innate Immunity Against Influenza Virus Infection

In natural influenza virus infections, the upper respiratory tract is the initial site of infection. The invading virus is removed primarily by multiple nonspecific mechanisms (such as the mucin layer, ciliary action and protease inhibitors), which can prevent effective viral attachment to epithelial cells and viral uncoating.[9] Influenza virus that succeeds in passing through this primitive defense line is recognized by the innate immune system. The innate immune system consists of several classes of pattern-recognition receptors, including Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors. Genomic viral single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) produced during viral replication have been implicated as the molecular signals that trigger Type-I interferon (IFN) production. Following virion endocytosis by plasmacytoid dendritic cells (pDCs), virus genomic RNAs are recognized in endosomal/lysosomal compartments by TLR7 coupled with the cytosolic adaptor molecule MyD88.[25,26] The TLR7 signaling pathway activates interferon regulatory factor (IRF) 7, resulting in the production of vast amounts of IFNα/β.[27,28] dsRNAs produced during viral replication are recognized by TLR3, which is expressed in human alveolar and bronchial epithelial cells and in conventional dendritic cells.[29-31] TLR3 signaling via the cytosolic Toll/IL-1 receptor domain-containing adaptor (TRIF) protein activates IRF3, resulting in the production of IFNβ.[32-34] In addition, these signaling pathways induce the production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and Interleukin-6 (IL-6), through activation of the transcription factor NFκB. It is well known that the immune system has evolved redundant mechanisms for innate viral recognition. In contrast to the TLRs, which recognize viral nucleic acids in the endosomal/lysosomal compartments, the RLRs detect viral replication within the cytosol of infected cells. Two cytoplasmic helicases, retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), were initially identified as sensors that can recognize the synthetic dsRNA polyinosine-polycytidylic acid (poly[I:C]).[35-37] RIG-I and MDA5 are expressed in both immune cells and non-immune cells (such as fibroblasts) and induce type-I IFN production via IFNβ promoter stimulator-1 (IPS-1), which is localized on the surface of mitochondria.[38] RIG-I is essential for the production of type-1 IFNs in response to RNA viruses, including paramyxovirus, influenza virus and Japanese encephalitis virus, whereas MDA5 is critical for the detection of picornavirus.[39] Moreover, it has been shown that RIG-I detects 5′-triphosphate ssRNA, such as that which composes the influenza virus genome,[40,41] and short (~1 kb) dsRNAs.[42] By contrast, MDA5 preferentially detects longer (>2 kb) dsRNAs, like poly(I:C).[42]

Another type of cytoplasmic immune sensor for RNA is the NLR. Recently, it was shown that one member of the NOD-like receptor family, pyrin domain containing 3 (NLRP3), is activated following influenza virus infection.[43-46] NLRP3 forms a complex, collectively called an inflammasome, with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1. Activation of Caspase-1 through NLRP3 and ASC is required to convert pro-IL-1β, pro-IL-18 and pro-IL33 into mature cytokines. IL-1β and IL-18 are potent pro-inflammatory cytokines, and IL-33 promotes immune responses mediated by Th2 cells. During influenza virus infection, the activation of the inflammasome is modulated by the pH change in the Golgi apparatus that occurs due to the influenza M2 protein, which functions as a proton-selective ion channel.[44] Interestingly, it was revealed that the NLRP3 inflammasome is stimulated by aluminum hydroxide gel, which is an approved adjuvant for a several vaccines.[47,48]

Recognition of viral infection by the innate immune system bridges the transition between the innate and adaptive immune responses. This is a particularly important aspect of innate immunity, especially in the context of vaccine development. These findings and observations have significant implications for the design of effective adjuvants.

Mucosal Immunity

Generally, antigen-presenting cells (APCs), including the macrophages and DCs described above, are essential in the induction of the adaptive immune response.[49,50] Exogenous viral antigens, such as intact viruses, inactive viral particles, infected cells and apoptotic cells, are taken up by APCs through endocytosis and provide a potential source of peptides that can bind to the major histocompatibility complex (MHC) class I or II molecules in the APCs.[51] Naïve $CD4^+$ T lymphocytes that recognize peptides presented by MHC class II molecules differentiate to Th1 or Th2 lymphocytes in response to particular cytokines (IL-12 and IFNγ or IL-4, respectively). Th1 cells secrete IFNγ and IL-2, which help induce IgG2a antibody (Ab) production by B cells and also enhance the proliferation of $CD8^+$ cytotoxic T lymphocytes (CTLs),[52-54] while Th2 cells secrete IL-4 and IL-5, which help induce IgA, IgG1 and IgE Ab production by B cells.[52] The Abs produced during these processes contribute to viral neutralization (NT) by binding viral antigens. CTLs recognize the antigenic peptides presented by MHC class I molecules on virus-infected epithelial cells and destroy these infected cells, mainly by releasing perforin and granzyme.[55-57]

In adaptive immunity, the mucosal immune system plays an important role in preventing influenza virus infection in the upper respiratory system. In addition, secretory IgA (s-IgA) and IgG Abs and $CD8^+$ CTLs are involved in recovery from influenza following viral infection in naïve mice. The most notable benefit of the mucosal immune system is the presence of cross-reactive s-IgA Abs, which play a fundamental role in mucosal immunity. IgA-producing B cells disseminate to the lamina propria underneath the mucosal epithelium via general circulation and are responsible for the s-IgA Ab production in mucosal secretions. Dimeric IgA Abs are joined by a J-chain and bind to a polymeric Ig receptor (pIgR) on the basolateral surface of the epithelial cells. The antibodies are then carried to the apical surface by transcytosis where they combine with the extracellular region of pIgR, a secretory component that is cleaved by a specific protease to generate s-IgA.[58,59] Blocking dimeric IgA transcytosis in pIgR-knockout mice immunized intranasally with an adjuvant-combined influenza vaccine resulted in a failure to protect against challenge with a heterologous influenza virus.[60,61] In addition, dimeric IgA can inhibit viral assembly within epithelial cells during Ab transcytosis.[59,62]

While parenteral vaccination with inactivated virus can induce immune responses, natural influenza virus infection is superior in generating cross-protection against infection by variant viruses that have mutations within HA, which compose a particular subtype of the A-type virus in humans.[63-65] Parenteral vaccination with inactivated virus can induce systemic IgG Ab responses but not s-IgA Ab in the mucosal area, in contrast to natural influenza virus infection. This result highlights the importance of cross-protective s-IgA Abs. Mucosal immune responses are induced in mucosal-associated lymphoid tissues. Subsequent infection then induces a secondary IgA Ab response in the local lymphoid tissue. This T cell-dependent induction of an IgA response to influenza virus infection can be generated in the absence of cognate interaction between $CD4^+$ T cells and B cells through MHC class II.[66] Thus, s-IgA Abs are involved primarily in the prevention of influenza virus infection in the upper respiratory tract.

Vaccines

Currently available vaccines. The vaccines currently approved for human use are divided largely into two groups: the parenteral inactivated vaccines, which include split-products, subunit vaccines and whole-virion vaccines; and the live attenuated virus vaccines. Subcutaneous administration of inactivated influenza vaccines is an effective strategy in an epidemic caused by a homologous virus, as these vaccines induce specific serum IgG Abs. However, this would be less effective in an epidemic caused by a heterologous virus.[63-65,67] A trivalent inactivated influenza vaccine containing ether disrupted split-viruses is approved for use in individuals ≥6 months old, including those with chronic medical conditions.[68] Recently, Koyama et al. demonstrated that a formalin-inactivated whole virus vaccine was more immunogenic than an ether-split vaccine.[69] Although the ether-split vaccine had no effect on the innate immune system, which is required for the induction of adaptive immunity, the formalin-inactivated whole virus vaccine was able to activate pDCs but only through TLR7. These results suggest that, by itself, the split-product has a very weak immunogenicity.

Among the live attenuated vaccines, one cold-adapted, live attenuated influenza virus (LAIV) vaccine (Flumist®; MedImmune, Gaithersburg, USA) was licensed in the USA in 2003. This vaccine can mimic the natural course of influenza virus infection and provides cross-protective immunity against variant types of viruses by inducing not only s-IgA and IgG Abs, but also CTL responses.[64,65,70] Currently, this LAIV vaccine is licensed for use among healthy individuals aged 2 to 49 years, but is not approved for use in those that are immunodeficient or for pregnant women. Safety has not been established in persons with underlying medical conditions that confer a higher risk of influenza complications.[68] Unfortunately, the major high-risk groups for influenza complications, namely infants and the elderly, are therefore unable to obtain this vaccine. Thus, although intranasal administration of a LAIV vaccine has the advantage of inducting a protective immune response against influenza virus infection, its usage has been limited due to safety concerns.

Development of adjuvant-combined intranasal influenza vaccines. A large number of studies have shown that a majority of the protective immunity induced by influenza virus infection is due to s-IgA and IgG Abs in the respiratory tract. S-IgA Abs are carried to the mucus by transepithelial transport, and serum IgG Abs are transported from the serum to the mucus by diffusion.[58,60,61,64,65] In the upper respiratory tract, s-IgA Abs prevent viral infection, while IgG Abs support s-IgA-mediated protection by neutralizing newly synthesized viruses.[71,72] In the lung, IgG Abs play a dominant role in protection.[71-74] As described above, intranasal administration of a LAIV vaccine is better able to induce cross-protective immunity against influenza virus infection, including the production of cross-reactive s-IgA Abs at the mucosal surface, systemic IgG Abs and CTLs. However, this

vaccine has some limitations in its clinical application. Intranasal administration of an adjuvant-combined inactivated vaccine may overcome these limitations. To date, there have been many attempts to develop an inactivated vaccine that can be intranasally administered. Since inactivated vaccine alone is insufficient to elicit proper immune responses at the mucosal surface due to its weak immunogenicity, adjuvant is required to enhance the induction of these immune responses. An adjuvant is a compound that can promote and modulate the immunogenicity of a given vaccine.

