



Report of meeting on the development of influenza vaccines with broad spectrum and long-lasting immune responses, World Health Organization, Geneva, Switzerland, 26–27 February 2004

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The immunogenicity of currently available influenza vaccines – the inactivated influenza vaccine (IIV) and the live attenuated influenza vaccine (LAIV) – is measured by the level of antibodies raised against the two major viral surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA) in serum and nasal secretions. Because the antigenicity of these two viral proteins is constantly changing, the influenza vaccines are updated every year to contain the HA and NA influenza antigens currently circulating. When the

vaccines contain antigens that are well matched with those in the circulating viral strains, they are very effective in preventing influenza in the majority of the population.

However, when those antigens are not well matched, the protection that follows vaccination is reduced.

Because the manufacturing and distribution process takes months, vaccine strains must be selected many months in advance of the influenza season to provide sufficient time to prepare and distribute the vaccine, a requirement that does not allow for the inclusion in the vaccine of viral strains that are identified too late to include in the manufacturing process. Other limitations of current vaccines include recommendations for yearly administration – a recommendation that will be hard to implement in industrialized countries, and even more difficult for developing countries – as well as their reduced protection in the elderly, the population at highest risk for influenza-related morbidity and mortality.

On 26–27 February 2004, the World Health Organization held a meeting on the “Development of influenza vaccines with broad spectrum and long-lasting immune responses” at its headquarters in Geneva, Switzerland. The main goal of this meeting was to examine available data and identify critical scientific issues and gaps in knowledge that need to be addressed to accelerate the development of new influenza vaccines capable of inducing broad spectrum and long-lasting immunity. Such vaccines would need to induce cross-protective immune responses against divergent influenza viruses and be acceptable for use in developing countries. This is an important and timely initiative, especially in light of the current avian influenza outbreak in Asia and the serious concerns from international health authorities that one of the influenza viruses, newly emerged from the animal reservoir, might gain the ability to spread between humans and result in a pandemic.

An influenza vaccine with a wide breadth of protection could protect against antigenically variant influenza viruses within a subtype; it could also protect the population, at least partially, from the antigenically novel viruses that cause pandemics. The components of such a vaccine would not need to be updated yearly because they

would protect against virus strains not perfectly matched with the vaccine, making the manufacturing process more flexible and less time constrained. The availability of vaccines that protect against a broad spectrum of influenza virus and that induce a long-lasting immunity would permit the current yearly vaccination strategy to be revised to a schedule that is more feasible to implement in developing countries. Finally, a broad spectrum influenza vaccine might provide increased efficacy in the elderly.

Scientists from around the world presented a variety of options for enhancing the broad spectrum and long-lasting immune responses to vaccines. Initial presentations focused on general principles and problems to be addressed in the development of new and improved vaccines. The role of antihaemagglutinin (anti-HA) antibodies was reviewed, and the value of anti-HA antibodies as a surrogate for assessing significance of antigenic variation was discussed. The existence of heterotypic immunity to infection, with variants related to the vaccine virus, was emphasized and it was noted that increasing doses of HA correlate with increasing magnitude, duration and cross-reactivity of anti-HA immunity. The roles of immunity to NA and of anti-NA antibody in reducing the intensity and preventing infection were noted. The slower rate of antigenic variation of the NA, in combination with the established role of the level of anti-HA antibody in immunity, indicate a need to focus on induction of optimal antibody responses to these two antigens for the contribution they can make to an increased duration of immunity to antigenic variants within a subtype. A role for cytotoxic lymphocytes (CTL) and anti-HA and NA antibody in reducing the intensity and duration of viral shedding was considered. The role of M1, NP, and possibly other invariant antigens in induction of CTL responses was noted, as well as the potential of antibody to the invariant M2 protein. The importance of these invariant antigens in vaccines that could convey long-lasting immunity and immunity to pandemic viruses with novel HA and NA antigens, but antigenically conserved NP, M1, and M2 proteins, was provided as support for pursuing vaccine approaches that optimize responses to these conserved antigens.

The deficiencies of current inactivated influenza vaccines were reviewed. Among the deficiencies highlighted were the dependence on eggs and the difficult selection, manufacturing, formulation, testing, and distribution process. Furthermore,

immunogenicity is relatively low for young children and the elderly, annual vaccination is recommended, vaccine is given parenterally, and protection is less than desired.

To combat many of the disadvantages of current vaccines and to identify options for improvement, the potential for mucosal delivery was discussed. Intranasal administration seems promising because administration of antigen to the mucosa is required for optimal IgA antibody responses and because the route can stimulate both mucosal and systemic immune responses. A mucosal delivery method may facilitate use of vaccine, particularly in developing countries. Evidence for greater heterotypic immunity of IgA antibody and for vaccines given by the nasal route was presented. The potential value for aerosol vaccination that would deliver vaccine to both the upper and lower respiratory tracts was discussed but the numerous hurdles to be surmounted for any acceptable method, including an optimal delivery system, concerns for inducing tolerance, a proper balance of IgA and IgG antibodies, and the need for adjuvants for optimising responses were emphasized. Mucosal delivery took a recent setback with regulatory withdrawal of an approved intranasally-administered influenza vaccine containing virosomes and an *E. coli* enterotoxin because its use was associated with an increased risk of Bell's Palsy. Nevertheless, the potential value of a mucosal delivery method is sufficient reason to continue study of this approach for vaccine delivery.