Cholera toxin B subunit containing a trace amount of whole toxin (0.1%) (CTB*) has been used as an adjuvant to enhance mucosal immune responses following intranasal vaccination. In studies, this adjuvant has induced effective cross-protection in the upper respiratory tract against variants (drift viruses) within the influenza A virus subtype and against variants of the B viruses.[75-78] The strong cross-protection observed in the upper respiratory tract was provided mainly by s-IgA Abs, whereas the weak cross-protection documented in the lower respiratory tract was provided by IgG Abs.[77-79] Although CTB* is an effective adjuvant for producing s-IgA Abs, it also has some side effects, including nasal discharge. Studies suggest that alternate nasal influenza vaccine adjutants, such as *Escherichia coli* heat-labile enterotoxin (LT), which is structurally and functionally similar to cholera toxin (CT), may also be clinically unsafe. An intranasal virosomal vaccine adjuvanted with LT has been linked to several cases of transient Bell's palsy (facial paralysis).[80] Therefore, many attempts have been made to identify an alternative adjuvant for intranasally administered inactivated vaccines. Other candidates include synthetic dsRNAs,[81-83] chitin or surf clam microparticles,[84,85] and natural killer T-cell-specific glycolipid ligand.[86]

Intranasal administration of an inactivated ether-split vaccine combined with synthetic dsRNA poly(I:C), an agonist of TLR3 and MDA5,[31,42] confers effective cross-protection in the upper respiratory tract against variants of influenza A- or B-type viruses.[81] Several viruses produce dsRNA during replication. Thus, synthetic dsRNA probably acts as molecular mimic of viral infection. Upon recognition of dsRNA, mammalian TLR3 induces the production of pro-inflammatory cytokines[31] and IFNβ,[32-34] that, in turn, enhance the primary Ab response to subcutaneous immunized soluble material.[87] In mice, intranasal administration of an ether-split vaccine from A/PR8 (influenza virus strain H1N1) together with a poly(I:C) adjuvant induced strong anti-HA s-IgA and IgG Ab responses in nasal wash and serum samples, respectively. In addition, intranasal administration of a vaccine for either A/Beijing (H1N1) or A/Yamagata (H1N1) and poly(I:C) conferred complete protection against A/PR8 virus challenge in a mouse model of nasal infection, suggesting that intranasal vaccination combined with poly(I:C) adjuvant confers cross-protection against variant viruses. Although 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 mRNA was upregulated upon intranasal administration of a split vaccine and poly(I:C) in nasopharyngeal-associated lymphoid tissue.[81] However, it has been reported that poly(I:C) induces a number of side effects in humans, including renal failure and hypersensitivity.[88] The most promising mucosal adjuvant candidate is PolyI:PolyC_{12}U (Ampligen®), a analogue of poly(I:C) that contains uridine residues, resulting in unique "mismatched" dsRNAs capable of undergoing accelerated hydrolysis.[89] The safety of Ampligen® has been tested in clinical trials, including a double-blind, placebo-controlled Phase III clinical trail for chronic fatigue syndrome.[89-91]

The adjuvant effect of Ampligen® was evaluated by testing the protection induced by intranasal administration of an inactivated vaccine (A/Vietnam; H5N1) together with Ampligen® against homologous and heterologous (A/Hong Kong and A/Indonesia) H5N1 influenza virus challenge (**Fig. 1**).[83] Two groups of mice were immunized either intranasally or subcutaneously with 1 μg of A/Vietnam vaccine and 10 μg of Ampligen® and then challenged by intranasal administration of 1,000 PFU of H5N1 influenza virus two weeks after the final immunization. A third group of control mice were immunized intranasally with 10 μg of Ampligen® alone. All of the mice immunized intranasally with vaccine and Ampligen® completely cleared virus in the nasal cavity following homologous virus challenge. By contrast, significantly higher levels of virus were detected in nasal wash samples collected from mice immunized subcutaneously with vaccine and Ampligen®. All mice of both groups survived after homologous A/Vietnam viral challenge. In the heterologous virus challenge group (A/Hong Kong or A/Indonesia infection), virus titers in the nasal wash of the intranasal vaccination group were significantly lower than those in the subcutaneous vaccination group. Although intranasally immunized mice survived a potentially lethal infection with A/Hong Kong or A/Indonesia viruses, most subcutaneously vaccinated, influenza virus-challenged mice died (**Fig. 1**). These results clearly indicate that intranasal administration of a H5N1 vaccine together with Ampligen® adjuvant is more effective than subcutaneous vaccination against homologous and heterologous H5N1 influenza virus challenge.

In a separate study, mice were immunized three times intranasally or subcutaneously with a trivalent inactivated influenza vaccine licensed in Japan for the 2005–2006 season together with Ampligen®. The vaccine included A/New Caledonia (H1N1), A/New York (H3N2) and B/Shanghai viral strains. The immunized mice were challenged with A/Vietnam, A/Hong Kong or A/Indonesia H5N1 influenza viruses two weeks after the final immunization. Mice immunized intranasally generated cross-reactive mucosal IgA and serum IgG Abs, showed reduced levels of H5N1 in nasal wash samples, and demonstrated higher survival after H5N1 virus challenge compared to non-immunized animals. Subcutaneous vaccination failed to induce a cross-protective IgA Ab response and did not afford protection against lethal H5N1 viral infection.

The mucosal adjuvant activity of Ampligen® has also been demonstrated in non-human primates (cynomolgus macaques).[92] Intranasal vaccination with an inactivated A/Vietnam vaccine combined with Ampligen® induced vaccine-specific IgA Ab responses in the saliva and IgG Ab responses

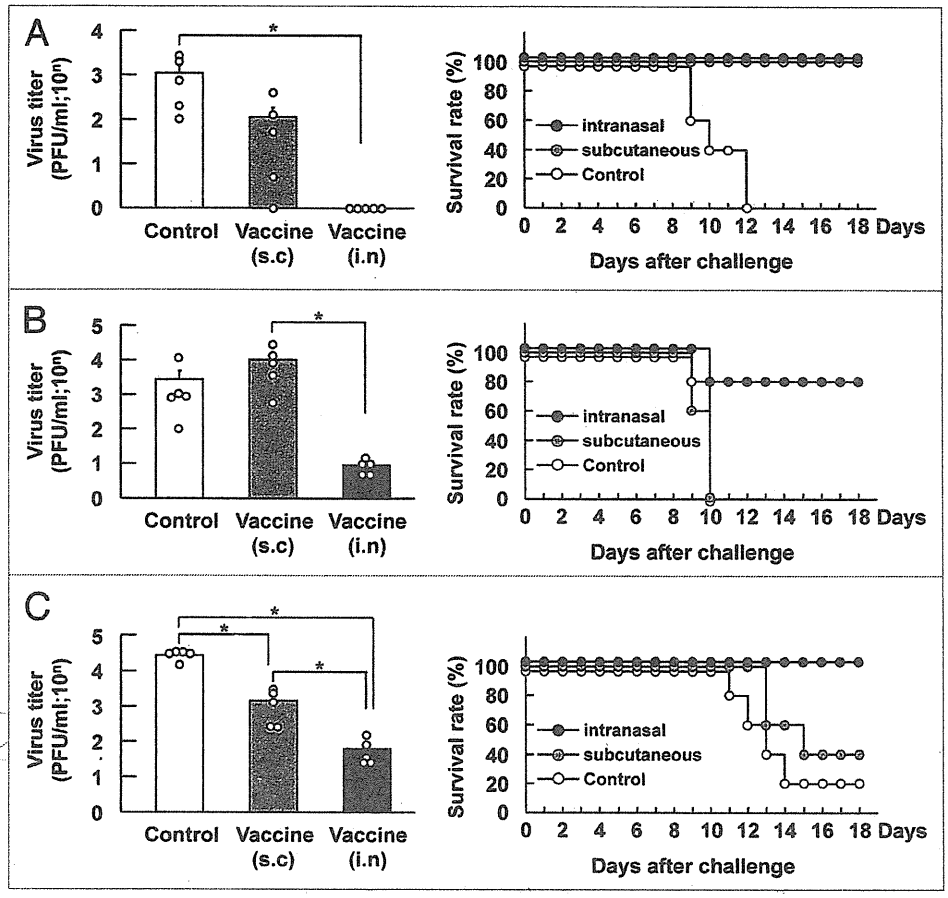

Figure 1. H5N1 virus titers in nasal wash samples, and the survival rates after lethal challenge with homologous A/Vietnam, heterologous A/Hong Kong or heterologous A/Indonesia viruses. Mice were immunized intranasally (black bar) or subcutaneously (gray bar) with vaccine and Ampligen® and then challenged by intranasal administration of 1,000 PFU of A/Vietnam (A), A/Hong Kong (B) or A/Indonesia (C) virus 14 days after the final immunization. Nasal wash samples were collected 3 days post-infection, and virus titers were measured by plaque assay. Each bar represents the mean ± SD from five mice, and the open circles indicate individual animals. For statistical analysis, viral titers were compared to those from control mice (open bar) that received intranasal administration of 10 μg of Ampligen® alone. Survival rates were monitored for 18 days. *$p < 0.05$. 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; 9:1333–40.

in serum and succeeded in reducing viral titer after homologous virus challenge. Vaccine-specific Ab responses induced by intranasal immunization persisted at least for one year and were sufficient to protect monkeys from homologous virus challenge one year after immunization (Ainai A, et al., unpublished data). These results suggest that the intranasal administration of an inactivated vaccine together with the synthetic dsRNAs, poly(I:C) and Ampligen®, induces long-lasting s-IgA and IgG Ab responses at mucosal surfaces and in serum, respectively, and that the mucosal immunity induced by intranasal vaccination is sufficient to prevent homologous and heterologous virus infection.