A variety of adjuvants is available for improving immune responses to vaccine and some of them were considered. An overview presentation identified the types and classes of adjuvants and emphasized that the choice of adjuvant depends on the proposed antigen, the type of immune response required and the target population. Virosomal vaccines containing IL-2 or CpG as an adjuvant have induced enhanced immune responses in clinical trials. In mice, cationic liposomes containing antigens are highly immunogenic without added adjuvants. ISCOMS can induce TH1 and TH2 responses and have induced long-lasting immunity and increased immune responses in both young and elderly populations.

Vaccines that specifically target conserved viral proteins were presented. Immunization with the M2 protein has induced homotypic and heterotypic immunity to infection of

antigenic variants within a subtype, as well as to different influenza A subtypes. DNA vaccines inducing responses against the surface antigens induce homologous protection in animal models while DNA vaccines against conserved internal proteins (NP, M1) induce broad heterotypic immunity that includes influenza A viruses of a different subtype.

Influenza virus infection can induce both broad spectrum and long-lasting immunity against reinfection with an influenza virus within a subtype; this suggests that live attenuated influenza vaccines (LAIV) could theoretically be able to do this. In mice, ferrets and humans, LAIV have induced potent prevention against both matched and antigenically drifted viruses. Children, in particular, appear to benefit from LAIV but evidence for superiority over inactivated vaccines for adults is mixed.

Guidance in the use of animal models for vaccine development was presented. A sequence of models used at one site is mice, followed by ferrets and possibly non-human primates, before proceeding to clinical trials in humans.

A number of regulatory issues relating to new vaccines were cited. As new vaccines aimed at inducing specific immune responses are developed there will be a need for convincing data for regulatory authorities a) on new correlates of immunity and b) that demonstrate that cross-reactive and long-lasting immunity is induced. Efficacy studies in humans that provide this information are likely to be required for licensing in both Europe and the United States of America. For these developments, it will be necessary to establish new reagents and guidelines for their use. In so doing, it is important that international harmonization of regulatory requirements be promoted.

A final item emphasized is the important role that the WHO must play in facilitating international cooperation in the development of new and different vaccines and in promoting an understanding of the importance of influenza and the need for vaccination in all countries, including developing countries. Recommendations from meeting participants and abstracts of presentations are included below.

General recommendations from meeting participants

- Increase awareness of the impact of influenza in general populations so as to increase vaccine demand, providing enhanced incentives for manufacturers to increase vaccine production and invest resources in development of more effective influenza vaccines.
- Expand the understanding of how different components of the immune system induce protection against influenza following immunization. Research on this area should include the elucidation of immunological factors responsible for heterotypic and heterosubtypic protection and specifically the role of CTL-based immunity in humans.
- Conduct clinical studies comparing the efficacy of LAIV and IIV vaccines in children and adults. Include assessments of potential correlates of immunity other than serum anti-HA antibody and of the value of each type of vaccine for induction of heterotypic immunity in the different age groups.
- Continue studies towards development of novel influenza vaccines that target conserved influenza proteins (e.g. M1, M2 and NP).
- Reassess efficacy of DNA-based influenza vaccines by using adjuvants and delivery systems. These vaccines have shown efficacy in pre-clinical studies and are an attractive alternative to the current vaccine technologies because they are easy and fast to manufacture in large scale.
- Conduct additional studies to further evaluate immunogenicity and efficacy of the various adjuvant systems in pre-clinical and clinical studies.
- Compare delivery systems of influenza vaccines – including intramuscular and intranasal, aerosol and transcutaneous routes – for safety, immunogenicity and efficacy of influenza vaccines in controlled clinical trials.
- Improve vaccine-induced immune responses in the elderly. Approaches include further evaluation of higher vaccine dosages and the use of adjuvants.

- Continue attention to both short-term and long-term occurrence of adverse events in clinical trials that evaluate new vaccine approaches.

Recommendations for WHO

- Promote appreciation of influenza as a significant medical problem in both developed and developing countries.
- Provide a forum for coordination of international efforts in development of influenza vaccines that induce broad spectrum and long-lasting immunity.
- Enlist vaccine and public health scientists from developing countries in the research on development of new influenza vaccines and planning programmes for control of influenza by vaccination.
- Continue to review and update international biological standardization activities, including development of standardized assays and reagents for evaluation of cross-protective efficacy of influenza vaccines.
- Provide a forum for review and coordination of decisions, seed selection and preparation, manufacturing efforts and delivery of vaccines for pandemic influenza.
- Coordinate international harmonization of regulatory requirements to facilitate vaccine development and licensing, including genetically-modified organism regulations where appropriate.
- Conduct a review and update of live-virus vaccines.
- Coordinate efforts to address intellectual property issues for using reverse genetics for vaccine manufacturing.
- Provide assistance and advice to appropriate persons and agencies regarding funding sources for influenza vaccine efforts. Publicize funding opportunities available through the National Institute of Allergy and Infectious Diseases of the National Institutes of Health in the United States of America.

Abstracts

Cross protection against drifted H3N2 induced by cold adapted, live attenuated influenza vaccine – trivalent (CAIV-T)

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Successful use of the currently-licensed inactivated vaccine to prevent influenza requires close antigenic match between the circulating viruses and the antigens contained in the vaccine. Thus, the ability to predict epidemic strains is inherently important in the use of these inactivated vaccines. With the use of live attenuated vaccines, evidence is accumulating that broad immune responses are generated, and these antibody responses may be primarily focused in the mucosal secretions [1]. Mucosal immunity coupled with broadly reactive immunity suggests that live attenuated intranasal influenza vaccines may have significant advantages over the inactivated vaccine [1, 2, 3, 4, 5].

CAIV-T consists of approximately 10^7 TCID₅₀/dose of each of influenza A/H1N1, influenza A/H3N2 and influenza B strains. The exact strains are updated each year to antigenically match the antigens recommended by national health authorities for influenza vaccine. CAIV-T is sprayed into the nose using a simple syringe-like device that delivers 0.25 ml volume of a large particle aerosol into each nostril for a total volume of 0.5 ml. The device is easy to use and adults can self administer the spray. Children require minimal assistance or very brief restraint to allow administration of the vaccine intranasally.

One of the potential advantages of a live influenza vaccine is that it might be expected to stimulate broader immunity against antigenic drift strains. The basis for this expectation is that a live replicating vaccine virus would present more antigen to the immune system than the inactivated vaccine and a complete complement of all influenza virus antigens in their native configurations. As a result, serum antibodies, secretory antibodies in the

upper respiratory tract and neutralizing antibodies would be produced to both surface proteins, the haemagglutinin and neuraminidase. Highly conserved antigens such as the matrix and nucleoprotein may also be presented in an immunologic context appropriate for stimulation of cross-reactive cytotoxic T cells and antibodies. Immune responses to native HA and NA antigens, as well as internal proteins, probably account for the observation that natural infection with a new pandemic strain induces some protection against drift strains that arise during the subsequent influenza seasons [6, 7].

At least four field efficacy-trials have demonstrated that immunization with CAIV can protect against antigenically drifted influenza strains, in addition to providing protection against homologous influenza strains [3, 5, 7, 8]. In the paediatric efficacy trial of CAIV-T, the drifted variant influenza A/Sydney/5/97 (an H3N2 virus) caused the majority of disease in year 2 of the study [3]. CAIV-T contained A/Wuhan 359/95 as its H3N2 antigen.

A/Wuhan and A/Sydney were significantly different antigenically as determined by ferret antisera. CAIV-T was 86% effective in preventing culture-confirmed influenza due to A/Sydney/5/97. In that same year an effectiveness study of CAIV-T in adults demonstrated significant reduction in illness and in illness-associated work loss and illness associated healthcare requirements among vaccinated adults versus placebo recipients [5].

The weight of evidence is that both natural infection [3] and immunization with CAIV [3, 5, 7, 8] can provide protection against drifted strains. This protection is a major advantage of a live influenza vaccine since drift strains might arise unexpectedly in some seasons. To address the issue of whether CAIV-T stimulated immunity that was cross-reactive with antigenic drift strains, serum specimens obtained during the 1996–97 paediatric efficacy trial were tested in the laboratory in HAI assays against a variety of H3N2 drift strains. These strains had been isolated during influenza seasons immediately preceding or following the 1996–97 efficacy trial and compared with serum of younger children immunized with TIV containing the A/Wuhan/359/95 antigen. Antibody responses were more broad following CAIV-T than with two doses of inactivated

vaccine; note that this comparison was not a double-blind study and the ages of the children given inactivated vaccine were younger than the children given CAIV-T. Nevertheless, these results provide an important hypothesis for further work. These results support the conclusion that CAIV-T stimulated a cross-reactive antibody response in children, and might be expected to provide a high level of protection against antigenic drift strains in an influenza season in which there was a similar sub-optimal match between the vaccine strain and the epidemic strain.

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Immunity to influenza after infection or vaccination

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Influenza is a recurring illness resulting from infection and reinfection with an influenza virus. The major reason for these recurring infections is antigenic variation of the haemagglutinin (HA) and neuraminidase (NA) surface antigens of the influenza virus that circumvents pre-existing host immunity. The circumstance for these recurring infections is the occurrence of an influenza epidemic annually so that pre-existing immunity is frequently challenged.

Immunity to infection and illness from an antigenically similar influenza virus is potent and persists for decades. Immunity to infection, with a related but distinct antigenic variant of influenza virus, is reduced with time and degree of antigenic variation

exhibited by the viruses causing the different epidemics. The degree of immunity to the same (homotypic) virus and also to an antigenically different (heterotypic) virus is directly related to the magnitude of serum anti-HA antibody at the time of the exposure. These antibodies persist for decades.