Enhancement of mucosal adjuvant activity. For large scale or global application of an effective vaccine, it is desirable to induce strong immune responses using a small vaccine dose due to the normally limited amount of commercially produced vaccines. In an attempt to increase the mucosal immune responses, zymosan was combined with poly(I:C) as a mucosal adjuvant for intranasal immunization of an ether-split A/PR8 vaccine.[93] Nasal IgA and serum IgG Ab responses in mice immunized intranasally with vaccine and both poly(I:C) and zymosan were significantly increased relative to the responses in mice intranasally vaccinated only with vaccine and poly(I:C) (Fig. 2). Zymosan, a cell wall extract from *S. cerevisiae* that contains β-glucan (55%), mannan, protein, lipid, chitin and an unknown TLR2 ligand, has been used for over 50 years as a model microorganism to investigate phagocytosis and the inflammatory response both in vivo and in vitro.[94-96] β-glucan is recognized by Dectin-1, a C-type lectin that induces phagocytosis of microbial agents by DCs or macrophages.[97-99] Dectin-1 signaling is modulated by spleen tyrosine kinase (Syk) and promotes NFκB activation.[100,101] Zymosan alone co-activates Dectin-1/Syk and TLR2/MyD88 signaling pathways, resulting in a synergistic increase in the production of IL-12 and TNFα.[95] In addition, simultaneous or sequential stimulation

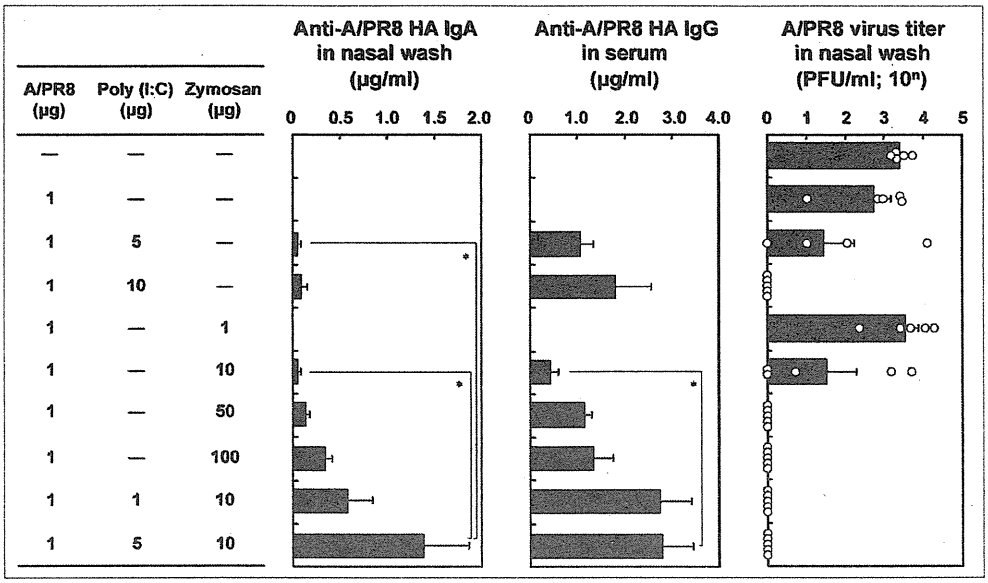

Figure 2. Figure 2. Enhancement of antibody responses and reduction of viral titers in mice vaccinated intranasally with a A/PR8 vaccine combined with both poly(I:C) and zymosan. BALB/c mice were immunized intranasally on days 0 and 21 with an A/PR8 HA vaccine (1 μg) in the presence of poly(I:C) (1, 5, 10 μg) or zymosan (1, 10, 50, or 100 μg) alone, or with both adjuvants. Two weeks after the second immunization, mice were subjected to challenge by intranasal infection with A/PR8 virus in the nasal cavity (1,000 PFU, 2 μl into each nostril). Nasal wash and serum specimens were collected 3 days after virus challenge. A/PR8 HA-specific antibodies in nasal wash and serum specimens were measured by ELISA. Viral titers in the specimens were determined by plaque assay. Bars represent the mean ± standard error. Open circles indicate values for individual mice. * $P < 0.05$. The data shown has been already published, and was reformatted for this review. Reproduced with permission from Ainai A et. al. Zymosan enhances the mucosal adjuvant activity of poly(I:C) in a nasal influenza vaccine. J Med Virol 2010; 82:476-84.

of MyD88-dependent and TRIF-dependent signaling pathways by their respective ligands induces a synergistic increase in the production of TNFα, IL-6 and IFNβ.[102] Reflecting these mechanisms, vaccine uptake and the production of pro-inflammatory cytokines and type-I IFN (IFNβ) were significantly increased by zymosan and poly(I:C) coadministration in cultures of bone marrow-derived DCs in vitro.[93] These immunological events were likely responsible for the significant induction of s-IgA and IgG that were observed in nasal wash and serum samples, respectively. Moreover, intranasal immunization with a A/PR8 vaccine together with both poly(I:C) and zymosan effectively reduced viral titer after challenge with the pandemic A(H1N1) 2009 virus (Fig. 3), even though the antigenicity of the HA molecule of this virus is largely different from that of seasonal influenza viruses.[1-3] These results suggest that using a combination of ligands on different innate immune sensors as adjuvants synergistically induces higher amounts of cross-protective Abs following intranasal vaccination against influenza virus.

Conclusions

In 2009, we experienced the first pandemic in the 21st century, and it was induced by an unpredicted strain of influenza virus. In light of the fact that it is difficult to know when a pandemic will occur or which strain of virus will cause it, it is in our best interest to develop broadly effective and safe vaccines against influenza virus. A broadly effective vaccine requires the induction of mucosal immunity, as mucosal s-IgA plays an important role in cross-protection. Vaccines designed to induce mucosal immunity are necessary for mitigating any new influenza pandemic. As described in this review, one of the requirements of inducing mucosal immunity is the administration of the vaccine at mucosal sites, such as the nasal mucosa. For this reason, intranasal administration of an inactivated vaccine plus adjuvants and live attenuated vaccines are promising candidates for inducing cross-protective immunity against variant influenza viruses. However, for safety reasons, the ideal candidate for the induction of cross-protective mucosal immunity may be an inactivated vaccine. Our studies revealed that the synthetic dsRNAs, poly(I:C) and Ampligen®, possess mucosal adjuvant activity following intranasal administration of an inactivated vaccine. These synthetic dsRNAs are sensed by TLR3 and MDA5 in DCs, which have a key role in orchestrating the innate immune system and adaptive immunity. S-IgA Abs induced at the mucosal surface by intranasal immunization with a vaccine plus Ampligen® were sufficient to reduce virus titers in nasal wash samples following homologous or heterologous virus infection. Moreover, the production of cross-protective s-IgA Abs in the nasal wash was synergistically enhanced by the addition of another pathogen recognition receptor ligand adjuvant in addition to dsRNAs. Thus, intranasal administration of an inactivated vaccine combined with mucosal adjuvants represents a promising approach for the development of safe and effective vaccines that may limit the impact of a potential influenza pandemic.

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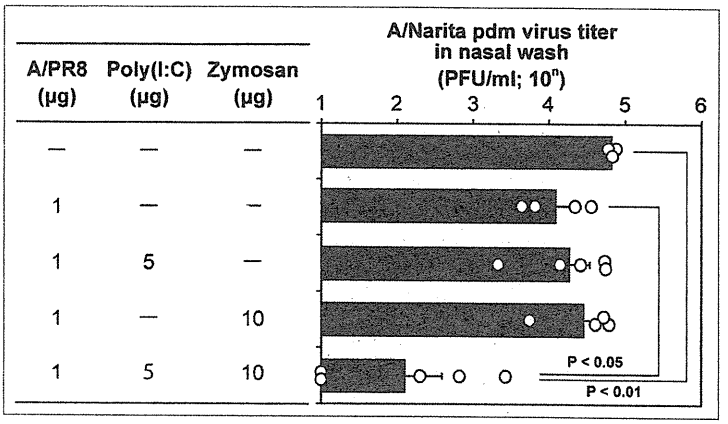

Figure 3. Figure 3. Reduction of pandemic H1N1 influenza virus titers in mice vaccinated intranasally with a A/PR8 vaccine combined with both poly(I:C) and zymosan. BALB/c mice were immunized intranasally on days 0 and 21 with an A/PR8 HA vaccine (1 µg) in the presence of poly(I:C) (5 µg) or zymosan (10 µg) alone, or with both adjuvants. Two weeks after the second immunization, mice were subjected to challenge by intranasal infection with A/Narita pdm virus [pandemic A(H1N1) 2009 virus strain] in the nasal cavity (1,000 PFU, 2 µl into each nostril). Nasal wash specimens were collected 3 days after virus challenge. Viral titers in the specimens were determined by plaque assay. Bars represent the mean ± standard error. Open circles indicate values for individual mice. * P < 0.05.

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Meeting Report

Improving influenza vaccine virus selection Report of a WHO informal consultation held at WHO headquarters, Geneva, Switzerland, 14–16 June 2010

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Executive summary

- For almost 60 years, the WHO Global Influenza Surveillance and Response System (GISRS) has been the key player in monitoring the evolution and spread of influenza viruses and recommending the strains to be used in human influenza vaccines. The GISRS has also worked to continually monitor and assess the risk posed by potential pandemic viruses and to guide appropriate public health responses.
- The expanded and enhanced role of the GISRS following the adoption of the International Health Regulations (2005), recognition of the continuing threat posed by avian H5N1 and the aftermath of the 2009 H1N1 pandemic provide an opportune time to critically review the process by which influenza vaccine viruses are selected. In addition to identifying potential areas for improvement, such a review will also help to promote greater appreciation by the wider influenza and policy-making community of the complexity of influenza vaccine virus selection.
- The selection process is highly coordinated and involves continual year-round integration of virological data and epidemiological information by National Influenza Centres (NICs), thorough antigenic and genetic characterization of viruses by WHO Collaborating Centres (WHOCCs) as part of selecting suitable candidate vaccine viruses, and the preparation of suitable reassortants and corresponding reagents for vaccine standardization by WHO Essential Regulatory Laboratories (ERLs).
- Ensuring the optimal effectiveness of vaccines has been assisted in recent years by advances in molecular diagnosis and the availability of more extensive genetic sequence data. However, there remain a number of challenging constraints including variations in the assays used, the possibility of complications resulting from non-antigenic changes, the limited availability of suitable vaccine viruses and the requirement for recommendations to be made up to a year in advance of the peak of influenza season because of production constraints.
- Effective collaboration and coordination between human and animal influenza networks is increasingly recognized as an essential requirement for the improved integration of data on animal and human viruses, the identification of unusual influenza A viruses infecting human, the evaluation of pandemic risk and the selection of candidate viruses for pandemic vaccines.
- Training workshops, assessments and donations have led to significant increases in trained laboratory personnel and equipment with resulting expansion in both geographical surveillance coverage and in the capacities of NICs and other laboratories. This has resulted in a significant increase in the volume of information reported to WHO on the spread, intensity and impact of influenza. In addition, initiatives such as the WHO Shipment

Fund Project have facilitated the timely sharing of clinical specimens and virus isolates and contributed to a more comprehensive understanding of the global distribution and temporal circulation of different viruses. It will be important to sustain and build upon the gains made in these and other areas.

- Although the haemagglutination inhibition (HAI) assay is likely to remain the assay of choice for the antigenic characterization of viruses in the foreseeable future, alternative assays – for example based upon advanced recombinant DNA and protein technologies – may be more adaptable to automation. Other technologies such as microtitre neuraminidase inhibition assays may also have significant implications for both vaccine virus selection and vaccine development.
- Microneutralization assays provide an important adjunct to the HAI assay in virus antigenic characterization. Improvements in the use and potential automation of such assays should facilitate large-scale serological studies, while other advanced techniques such as epitope mapping should allow for a more accurate assessment of the quality of a protective immune response and aid the development of additional criteria for measuring immunity.
- Standardized seroepidemiological surveys to assess the impact of influenza in a population could help to establish well-characterized banks of age-stratified representative sera as a national, regional and global resource, while providing direct evidence of the specific benefits of vaccination.
- Advances in high-throughput genetic sequencing coupled with advanced bioinformatics tools, together with more X-ray crystallographic data, should accelerate understanding of the genetic and phenotypic changes that underlie virus evolution and more specifically help to predict the influence of amino acid changes on virus antigenicity.
- Complex mathematical modelling techniques are increasingly being used to gain insights into the evolution and epidemiology of influenza viruses. However, their value in predicting the timing and nature of future antigenic and genetic changes is likely to be limited at present. The application of simpler non-mechanistic statistical algorithms, such as those already used as the basis of antigenic cartography, and phylogenetic modelling are more likely to be useful in facilitating vaccine virus selection and in aiding assessment of the pandemic potential of avian and other animal influenza viruses.
- The adoption of alternative vaccine technologies – such as live-attenuated, quadrivalent or non-HA-based vaccines – has significant implications for vaccine virus selection, as well as for vaccine regulatory and manufacturing processes. Recent collaboration between the GISRS and vaccine manufacturers has resulted in the increased availability of egg isolates and high-growth reassortants for vaccine production, the development of qualified cell cultures and the investigation of alternative methods of vaccine potency testing. WHO will continue to support these and other efforts to increase the reliability and timeliness of the global influenza vaccine supply.
- The WHO GISRS and its partners are continually working to identify improvements, harness new technologies and strengthen and sustain collaboration. WHO will continue in its central role of coordinating worldwide expertise to meet the increasing public health need for influenza vaccines and will support efforts to improve the vaccine virus selection process, including through the convening of periodic international consultations.

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Introduction

The historic initiative to establish a global network to detect and identify new and potentially dangerous influenza viruses predates the adoption of the WHO Constitution in 1948. With memories of the 1918–1919 influenza pandemic still vivid, and the ever-evolving threat posed by influenza recognized, the WHO Global Influenza Surveillance and Response System (GISRS)† was formally established in 1952. Influenza thus became one of the first diseases to highlight the importance of international monitoring and collaboration in protecting human health.

Following the re-emergence of human cases of highly pathogenic avian H5N1 influenza in 2003 and the adoption of the International Health Regulations (2005), the GISRS was strengthened and its role in protecting public health enhanced. In addition to tracking the course and impact of annual influenza epidemics and monitoring the evolution of seasonal influenza viruses, the GISRS also acts as a global alert mechanism for the emergence of influenza viruses with the potential to cause a human pandemic. The Network provides support to both seasonal and pandemic influenza preparedness and response activities in areas such as diagnostics, vaccine development, virological surveillance and risk assessment. It also acts as the focus of WHO efforts to

† Former WHO Global Influenza Surveillance Network (GISN), which has been renamed as WHO Global Influenza Surveillance and Response System (GISRS) since 24 May 2011, when the World Health Assembly Resolution WHA 64.5 was adopted.

assist Member States in strengthening their national capacity for the surveillance, diagnosis, characterization and sharing of influenza viruses.

As a key player in global influenza risk assessment and response, the GISRS continues to evolve and expand, and as of December 2010 consisted of 135 National Influenza Centres (NICs) in 105 countries, six WHO Collaborating Centres (WHOCCs), 11 WHO H5 Reference Laboratories and four WHO Essential Regulatory Laboratories (ERLs). The GISRS also works to ensure the successful coordination of WHO activities with those of external agencies such as the Global Outbreak Alert and Response Network (GOARN), national regulatory authorities, academic and veterinary institutes, and the pharmaceutical industry.

The first formal WHO recommendations on influenza vaccine composition were issued in 1971. Since 1998, separate and appropriately timed recommendations for the Northern and Southern Hemispheres have been issued each year in February and September, respectively. These biannual recommendations are based upon the virological and epidemiological information generated by the GISRS and play a crucial role in the development, production and availability of effective influenza vaccines.

The continuing threat posed by avian H5N1, the aftermath of the 2009 H1N1 pandemic, the increased knowledge of influenza, and the development and availability of new technologies provide a timely opportunity to review the complex processes and issues involved in influenza vaccine virus selection and to identify potential areas for improvement. This WHO informal consultation represents the latest step in an ongoing process of GISRS strengthening and was convened with the following objectives:

- to review the current vaccine virus selection process, including its constraints and limitations;
- to identify opportunities for improving influenza surveillance and representative virus sharing;
- to assess the potential for improving the assays and technologies used for vaccine virus selection; and
- to assess the potential impact of new vaccine technologies on the vaccine virus selection process.

Participants were drawn from a broad and highly diverse range of institutes and sectors including the following: WHOCCs, NICs, WHO ERLs, WHO H5 Reference Laboratories, national regulatory authorities, public health agencies, academia, influenza vaccine manufacturers, and veterinary laboratories and organizations.

The GISRS vaccine virus selection process

The primary goal of the GISRS vaccine virus selection process (Annex 1) is to generate and analyse the data needed to recommend the influenza vaccine viruses that will most closely match the influenza viruses likely to be circulating during forthcoming influenza seasons. Current vaccine technologies and production schedules mean that decisions on vaccine composition have to be made almost a full year in advance of the peak of seasonal influenza activity. As a result, the process relies upon the earliest possible detection of emerging antigenic variants and the most up-to-date information on their potential future epidemiological significance. Information must therefore be collected year round on the continuous evolution and global circulation of human influenza viruses to provide a sound basis for the biannual WHO recommendations on the composition of influenza vaccines for use in the Northern and Southern Hemispheres. For countries in equatorial regions, epidemiological considerations influence which recommendation (February or September) individual national and regional authorities consider more appropriate.