Immunity after parenteral vaccination with inactivated vaccine (IV) can also be potent for infection with an antigenically similar virus and the degree of immunity is directly related to the magnitude of serum anti-HA antibody at the time of exposure. This has been repeatedly demonstrated in the use of inactivated vaccines for interpandemic influenza; it is less clear that this is true for pandemic influenza. Vaccination with IV can also convey immunity to challenge with a heterotypic virus; the degree of immunity conveyed is also directly related to the magnitude of serum anti-HA antibody. For both these circumstances, the magnitude of the serum anti-HA antibody detectable is directly related to the dose of HA antigen contained in the vaccine. For a 15 µg dose of HA, IV-induced immunity lasts for at least three years.

Both homotypic and heterotypic immunity are induced by cold-adapted attenuated influenza virus vaccines. The degree of homotypic and heterotypic immunity to natural influenza among healthy adults given the attenuated vaccine is about the same as that induced by IV containing 15 µg of HA. Available data suggest the attenuated vaccine is superior in young children.

Studies on the mechanisms for immunity to influenza in animals or humans have shown that antibody to the HA in serum and respiratory secretions is necessary to prevent infection. These antibodies as well as anti-NA antibody, antibody to the M2 protein and cell-mediated mechanisms can contribute to amelioration of the infection and illness. When present in high quantities, anti-NA antibody can prevent infection. Antibody to both the HA and NA conveys immunity to homotypic and heterotypic variants within a subtype while anti-M2 antibody, cytotoxic lymphocytes (CTLs) and other antigen-induced cell-mediated responses are effective against all influenza viruses within a type. CTLs contribute significantly to control and recovery from influenza infections in the lungs of mice. The suggestion of a similar effect of CTLs at the mucosal level has been

confirmed in adoptive immunization studies in mice [Mbawuiké I, unpublished data]. It is proposed that influenza vaccines designed to provide broad and long-lasting immunity should induce optimal quantities of all the humoral and cellular immune responses that are known to convey immunity to influenza.

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A universal human influenza A vaccine based on the conserved external domain of the M2-protein.

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Conventional influenza vaccines are produced from virus grown in embryonated chicken eggs or in tissue culture. These vaccines have a number of drawbacks and deficiencies, the most important being the difficulty to match the rapid virus changes due to drift and the unpredictable emergence of new strains with pandemic potential. Unlike haemagglutinin (HA) and neuraminidase (NA), which are responsible for the drift-and-shift phenomena, the external domain of the human influenza A tetrameric M2-protein (M2e) has remained remarkably constant since the first influenza virus stain was isolated in 1933. This despite numerous epidemics and three major pandemics in the previous century. M2-protein is hardly present on the virus, but is quite abundant on virus-infected cells. The reason M2e neither drifts nor shifts may be twofold. M2e is only 23 aa in length, and barely sticks out from the membrane where it is surrounded by giant HA and NA molecules. Hence it is hardly accessible by immune cells and, indeed, adults have no – or almost no – detectable M2e-antibodies. The second reason is a double genetic constraint. Both M1 and M2 are coded by influenza gene 7 by differential splicing. The first 9 aa of M2, including the initiating methionine, are shared with M1, where they play a different functional/structural role. The next 15 aa are coded by a sequence which, in a different reading frame, codes for the terminal part of M1. This constitutes again an important barrier against mutation. Although natural M2e is hardly immunogenic, we have previously reported that, by presenting the peptide in an appropriate structural configuration to the immune system, it becomes an effective immunogen and induces protection in mice against a potentially lethal virus infection [1]. Most of our experiments have been done with M2e genetically linked to hepatitis B core (HBc). We have previously shown that M2e-HBc, administered either i.p. or i.n., induces full protection against a potentially lethal infection. This protection is due to

antibodies (passive transfer) and is long-lasting. Moreover, M2e-HBc can easily and cheaply be produced in *E. coli*. More recent research has revealed that the M2e sequence can be either fused to the N-terminus of the HBc subunits, or inserted in the major immunodominant loop (a HBc particle is formed by 240 subunits, arranged as dimers; two loops form a spike) [2]. RG-529-AF (Corixa Co.) is a well-defined, synthetic compound of similar structure and function to phospholipid A. It has been used in several clinical trials. M2e-HBc vaccine, in combination with RG-529-AF as adjuvant, provided complete protection against a potentially lethal viral infection upon i.p. administration. Likewise, complete protection was obtained after i.n. administration of the M2e-vaccine in combination with the non-toxic enterotoxin mutant LTR192G (provided by Dr John Clements, Tulane University). A remarkable enhancement of immunogenicity was obtained by using a construct containing three M2e-sequences at the N-terminus of the HBc-subunit. HBc-particles are well known to be highly immunogenic. But, with the recent (M2e)₃-HBc construct, the immune response against M2e was even higher than against HBc.

We have previously reported that immunization with recombinant NA protects as efficiently against an influenza infection as recombinant HA. But the problem with NA is its instability. It dissociates into dimers and monomers. The immunogenicity decreases considerably in the order tetramer>dimer>monomer. However, we have been able to stabilize the tetramer form by linking NA (minus membrane and intracellular domain) to a modified leucine-zipper motif [3]. This stabilized tetrameric NA retains its high immunogenicity and also its enzymatic activity. M2-protein is also a tetramer and likewise we have been able to produce tetrameric M2e by fusion to the modified leucine-zipper motif. This molecule presumably mimics the native M2e-domain as present on the infected cells.