Role of National Influenza Centres

National Influenza Centres (NICs) play a vital role in this complex process. Their core activities include collating epidemiological information, diagnosing cases of influenza A and B infection, and identifying the subtype or lineage of the viruses responsible. The primarily molecular diagnosis of infection using RT-PCR techniques is based upon standardized primers and probes provided by the GISRS. Viruses must also be isolated to allow their antigenic identification using the type- and subtype-specific reference reagents provided in annually distributed WHO kits. Further detailed characterization may include sequence analyses to monitor genetic changes and assessment of virological traits such as resistance to antiviral drugs. Sequence data are shared within the GISRS using public databases such as GenBank and GISAID EpiFlu. NICs in some settings then attempt to relate potentially important virological changes observed with clinical and epidemiological information and trends and may even conduct serological studies to evaluate the immune status of the population.

Weekly reports on the virological characteristics and epidemiology of circulating viruses are submitted to the WHO FluNet – an internet-based data-query and reporting tool. Information on the virus subtypes and lineages is collated, together with observations of potential clinical or epidemiological importance, and regular summaries of the geographical spread, intensity and impact of influenza are produced by WHO.

If human infection with an avian or other animal influenza virus is suspected, a suitably equipped NIC or other national influenza reference laboratory can conduct preliminary diagnostic testing using RT-PCR protocols and/or reagents for H5, H7 and H9 subtypes provided by WHO. Such RT-PCR testing does not require high-level biocontainment facilities. However, it is expected that the detection of any unusual influenza A virus distinct from known

circulating viruses, especially one suspected to be of animal origin or unsubtypable using current WHO reagents, will immediately be reported to WHO and collaboration urgently initiated with a WHO Collaborating Centre (WHOCC). If the required laboratory biosafety facilities and procedures are not available, then virus isolation should not be attempted in the national laboratory and the sample should be promptly sent to a WHOCC.

Role of WHO Collaborating Centres

The routine and timely sharing of representative circulating influenza viruses and unusual viruses with a WHOCC is an essential step in the vaccine virus selection process. The criteria for forwarding viruses include their temporal, geographical and age-group distribution, severity of cases and virological characteristics such as unidentified subtype and antiviral drug resistance. WHOCCs are then responsible for the systematic antigenic characterization of the thousands of viruses forwarded each year by NICs and other laboratories, and for the detailed genetic characterization of a selected subset. Such detailed antigenic and genetic characterization is a necessary step in monitoring virus evolution and detecting any distinct antigenic variants that may necessitate updating the seasonal vaccine composition. The process also allows for the identification and characterization of animal viruses causing sporadic human infections, assessment of the risk they pose and the potential development of candidate vaccine viruses as part of pandemic preparedness.

Antigenic characterization

Of prime importance in immunity to influenza is the production of antibodies to the virus haemagglutinin (HA) protein. Such antibodies can neutralize the infectivity of viruses, and their level in the blood has been shown to correlate with the level of protection against infection with a homologous virus. As a result, influenza vaccine virus selection has primarily been based upon the antigenic characterization of virus HA using the haemagglutination inhibition (HAI) assay. HAI tests provide a visual readout of the ability of specific antibodies to prevent the attachment of HA to red blood cells (RBCs) and thus prevent their agglutination. Antigenic drift in the HA of circulating viruses in response to host immunity reduces the effectiveness of vaccines and is therefore the major consideration when recommendations are made on the composition of influenza vaccines.

The HAI test is likely to remain the assay of choice for the antigenic characterization of virus HA for the foreseeable future. Strain-specific antisera are produced by infecting previously unexposed ('naive') ferrets with either vaccine viruses, reference viruses representative of circulating viruses or viruses that appear in HAI tests to be potential antigenic variants. The resulting sets of reference viruses and antisera are then used to evaluate the antigenic characteristics of the HAs of recent isolates. Where antigenic differences are detected, these are likely to affect human immunity against the new variants. The HAI test is a surrogate for the more complicated and time-consuming virus neutralization assay used to clarify antigenic relationships when observed variations in HAI titre reflect, for example, changes in receptor binding rather than differences in antigenicity.

Genetic characterization

A subset of between 10% and 20% of all viruses received is selected for genetic sequencing and more detailed analysis – principally of their HA and NA components. This subset is selected to include representative circulating viruses, as well as apparent antigenic variants and viruses from severe or fatal cases. Phylogenetic analyses are carried out to better understand the evolution of circulating viruses, their degree of genetic heterogeneity and the emergence of new genetic clades. Antigenic or other phenotypic variants may thus be defined in terms of separate genetic clades with distinct amino acid signatures. Relating the locations of amino acid substitutions to antigenic, receptor-binding or glycosylation sites on the 3D structure of the HA molecule then helps to identify the individual substitutions associated with phenotypic (antigenic) changes. Identifying such amino acid signatures also facilitates global monitoring of the emergence, distribution and impact of different genetic variants. This is particularly helpful when data on emergent variants are limited at the time of a WHO vaccine consultation. Comparisons of the sequences found in clinical specimens and virus isolates are also useful in revealing amino acid substitutions which result from passage in different substrates, mainly MDCK cells and eggs. Up-to-date sequence data are shared within GISRS and made publically available via the GISAID EpiFlu database.

Complete genome sequencing is necessary to identify animal (including avian) viruses causing human infection and is important in detecting the emergence of reassortant viruses among co-circulating human viruses or between human and animal viruses. WHOCCs maintain panels of reference reagents for all influenza A subtypes. These include H5 (especially H5N1), H9 and H7 avian viruses and various H1N1 and H3N2 swine viruses, as well as viruses present in other animals such as horses and dogs.

Studies using human sera

WHOCCs also collaborate with the WHO ERLs in serological studies of representative human sera from previously vaccinated individuals. Sera are provided by vaccine manufacturers and are used in HAI tests to assess whether or not the antibodies induced by current vaccines are likely to be effective against currently circulating viruses. The results

provide important supplementary evidence for vaccine composition decisions.

WHO recommendations on influenza vaccine composition

The principal criteria used to decide whether or not to recommend changes to influenza vaccine components include:

- the emergence of an antigenically and genetically distinct variant among circulating viruses (including a novel influenza A virus with the potential to cause a pandemic);
- evidence of the geographical spread of such a distinct variant and its association with outbreaks of disease, indicating its future epidemiological significance;
- the reduced ability of existing vaccine-induced antibodies to neutralize the emergent variant; and
- the availability of suitable candidate vaccine viruses.

To facilitate collaborative studies by the WHOCCs and WHO ERLs and ensure that appropriate potential candidate vaccine viruses are identified in advance of the WHO vaccine composition consultation, the most recent virological and epidemiological data are shared and discussed via teleconferences held 6 and 2 weeks before the WHO consultation. A summary of each teleconference is promptly distributed to keep all NICs and vaccine manufacturers informed of the developing situation. In addition, potential candidate vaccine viruses are provided to manufacturers.

During the formal biannual consultations, the technical advisory group considers the cumulative antigenic and genetic data on the viruses characterized by WHOCCs. The data are set against the broader epidemiological context collated by WHO and are supported by serological data from WHOCCs and WHO ERLs, as well as by additional information provided by NICs. HAI data obtained in the different centres using a wide variety of reference viruses and ferret antisera are correlated using common reference reagents. In recent years, antigenic cartography has been used to collate and statistically visualize the degree of antigenic variation. The interpretation of HAI data may, however, be complicated by the influence of changes in the receptor-binding properties of natural viruses or by the selection of variants during isolation and passaging in different cell or egg substrates. Comparisons with sequence data are made to relate any differences in antigenicity with specific HA genetic clades and to more precisely define the identity of antigenic variants. The results of virus neutralization tests, which usually correspond to those of HAI tests, are used to clarify the true antigenic relationships between different viruses.

If the antigenic data, supported by genetic and serological data, indicate that a new antigenic variant is spreading globally, then a change in that component of the seasonal vaccine is considered to be warranted. The implementation of a recommendation to update a vaccine component is, however, contingent upon the availability of suitable vaccine viruses. Only after all the factors have been taken into account is a decision taken on whether or not to recommend a change in influenza vaccine virus composition. The decision is announced at an Information Meeting immediately following each WHO consultation and published on the WHO web site and in the WHO Weekly Epidemiological Record.

Since the re-emergence of human cases of highly pathogenic H5N1 avian influenza in 2003, WHO has also regularly reviewed the available antigenic and genetic data on human and avian viruses in relation to the epidemiology of H5N1 influenza among birds. To support the development of safe and effective human H5N1 vaccines, WHO has coordinated the development of a number of candidate attenuated vaccine viruses (Annex 1) and made them available to vaccine producers. Clinical trials have been conducted to evaluate the immunogenicity of different H5N1 vaccine formulations and the breadth of antibody responses elicited. In addition, as part of pandemic preparedness, WHO has coordinated the ongoing development and updating of an inventory of H2, H7 and H9 candidate vaccine viruses.

Vaccine development considerations

Important constraints on the vaccine virus selection process include the tight timelines involved (Annex 1), particularly in the Northern Hemisphere, where since recent years seasonal influenza activity tends to start increasing in middle or late January in general. As a consequence, decisions often have to be made relatively early in the influenza season. In addition, post-infection ferret antisera against potential antigenic variants are urgently required to define their antigenic relationships to previously circulating viruses. Panels of recent isolates must also be prepared to assess the degree to which they are neutralized by antibodies in the sera of previously vaccinated individuals. Finally, potential new candidate vaccine viruses must be prepared and evaluated for their suitability in vaccine production. Ensuring the timely availability of viruses with suitable growth properties is a crucial step in ensuring that sufficient quantities of vaccine can be produced in time for administration prior to the next influenza season. Although cell culture has steadily replaced the use of embryonated eggs for the primary isolation of viruses, candidate vaccine viruses must still be isolated directly in eggs according to current regulatory requirements. The limited availability of egg isolates, particularly of recent H3N2 viruses which generally grow poorly in eggs, has led to the establishment of Cooperative Research and Development Agreements (CRADAs) and similar agreements between the vaccine industry and a number of WHOCCs to increase the availability of egg isolates for vaccine use.

The GISRS vaccine virus selection process necessarily involves a series of collaborative steps, including the selection of prototype antigenic variants and suitable vaccine viruses, and the provision of standardizing reagents by the WHO ERLs. The process thus impacts directly upon the subsequent authorizing of vaccine composition by national and regional regulatory authorities and upon the large-scale production of vaccine by manufacturers. Mismatches have occasionally occurred as a result of the emergence of variant strains shortly after the recommendations have been made, highlighting one of the unavoidable consequences of current vaccine development and production constraints. Nevertheless, retrospective studies have shown that with very few exceptions WHO vaccine virus recommendations have closely matched the influenza viruses that have circulated during the following influenza season. In addition, following the out-of-season emergence of the pandemic A(H1N1) 2009 virus, this closely integrated system demonstrated its unique ability to very rapidly orchestrate the development and provision of appropriate (suitably attenuated) candidate vaccine viruses for pandemic vaccine production.

Improving influenza surveillance and representative virus sharing

Global influenza surveillance has always presented a major challenge as it is a highly demanding public health need with a significantly uneven distribution of surveillance capacity worldwide. Since the outbreak of severe acute respiratory syndrome (SARS) in 2003, the re-emergence of H5N1 infection in humans and the 2009 H1N1 pandemic, it has become ever clearer that surveillance and the prompt sharing of viruses and information are central to the broad range of influenza preparedness and response activities.

Enhancing NIC surveillance capacity

Although the known impact and the awareness of seasonal influenza vary in different parts of the world, the threat posed by avian H5N1 viruses has galvanized influenza surveillance efforts in all countries. Improving surveillance and acquiring the capacity to detect and report unusual cases of influenza are essential components of global pandemic planning and are enshrined in the International Health Regulations (2005). Successful efforts to increase the capacity of NICs and other laboratories have been made, and in a number of settings the development, revision and adoption of guidelines on strengthened national, regional and global surveillance and collaboration is under way.

Global influenza surveillance has also been strengthened through expanded geographical coverage and the collection of more data of better quality. For example, in Africa there are now 25 influenza laboratories in 21 countries, including 12 recognized NICs, almost all of which have the capacity to conduct RT-PCR diagnosis of influenza infection. In less than two years, the percentage of African countries with an NIC increased from 17% to 26% with the number of countries with no influenza laboratory markedly decreasing.

Global, regional and national training workshops, assessments and donations have all led to significant increases in trained personnel, equipment procurement and laboratory capacity, resulting in the increasingly widespread use of molecular techniques such as real-time RT-PCR and genetic sequencing. Recent WHO capacity-building activities have included BSL-3 training courses for NICs to promote safe practices when working with highly pathogenic influenza viruses, and courses on virus isolation, gene sequencing and antiviral resistance detection. Increased participation in both internal and external quality assurance programmes such as the WHO external quality assessment project (EQAP) has contributed to marked improvements in laboratory proficiency.

These and other efforts enabled a more effective response to the emergence of the 2009 H1N1 pandemic in many countries. However, the pandemic also revealed significant limitations in the analysis and integration of epidemiological and virological surveillance data. In addition, few early seroprevalence surveys were conducted to allow for the timely assessment of the extent and impact of the pandemic. The pandemic also revealed significant gaps in laboratory infrastructure and personnel, equipment procurement and funding, particularly in developing countries. Improvements and training in areas such as web-based integration and analyses of clinical, epidemiological and virological data are being implemented but care must be taken to ensure that such activities are not conducted at the expense of detection, characterization and virus-sharing activities in less well-resourced settings.

Identified research priorities in influenza surveillance and response include evaluation of the temporal and geographical circulation of influenza viruses and of the burden of influenza. In all settings, establishing a sound evidence base will support the development or updating of national, regional and global policies, plans and guidelines. This in turn could lead to greater acceptance of the use of influenza vaccines, particularly seasonal vaccines, and assist in the development of vaccination policies.

Virus and information sharing

The primary requirement of NICs will remain the prompt diagnosis of influenza infection and the timely sharing of clinical specimens and virus isolates – especially those obtained from unusual, severe or fatal cases – backed up by appropriate epidemiological and clinical information. Procedures should be in place to ensure that the increasingly predominant use of molecular diagnostic techniques, particularly real-time RT-PCR, does not adversely affect the timely isolation and for-

warding of viruses. Improved communication between NICs and WHOCCs on how best to facilitate prompt virus sharing, including discussion of the constraints faced, could improve coordination and avoid potential delays.

A more systematic approach to engaging NIC information and expertise would also lead to significant benefits. Such an approach is likely to be facilitated by a number of developments in the use of WHO web-based tools. For example, NICs with enhanced capabilities currently strengthen the collaborative characterization of viruses and aid early assessment of the significance of genetic and antigenic changes by sharing detailed virological information (especially HA sequences) on selected viruses, either directly or via public databases. As technologies advance, national patterns of seropositivity to circulating influenza viruses may also become available on a more timely basis and could thus guide vaccine use. This is particularly important given the increasing emphasis now placed on assessing vaccine effectiveness. Comprehensive NIC summary reports forwarded just prior to each WHO consultation also provide highly beneficial additional data to inform WHO recommendations on vaccine composition.

To overcome logistical and other obstacles to the safe and efficient shipping of clinical specimens and virus isolates to WHOCCs, a WHO Shipment Fund Project was established. The project provides support to NICs and other influenza laboratories in all countries by arranging the transport of specimens and isolates along a guaranteed cold chain, especially in settings where there are severe financial and infrastructural constraints. As a direct result of the project, and associated 'infectious substances shipping' workshops conducted in all WHO regions, there has been a significant increase in the number of countries sharing specimens and isolates, especially following the outbreak of the 2009 H1N1 pandemic. Furthermore, the expansion and harmonization of the information currently provided in the accompanying standard shipping form to include information such as clinical outcome, patient vaccination status or recent travel history would greatly enhance understanding of the epidemiological context associated with the spread of viruses.

Animal viruses

A better understanding of the diversity and evolution of animal influenza viruses is essential for evaluating the pandemic risk posed by subtypes currently causing sporadic human infections (such as H5N1 and H9N2) and informing the selection of candidate vaccine viruses. The emergence of H5N1 in particular led to the establishment in 2005 of the OIE–FAO Network of Expertise on Animal Influenza (OFFLU) – a worldwide network of approximately 20 laboratories and institutions that coordinates the global surveillance of animal influenza. A number of joint WHO-OFFLU technical initiatives on influenza at the human–animal interface have been conducted (including successful collaboration during the 2009 H1N1 pandemic) and reciprocal participation in annual meetings has taken place. There remains, however, considerable scope for improved coordination and collaboration with the animal influenza surveillance sector, especially in the collection and analysis of antigenic and genetic data, the timely exchange of representative viruses and reference reagents, and the conducting of serological studies of human exposure to zoonotic infection.

Influenza is an important disease of many avian and mammalian species with serious economic consequences for livestock industries and has potential adverse impacts on human food supplies. Despite this, animal influenza surveillance coverage is limited with a shortage of epidemiological data on the circulation of various viruses in different countries. Efforts are now under way to establish triggers for initiating enhanced surveillance that go beyond animal disease notification and sporadic human infections. Although there is increasing understanding of the interrelationships between animal and human influenza and the need for 'integrated' surveillance, full collaboration at both national and global levels is currently constrained by a number of practical, funding, regulatory and policy issues. Maintaining a regular dialogue based upon the mutual interests of the different networks will be an important public health activity and may also help to enhance the sustainability of animal influenza surveillance in particular settings. A more formal collaborative mechanism might allow for the improved integration of animal virus data into the WHO candidate vaccine virus selection process. Increased awareness of the content and extent of use of animal influenza vaccines would also aid understanding of their impact on virus evolution.

Improving the process of vaccine virus selection

A range of laboratory assays and other techniques provide the complementary information on changes in the antigenic and genetic characteristics of influenza viruses needed to select the most appropriate influenza vaccine viruses. However, inherent limitations in the biological assays used and significant variations in the results obtained by different laboratories complicate the collation and definitive interpretation of data.

Assays for characterization of antigenic properties and antibody responses

Because the HAI test outlined previously is a simple, rapid and reproducible surrogate assay for virus neutralization, it is widely used to measure the antigenic relationships between different viruses as well as antibody responses to

infection or vaccination. In addition, the test provides the basis of the only current quantitative correlate of protection against infection (serum HAI antibody titre ≥40) used to standardize inactivated vaccines. However, variations in the physical characteristics of RBCs obtained from different species and differences in the receptor-binding properties of different viruses influence both the sensitivity and the utility of the assay. Furthermore, changes in receptor-binding affinity or specificity associated with adaptation, antigenic drift or the isolation and passage of viruses in eggs and cell culture may also affect HAI titres. Standardization between laboratories has also proved difficult, and the assay is currently not suitable for use in a fully automated system.