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Vaccination of pigs with a DNA construct expressing an influenza M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus

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In mice, vaccines inducing antibodies to the extracellular domain of the M2 protein (M2e) can confer protection to influenza A infection [1]. Unlike the surface glycoproteins haemagglutinin and neuraminidase, this domain of M2 is highly conserved and is, therefore, a potential broad-spectrum immunogen. In this study, the protection conferred by vaccines inducing antibodies to M2e was evaluated in a challenge model for swine influenza in pigs. A protein resulting from the fusion between M2e and hepatitis B core protein (M2eHBc), with or without adjuvant, was evaluated. In addition, a DNA construct expressing a fusion protein between M2e and influenza nucleoprotein (M2eNP) was evaluated to see if the broad-spectrum protection conferred by antibodies could be further enhanced by T-helper cells and cytotoxic T-cells. All vaccines induced an antibody response against M2e, and the M2eNP DNA vaccine additionally induced an influenza virus-specific lympho-proliferation response. However, after challenge with a swine influenza virus (H1N1), no protection was observed in the vaccinated groups compared to the non-vaccinated control group. On the contrary, vaccinated pigs showed more severe clinical signs than the control pigs. The M2eNP DNA-vaccinated pigs

showed the most severe clinical signs and three out of six pigs died on days 1 and 2 post-challenge. These results indicate that antibodies to M2e, especially in combination with cell-mediated immune responses, exacerbate disease. Thus, clinical signs after infection should be observed closely in further studies using M2e as an immunogen, and caution should be exercised using M2e in humans. We hypothesize that the non-neutralising M2e antibodies, the NP-specific T-helper cells, and the extensive influenza virus infection all act together to stimulate macrophages in the lung, leading to an over-induction of cytokines (TNF- α) and severe clinical signs.

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Live attenuated vaccines – Overview

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FluMist – a live, attenuated, influenza vaccine (LAIV) – was licensed for use in the United States of America in time for the 2003–04 influenza season. Vaccines derived from the FluMist master donor strains have been previously shown to elicit durable antibody responses and to provide immunity to both homologous and antigenically

drifted strains. For this season, both injectable trivalent inactivated influenza vaccines (TIV) and FluMist contained A/Panama/2007/99 (H3N2). However, a large proportion of the H3N2 viruses isolated during the season in the United States was from the antigenically distinct A/Fujian/411/02-like (H3N2) lineage. Due to this disparity between the antigenicity of the predominant circulating strain and the vaccine strain, it was crucial to determine whether the current vaccines would elicit an immune response to these variants.

In order to address the heterotypic immunity elicited by these vaccines, ferrets were immunized with them then the antibody response and resistance to challenge with wild type viruses were evaluated. FluMist induced high rates of seroconversion to the homologous vaccine antigen as well as the drifted A/Fujian variants. In contrast, TIV did not elicit high levels of antibody to either of the antigens. In addition, the animals immunized with LAIV resisted challenge infection with either wt A/Panama or a wt variant of the A/Fujian lineage significantly better than unimmunized animals or animals receiving TIV.

WHO Initiative for Vaccine Research and its role in influenza vaccines research and development.

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The Initiative for Vaccine Research (IVR) is a structural component of the Department on Immunization, Vaccines and Biologicals (IVB) within the Family and Commune Health Cluster (FCH). IVR is composed of three teams, each headed by a coordinator: Research on Bacterial Vaccines, HIV Vaccine Initiative, and Vector-Born Pathogens and Acute Respiratory Infections [1]. The role of IVR is to provide a source of guidance and vision for the worldwide vaccine R&D efforts, to contribute to a global agenda with other

partners and identify the specific role of WHO/IVR, to advocate and coordinate clinical trials, to provide normative guidance, standards and reagents, to build capacity and provide facilities for technology transfer, and to encourage partnerships.

In 2002, the WHO Global Influenza Programme recommended the development of a novel generation of influenza vaccines [2]. On IVR request, experts from National Institute for Biological Standards and Control (NIBSC), UK and National Institute of Health (NIH), USA prepared a concept paper on options to develop new vaccines that induce broad spectrum and long-lasting immune responses to provide protection against divergent influenza viruses. New vaccines should overcome the problems related to annual intervention of the current vaccination strategy and be acceptable to developing countries. The vaccines should contribute to the control of epidemic situations and future pandemics. In 2003, development of a new generation of influenza vaccines was included in the IVR strategic plan.

There are several lines of research with the potential to develop influenza vaccines with a broad spectrum of protection. However, some studies are fragmented, depending upon funding and local resources; consequently progress may not be smooth. It is important for WHO to participate in the development of a new generation of influenza vaccine with a broad spectrum of protection.

The objective of the first meeting in the area of new influenza vaccines will be to evaluate the current status of studies in the cross-reactivity and cross-protection of influenza vaccines, to develop a research agenda, and to recommend activities for WHO.

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Aerosol delivery of vaccines: global overview.