A range of practical refinements such as attempts to develop 'synthetic' RBCs (for example using glycan-coated beads) have been unsuccessful. Given the currently limited knowledge of the principal natural receptors for influenza viruses, such approaches are unlikely to circumvent the virus-dependent shortcomings of assays based upon natural RBCs which are therefore likely to remain the primary approach to antigenic characterization for the foreseeable future. Recent developments based on the use of panels of recombinant HA do offer alternative or supplementary microtitre or microarray binding-assay formats for assessing antibody specificity and antibody inhibition of the HA-glycan receptor interaction. Although such approaches are relatively expensive and require a high degree of skill to implement, they are potentially highly suited to automation and in time may reduce the need for virus isolates. In addition, such formats can readily be adapted to incorporate biosensor technologies to provide more quantitative analyses of binding characteristics. A number of such assays are currently being validated using ferret and human antisera.

The contribution of antibodies against virus NA in conferring protection following natural infection or vaccination is still not well understood. Studies of NA antigenic variation have been limited, and the NA content of influenza vaccines is not currently standardized. Although neuraminidase inhibition (NAI) assays were conducted more routinely in the past, these were cumbersome to perform and were complicated by the relatively low levels of antibodies against NA in post-infection ferret sera and by interference from antibodies against HA. A number of different NAI microtitre assay formats have recently been developed. These have been used to correlate antigenic changes with sequence variations in the NA component, provide more precise information on the evolution of NA and assess NA antibody responses following vaccination. Improved understanding of antigenic drift in NA and of the role of anti-NA antibodies in conferring immunity might have significant implications for both vaccine virus selection and vaccine development.

Microneutralization (MN) assays – based on measuring virus replication, cell viability or NA activity – provide an important adjunct to HAI tests in antigenic characterization. MN assays are generally more sensitive and measure a broader repertoire of functional antibodies that neutralize viral replication, with potential advantages in the evaluation of human serological responses. In addition, comparisons of MN and HAI tests for measuring antibody responses in vaccinated individuals have shown a consistent degree of correlation and have confirmed the utility of MN assays in analyses of human antibody responses to H3 vaccine components. Techniques for simplifying assay formats and making them more readily applicable to the routine testing of low-titre viruses are under investigation, and efforts are under way to use MN assays for H1 and B viruses. This should facilitate the use of MN assays to overcome the variable nature of interactions between viruses and RBCs, and hence in interpreting 'anomalous' HAI results which complicate vaccine virus selection. Pseudotype virus neutralization assays may also offer some advantages in scale and standardization over conventional MN assays for measuring serological responses to particular viruses, especially highly pathogenic viruses. Furthermore, ongoing improvements in automation will potentially enable the more labour-intensive MN assay to be applied to large-scale serological analysis. Epitope mapping using genome fragment phage display libraries provides another powerful technique for further dissecting the fine specificity of antibody responses to vaccination and infection and should allow for a better assessment of the quality of a 'protective' immune response and aid the development of additional correlates of immunity.

Serological studies

To encourage the performance of seroepidemiological surveys to assess the impact of influenza in a population, countries should be supported in establishing well-characterized serum banks of age-stratified representative sera as a national, regional and global resource. Current advantages of the GISRS serological activities undertaken in support of vaccine virus selection include the use of shared serum panels and common antigens, with frequent consensus obtained from participating WHOCCs and WHO ERLs. Limitations include the large variability of HAI data, a requirement for antibody standards and a need for MN or other assays to resolve inconsistencies. The availability of antibody standards would not only enhance the comparability of serological data generated in different laboratories and countries but also facilitate the comparison of antibody responses to different vaccines.

Increasing attention to influenza vaccine effectiveness studies will lead to the availability of more real-time data for comparing clinical benefit with the degree of antigenic

relatedness of vaccine and circulating viruses. Such studies, especially those based upon laboratory-confirmed outcomes, should provide evidence of the specific benefits of vaccination. Consistent studies providing estimates of vaccine efficacy over successive influenza seasons should improve understanding of the effects of small rather than major antigenic differences between vaccine and circulating viruses on clinical outcomes and should help to allay concerns arising from a perceived vaccine mismatch caused by the emergence of virus clades exhibiting little or no antigenic drift.

Technological developments

Recent advances in high-throughput genetic sequencing could potentially lead to a greatly enhanced understanding of the genetic changes occurring in influenza viruses and the evolutionary interactions that occur between co-circulating viruses. In-depth analyses of the precise mechanisms involved in the evolution and epidemiology of influenza would require advanced bioinformatics tools to comprehensively mine the data produced. Such an approach should reveal, for example, the broader genetic changes that underlie antigenic variation in HA and thus allow for a better understanding of the relationship between genetic evolution and antigenic drift. Increased information from X-ray crystallography on the structural features of the HAs of recent viruses and specific mutants, together with developments in computer modelling, should assist in attempts to predict the likely influence of amino acid substitutions on the antigenic and receptor-binding properties of new variants. Further development of high-throughput laboratory systems for integrated and automated genetic and phenotypic analyses – from initial sample accession to data management – offers the intriguing prospect of a futuristic standardized virtual network for virus characterization in an epidemiological context. As such systems will have broad implications, not only for vaccine virus selection, but also for the organization and conduct of global influenza surveillance, it is extremely important that their development and deployment are integrated with the activities of the WHO GISRS.

Mathematical modelling

Numerous mathematical modelling techniques have now been used to gain insights into the mechanisms that underlie both the evolution and the epidemiology of influenza viruses. For example, exploratory models have been developed to generate and test various hypotheses to explain the relatively restricted diversity of influenza viruses in terms of constrained antigenic repertoire, and to explore the underlying nature of immunity. They have also been used to improve understanding of the extent of between-subtype and between-type competition and of the potential consequences of such interactions for trends in the incidence of seasonal influenza viruses.

Phylogenetic models have also been used to identify changes in selective constraints in relation to antigenic drift and inter-species transmission. When based upon the amino acid substitutions associated with mammalian host adaptation, such models may aid assessment of the pandemic potential of avian and other animal viruses. Phylodynamic modelling based upon available sequence data, supplemented with antigenic data, has already been successfully used to trace the emergence of new antigenic and genetic variants and track their geographical spread.

However, in the absence of greatly improved understanding of the underlying evolutionary and biological mechanisms and other processes involved, the capacity of current mathematical modelling techniques to predict the timing and nature of future antigenic and genetic changes is limited. The intrinsically stochastic nature of influenza evolution may make such predictive modelling extremely challenging. Where changes occur over short time scales, the application of simpler non-mechanistic statistical algorithms, such as those used as the basis of antigenic cartography, is likely to be more useful in facilitating vaccine virus selection than attempts to develop predictive models from the existing complex dynamical models of influenza evolution and transmission. Such predictive models might presently be better suited for use in understanding the possible long-term effects of vaccination, optimizing the timing and location of focused surveillance efforts and predicting the possible consequences of the emergence of a novel virus. Eventually, these models should be able to take advantage of integrated immunological and antigenic surveillance data to develop predictions of short-term dynamics in specific locations.

Impact of new vaccine technologies

All new influenza vaccine technologies have implications for vaccine virus selection and for regulatory and manufacturing processes. However, any potential requirement to tailor the virus selection process to specific types of vaccine is unlikely to be a crucial issue, especially if advances in vaccine technology and speed of production lead to greater flexibility in the timing of recommendations. Although live-attenuated vaccines are not yet universally licensed, the current vaccine composition recommendation process is used. However, antibody response is not a good correlate of protection for such vaccines and the identification of a true correlate might affect the requirement for annual updating. Several quadrivalent vaccines are also now under development that contain representative strains of the two influenza B virus lineages (B/Victoria and B/Yamagata) together with influenza A(H1N1) and A(H3N2) viruses.

This raises a number of issues that could affect vaccine supply, including the possibility of two poorly growing vaccine viruses; the likely variable impact of a fourth component on vaccine yields and timing of manufacture; the prioritization of influenza B lineage viruses in the context of both trivalent and quadrivalent vaccine production; and the need for a fourth set of reagents. Adjuvanted vaccines have been licensed with the primary aims of inducing better immune responses in certain age groups and allowing 'antigen sparing'. Although there has been no specific intention to provide a broader spectrum of immunity to circumvent the need for annual vaccine updates, different products are likely to show a different breadth of response. Providing recommendations in relation to product-specific cross-reactivity over successive influenza seasons is unlikely to be a feasible option for the WHO GISRS. In addition, various types of recombinant vaccines are now under development, including protein subunit, DNA, vector and VLP vaccines – none of which are presently licensed.

In the case of non-HA-based vaccines, different guidelines will apply and all such vaccines are likely to impact the current vaccine virus selection process in various ways depending upon their precise type and mechanism of protection. The level of protection afforded by immunity to NA is receiving continued interest. Currently, this component is included as part of the candidate vaccine virus and is selected on the basis of its sequence but not antigenicity. Standardization of the NA component would require antigenic characterization during the virus selection process, while antigenic changes in NA in the absence of a corresponding change in HA antigenicity may on its own necessitate the updating of vaccine composition. For all such vaccines, HA variant selection may become less crucial than it is for current vaccines.

Although high-growth reassortants have been used to manufacture influenza A vaccine components for many years, their yields have been variable and there is continued need to identify the molecular determinants of high yield to engineer a more reliable and reproducible production process. Reverse genetics, now used in the United States to produce virus reassortants for live-attenuated vaccines, has also been used to produce attenuated candidate H5N1 vaccine viruses suitable for inactivated vaccine manufacture. This approach was, however, less successful than classical reassortment in obtaining a suitable 2009 H1N1 pandemic vaccine virus, emphasizing the need for further investigation of the applicability of reverse genetics in the routine provision of suitable vaccine viruses.