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The aerosol delivery of vaccines via the nasal route offers the potential for needle-free delivery and mucosal protection. However, certain practical as well as scientific issues need to be addressed before this route of delivery can be applied in the global setting. Although there are promising lines of research, there have been significant recent setbacks [1]. The nasal portal is suitable for delivery of live, attenuated vaccines that can colonise and invade the respiratory epithelium. However, given the proximity of the meninges to the cribriform plate, it is likely that such organisms will be limited to viruses that are well attenuated. Over-attenuation may result in the loss of ability to colonize and invade, leading to failure of immunization. Delivery systems that manipulate particle size to enable penetration of the bronchioles allow for delivery of live organisms to the alveoli. Again, safety considerations probably limit this to attenuated viruses. The human respiratory tract immune system contains structured elements within the NALT, analogous to the Peyer's patches of the small bowel, together with the tonsils, adenoids and structured elements within the BALT. Whereas the upper, ciliated airways have a general "mucosal" phenotype, the lower airways become progressively more "systemic" in nature. In this context there is a switch from IgA to IgG effector functions. Within the NALT there also appears to be a differential processing of soluble and particulate antigens with regard to immunity or tolerance. Vaccine delivery systems must therefore determine what is the PURPOSE of aerosol delivery – merely as a passive portal of entry for therapeutic infection by a live organism, or as an active site of immune induction at the portal, the systemic compartment or distant mucosal portals? If the purpose is to induce local and distant mucosal portal immunity then issues of tolerance, short duration of IgA responses, and compartmentalization of the common mucosal immune system (even to the level of a unilateral response in the NALT) must be addressed [2]. Parenteral immunization with live or subunit vaccines is highly effective in inducing IgG and CTL

responses that can protect mucosal surfaces in certain cases (e.g. the lower airways or, in the case of polio, even the gut). However, to induce significant secretory mucosal IgA generally requires mucosal delivery of antigens. Although nasal immunization appears to prime and boost both systemic and mucosal responses (even in the genital tract), it requires adjuvants or carrier systems to overcome mucosal tolerance and duration of responses are very short. Adjuvants such as the cholera-related toxins have been associated with significant adverse events, [3] and carrier systems such as chitosan may induce significant levels of local adverse reactions. Delivery systems that target the lower respiratory tract bring issues of airway irritation, bronchospasm and allergic-type responses. Intranasal delivery equipment has turned out to be expensive, and may involve devices that are themselves subject to regulatory issues. Multiple-use, mass-dispensing equipment has been used successfully to immunize thousands of children with measles virus vaccines [4], but have issues of cost, reliability, practicality and spread of mucosally-transmissible diseases. While initial studies in animals and limited clinical trials provide ample evidence that aerosol delivery of live and subunit immunogens offers the potential for needle-free vaccines [5], further human-based work is required to prove that this offers a realistically affordable alternative to parenteral immunization, with significant reduction in reactogenicity and risks of cross infection, without loss of immunogenicity and immune memory. To this end further clinical trials of vaccine delivery systems and adjuvants, ideally using standardized antigens and read-outs of immunity and protection are required, preferably independent of commercial pressures.

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Cross-protection and cross-reactive immune responses

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To obtain efficient non-replicating vaccines, a delivery system harbouring key functions for immune induction is required. The immuno-stimulating complex (ISCOM) is an antigen-adjuvant delivery system in a 40 nm spherical particle which can be supplemented with a targeting device facilitating mucosal and systemic routes of administration. The ISCOM is versatile, characterized by the fact that all functions can be varied to tailor the particle for antigen and for immune response leading to protection.

ISCOMs induce protection and cross-protection against experimental infection with influenza virus in animal models both by mucosal and systemic immunizations. The mucosal route includes mucosal IgA and systemic IgG of all subclasses. The cell-mediated immune response is balanced. The ISCOM has the capacity to drive to Th1 type of response including a potent MHC class 1 CTL response by CD8 cells. The systemic immunization leads also to a balanced T helper cell response and CTL by CD8 cells. The protection induced by ISCOMs against experimental infection of homologous influenza virus is similar to that induced by conventional vaccines, including anti-HA, virus neutralising-antibodies and anti-N. Moreover, ISCOMs induce a potent CTL response of immune protective value.

ISCOMs containing H1N1 administered by the oral route, induce cross-protection in a murine model against challenge with H2N2 virus. The protection was correlated to a DTH response. By the systemic immunization, H1N1 envelope proteins incorporated into ISCOMs induced protection against respiratory challenge infection with influenza virus sub-serotypes H2N2, H5N1, H9N2 and H3N2. The correlate to protection was ascribed to CTL directed to epitopes in the H molecule. In contrast, the cross-protection induced by live influenza virus has been correlated to CTL epitopes in the nucleoprotein which seems to dominate over CTL epitopes in the H molecule. Also non-neutralising antibodies were protective, but required cellular components manifested with the supplement of macrophages in an *in vitro* system.

ISCOMs with influenza virus envelope proteins induce long-lasting immune response in horses and in primates in contrast to a short-lasting response induced by the conventional influenza vaccines. Such ISCOMs have, in a murine system, induced immune response of similar magnitude in old mice as in young adults in contrast to a conventional vaccine. The protection was found to correlate with the capacity to stimulate expression of CD86. Generally, elderly individuals respond less efficiently with expression of CD86 than young adults. ISCOMs, with the envelope protein F and G from respiratory syncytial virus (RSV), have also induced protection in newborn mice against challenge infection in the respiratory tract; likewise in calves with maternal antibodies against challenge with bovine RSV. Conventional vaccines failed in these experiments.

ISCOMs are on the veterinary market and in clinical trials in man. New – more efficient – ISCOM formulations, which are very well tolerated, have now been developed with the possibility to tailor for antigen and disease.

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Limitations of currently available influenza vaccines

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The first successful influenza vaccines were produced in the United States of America in the 1940s using formalin-inactivated influenza virus grown in embryonated hens' eggs. Despite the availability of influenza vaccines for more than half a century, annual epidemics of influenza still inflict an enormous socioeconomic burden globally – even in countries with high levels of vaccine uptake in people most at risk from the complications of influenza. More worrying, should a pandemic of highly lethal H5 influenza emerge within the next four to six months and candidate vaccines were developed and tested in the interim – even if the worlds' vaccine producers were working at full capacity and vaccine were used sparingly – vaccine would be available for less than 20% of the worlds' 6.35 billion population (assuming that each vaccine requires two 7.5 μ g doses). These two scenarios illustrate two important limitations of current influenza vaccines – first, their efficacy, while appreciable, is less than optimal in those targeted for vaccination; second, vaccine manufacturers are incapable of producing sufficient vaccine to protect mankind from pandemic influenza. These and other limitations of currently available, inactivated, influenza vaccines are enumerated below:

1. All of the current commercially available vaccines are prepared from the allantoic fluids of chick embryos inoculated with a specific updated type of influenza virus. Because of the large numbers of embryonated hens' eggs required, advanced planning must start nearly a year before vaccination [1]. The number of eggs required to produce the number of doses to be marketed must be anticipated well

in advance (up to six months) of the production cycle. The process is inflexible, providing no opportunity to scale-up vaccine production.

2. Chickens are susceptible to disease so an adequate supply of eggs and vaccine cannot necessarily be guaranteed [2]. In addition, there are increasing concerns about the presence of adventitious agents in embryonated hens' eggs, particularly retroviruses [3].
3. Once the variant to be included in the next season's influenza vaccine has been determined, a new variant may prevail, providing insufficient time and resources to prepare vaccines that closely match the circulating strain. In the decade 1987–1997, a good match between the WHO-recommended strains and those strains causing outbreaks in the world was achieved in respect of 23 (77%) of 30 circulating strains [4]. However, when considered on an annual basis, the epidemic strains were antigenically different from the vaccine strains during 5 of the 10 seasons.
4. Growth of human influenza virus in eggs can lead to the selection of variants that differ antigenically from the original [5]. Influenza viruses grown in mammalian cells detect neutralising and haemagglutination-inhibiting antibodies more frequently and to higher titres in post infection sera than do their egg-grown counterparts [6].
5. Manufacturers occasionally experience manufacturing problems, resulting in delays in distribution and potential shortages of influenza vaccine [7].
6. In the event of a pandemic, the necessary number of high quality fertile hens' eggs, and the capacity of manufacturers to process them, is unlikely to match global needs.
7. In the face of a pandemic threat, an expected minimum eight months will pass before new vaccine first begins to be distributed from manufacturers [8]. Thus, using current technology, the interval between the initial identification of a new

- pandemic strain and outbreaks may be insufficient to produce vaccine for the first wave of infection.
8. The strains recommended by WHO are chosen by virtue of their antigenic and genetic characteristics. However, there are often practical difficulties with their use as vaccine strains, either because of unacceptable passage history or poor growth in hens' eggs [4]. Accordingly, it is now normal practice to prepare candidate influenza A seed strains for vaccine production by genetic reassortment using the field strains chosen by WHO and an A/PR8/34 or PR8-like virus that grows to high-titre in embryonated eggs. The requirement for high growth reassortants can delay vaccine manufacture. Reassortants may be unstable and difficult to propagate [9]. Seed strains for producing influenza B vaccines are field isolates. No master B strain that improves the growth of influenza B in egg-based production systems has been identified yet [1].
 9. Although current egg-derived vaccines are highly purified, allergy to egg protein may be responsible for the rare occurrence of anaphylaxis following vaccination [10].
 10. Due to differing production processes by manufacturers, current influenza vaccines may contain trace amounts of antibiotics (neomycin and polymyxin) which are used to prevent bacterial contamination. Although influenza vaccines are highly purified, allergy to antibiotics may be responsible for the rare occurrence of anaphylaxis following vaccination [10].
 11. Most inactivated vaccines contain thiomersal, which is used as preservative, giving rise to theoretical concerns about vaccine safety [11] and potentially affecting uptake.
 12. Split and sub-unit vaccines are less immunogenic than whole virion products in immunologically-naïve populations [12–14].