Following the licensing of cell culture vaccines, the feasibility of isolating seasonal vaccine viruses in qualified[1] cell lines is being evaluated in a collaboration involving a number of WHOCCs and WHO ERLs under CRADAs with vaccine manufacturers. These studies should provide the basis for the introduction of a universal qualified cell culture system for providing mammalian cell-derived seasonal influenza candidate vaccine viruses. This would result in a greater choice of candidates, especially for recent H3N2 viruses, and may provide greater flexibility in responding to the 'late' emergence of a variant necessitating a vaccine composition change. Such virus isolates would not be subject to undesirable egg-selected changes and would potentially provide a better match to the natural virus. However, the relative merits of egg and cell culture candidate vaccine viruses have still to be rigorously evaluated. Guidance on quality assurance aspects has already been published by the European Medicines Agency (EMA). The finalization of new EMA regulatory guidelines may be accompanied by a WHO technical document on harmonizing regulatory approaches worldwide and the engagement of other regulatory authorities in vaccine-manufacturing nations.

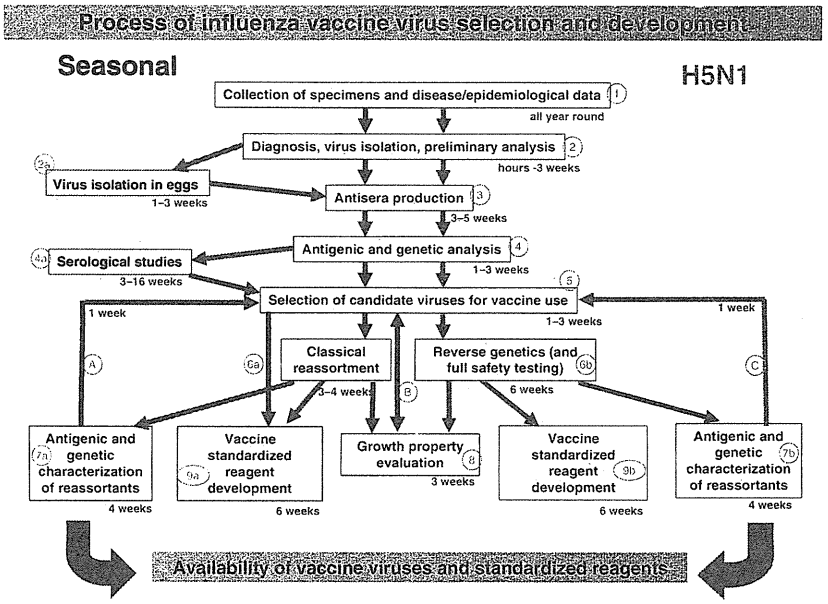
Vaccine manufacturers and the WHO ERLs are also collaborating in an evaluation of cell culture-based reagents for use in single radial immunodiffusion (SRID) potency testing, due for completion in early 2011. In addition, despite international consensus on the key quality specifications for 2009 H1N1 pandemic influenza vaccines, reagents to calibrate the majority of candidate vaccines using conventional potency tests only became available immediately prior to the initiation of clinical trials. In some cases, candidate vaccines were available ahead of the reagents. Although national authorities proved flexible in accepting the use of validated alternative potency tests to allow clinical trials to proceed, newer methods such as high-performance liquid chromatography (HPLC) and mass spectrometry are now being evaluated.

Conclusions and future perspective

The GISRS has a long history of success in recommending influenza vaccine compositions that have closely matched the combination of viruses circulating during subsequent influenza seasons. Based upon the voluntary participation of its many constituent partners, the GISRS enjoys strong institutional and governmental support.

Global influenza surveillance is the foundation of the vaccine virus selection process. Efforts to enhance and strengthen national, regional and global laboratory capacity for virological surveillance and representative virus sharing must continue. As part of this, improved integration of virological and disease surveillance data will be a key aim and will help to build the foundations for future studies of the impact and burden of influenza worldwide.

[1] Defined as cell lines accepted by regulatory authorities as suitable substrates for vaccine manufacture.



To strengthen the pandemic influenza preparedness, collaboration between the GISRS and veterinary laboratories and organizations such as OFFLU in relation to zoonotic influenza infection has been greatly enhanced and has included the development of appropriate candidate human vaccine viruses from animal viruses. However, there remains considerable scope for improvement in this area, including the more timely exchange of information, viruses and reagents, and strengthened technical collaboration at all levels.

Although antigenic characterization of promptly forwarded virus isolates will remain the central criterion for selecting influenza vaccine viruses in the foreseeable future, technological developments (such as advanced recombinant DNA and protein technologies, and high-throughput sequencing and advanced bio-informatics tools) will inevitably impact current GISRS surveillance and virus selection activities. In the interests of global public health, it will be important to integrate into the GISRS system appropriate information and data generated by various networks using emerging technologies.

Antigenic cartography has been adopted by the GISRS in recent years as a means of integrating HAI data from different laboratories to allow for statistical comparison and visual display. The development of new statistical algorithms to complement the use of antigenic cartography may further facilitate vaccine virus selection.

Greater emphasis should be placed on conducting human serological studies which incorporate the use of antibody standards to improve the comparability of results. Such studies would improve current understanding of the prevalence and spread of influenza, and complement the development of improved epidemiological models. Greater collaborative effort is needed to generate randomly sampled, representative and integrated serological, epidemiological and evolutionary data that provide snapshots of host and viral populations suitable for modelling hypotheses on virus evolution and host immunity. The application of advanced techniques for dissecting the fine specificity of antibody responses to vaccination and infection should also lead to improvements in understanding the quality of a 'protective' immune response and aid in the development of additional correlates of immunity.

Recent collaboration between the GISRS and external partners including academic institutions and vaccine manufacturers has resulted in the increased availability of egg isolates and high-growth reassortants. New approaches to the generation of high-growth vaccine viruses involving the use of reverse genetics and qualified cell cultures will continue to be evaluated and developed, as will alternative methods of vaccine potency testing. WHO will continue to support these and other efforts to increase the reliability and timeliness of global influenza vaccine supply.

New vaccine types currently under development may allow more flexibility in the timing of recommendations on vaccine virus composition. Conversely, alterations to the virus selection process and additional information may be needed in relation to new-generation vaccine types with different compositions and mechanisms of protection.

The WHO GISRS vaccine virus selection process lies at the heart of global efforts to address the constantly evolving threat posed by influenza. For decades, this highly collaborative and complex process has ensured a continued supply

of effective seasonal vaccines and was able to respond very rapidly to the emergence of the 2009 H1N1 pandemic. If the current limitations and constraints inherent in the process are to be overcome, ongoing efforts by the WHO GISRS and its partners must continue to identify improvements, harness new technologies and strengthen collaboration. WHO will continue in its central role of developing and coordinating worldwide expertise to meet the increasing public health need for influenza vaccines and will support this process through the convening of periodic international consultations on improving influenza vaccine virus selection.

Annex 1: Process of influenza vaccine virus selection and development[2]

The diagram shows that the individual steps in the selection of candidate vaccine viruses and development of standardizing reagents for seasonal influenza and for a potential H5N1 influenza pandemic are essentially equivalent. For seasonal vaccines *the timelines* are:

- Steps 1–4: the collection, isolation and thorough antigenic and genetic characterization of recent virus isolates continues *throughout the year*;
- Step 4a: comparisons of the recognition of representative recent viruses by vaccine-induced antibodies in human sera are conducted *2–3 weeks before the biannual WHO vaccine consultation meetings*;
- Steps 5, 6a and 7a: candidate viruses for vaccine use are reviewed and selected, and high-growth reassortants prepared and characterized *following identification of (potential) antigenic variants* – these steps are not solely dictated by the recommendations of the WHO biannual vaccine virus consultations.
- Step 8: Evaluation of their growth properties is conducted in a timely manner around the time of the WHO vaccine virus consultations and *prior to authorization of vaccine composition by national authorities.*
- Step 9a: Preparation of the standardizing reagents for new vaccine components is initiated *once the particular vaccine virus has been selected following the WHO recommendation.*

Annex 2: Declaration of interests

The WHO Informal Consultation for Improving Influenza Vaccine Virus Selection, 14–16 June 2010, was organized by the Virus Monitoring and Vaccine Support (VMV) Unit of WHO, with participation from WHO Collaborating Centres on influenza, ERLs, National Influenza Centres, national control laboratories, national regulatory authorities, academic and veterinary institutions, influenza vaccine manufacturers and other collaborating organizations.

In accordance with WHO policy, all members of the writing group that assisted WHO in the development of this meeting report had completed the WHO Declaration of Interests for WHO experts. These declarations were then evaluated by the WHO Secretariat prior to the consultation.

The members of the writing group declared the following personal current or recent (within the last 4 years) financial or other interests relevant to the subject area:

Institution	Representative	Personal interest
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NIC, Argentina	Dr Jorge Augusto Camara	None
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HPA, UK	Dr Maria Zambon	Honoraria for speaking at educational meetings

The interests declared by Drs Levandowski, Monto and Zambon were reviewed by the WHO Secretariat and were considered not to present a conflict of interest with their role in the writing group.

Annex 3: Further reading

The GISRS vaccine virus selection process

Barr IG *et al.* Epidemiological, antigenic and genetic characteristics of seasonal influenza A(H1N1), A(H3N2)

[2] For further information see: http://apps.who.int/gb/pip/pdf_files/Fluvaccvirusselection.pdf