13. Whole virion vaccines frequently cause febrile and other systemic reactions, especially in young children after the first injection [15–17].
14. Influenza vaccine is evidently poorly antigenic in infants less than six months of age [18], though few studies have been conducted in very young children.
15. Because of inconsistency of protection and poor immunogenicity, two doses of current inactivated vaccine are required for young immunologically naive children [19], adding to the logistical problems of vaccination.
16. Even when the current influenza vaccine contains one or more antigens administered in previous years, annual revaccination is recommended – particularly in the frail elderly – because immunity declines during the year after vaccination [20,21].
17. Current influenza vaccine formulations should be stored at temperatures of 2–8°C. The stability of current inactivated vaccines in the higher and fluctuating temperatures that are encountered in tropical and sub-tropical countries, with unreliable power supplies, is unknown. Annual influenza vaccination is becoming an increasingly important aspect of public health programmes in rapidly developing countries, including parts of Africa and South America [22]. Breakdowns in the vaccine cold-chain may impair vaccine efficacy.
18. The need to give inactivated vaccines by injection is a barrier to self-administration. Children and their parents and many others from vaccine target groups are reluctant to receive vaccines by intramuscular injection, and the inconvenience and problems of access associated with parenteral vaccination also adversely affect vaccine uptake [23–27].
19. Parenteral vaccine delivery has important cost implications (trained staff, facilities, vaccine storage, medication for allergic reactions, additional off-site delivery costs, costs to patients and carers).

20. Parenteral administration of inactivated vaccine provides reduced efficacy against heterologous virus infections (i.e., drift variants within the same virus subtype) – as demonstrated, for example, by the experience in 1947 when a new influenza A (H1N1) variant, A/FM/1/47, spread throughout the world and the then current vaccines were ineffective and, also, by virus challenge studies of volunteers who were previously immunized with various inactivated influenza vaccines [28].
21. The mucosal secretory (SIgA) response to conventional injected vaccines is poor [29–32] and, thus, they do not prevent the initial replication of virus in the respiratory tract nor heterosubtypic immunity related to SigA.
22. Conventional parenterally-delivered vaccines generally fail to induce cross-reactive cytotoxic T cell responses or to produce inferior responses in comparison to adjuvanted formulations [33–37].
23. Due to the narrow spectrum of immunity provided by current vaccines, vaccine must be delivered shortly before each annual outbreak – this poses logistical problems, especially in years with early or late epidemics.
24. Influenza vaccination is contraindicated in people who have had an anaphylactic reaction to a previous dose or are known to have anaphylactic sensitivity to eggs. It is also prudent to avoid vaccination in people who previously developed Guillain-Barré syndrome during a period of six weeks after vaccination [38].
25. Current inactivated influenza vaccines cause local soreness in up to 40% more recipients than placebo [39–42]. Despite the excellent safety record of influenza vaccine, influenza vaccine is perceived to cause unacceptable local and systemic reactions, including influenza itself.
26. Recent studies in North America show that about 85% of long-term care residents and 30% of adults and children with medical conditions receive vaccine annually [38]. Studies of health-care workers in hospitals and long-term care facilities have shown vaccine uptake rates of only 36% [38]. While low rates of vaccine

- utilization are multifactorial in origin, patient surveys highlight problems of inconvenience, difficulty in getting to the vaccination centre, cost, fear of needles, fear of local and systemic reactions, belief that vaccine would compromise an underlying chronic illness, fear of acquiring influenza from the vaccine, and concern over vaccine efficacy as reasons for not accepting vaccine [24,24,26].
27. The protection afforded by influenza vaccine is appreciable, yet incomplete [43], and varies according to the age and health status of the recipient and the similarity between the vaccine strain and the virus in circulation. Outbreaks are not uncommon in nursing homes, despite high levels of vaccine coverage.

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Animal model systems for investigation of vaccine efficacy

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Experimental animal model studies commenced during the Great Pandemic of 1918 and experiments in primates gave the first indication of a filterable virus as the cause of the outbreak [1]. But the definitive experiment with a drifted variant of the Spanish influenza, isolated in London in 1933, proved conclusively that influenza was viral and could be transmitted to the ferret and hence back to man [2]. Other workers described adaptation of the virus to mouse lung whilst a neurovirulent virus was derived in the same species [3]. At this early time a variety of small mammals was screened for model infections including guinea pigs and even hedgehogs. The early classic works, particularly in the ferret, established pathogenicity of the disease and also transmission from animal to animal by droplet nuclei [4]. Finally both the early and later investigators [5] found that co-infection of virus and pneumococcus and other bacteria enhanced virulence and infectiousness. Also investigated was natural influenza in pigs [6] and embryonated hens eggs [7], ducks [8] and pelagic birds [9].

Modern animal model systems have not changed significantly from these classic studies except that new technology such as telemetry is now used to continually monitor temperature changes whilst video cameras can quantify animal health and movement

objectively. The ferret remains the key model system because all influenza viruses, including avian such as H5 can infect this animal directly, without prior serial passage or prior growth in mammalian cells. Multiple quantitative parameters such as nasal virus, weight loss, temperature gain and movement can be measured in groups of ten ferrets to give statistically valid data. The classic ferret model provides precisely predictive data for new vaccines [10, 11] more classic live attenuated or killed vaccines [12, 13, 14] and virus virulence [15, 16] as well as acting as the penultimate screen before tests in humans including transmission [17].

The mouse model of necessity is required for studies of T cell responses and serious pneumonia [18]. Macaques [19] are used to establish details of pathology but not for initial vaccine studies per se.

The most pertinent animal model of course is the human volunteer infected in a totally closed quarantine unit originally in the UK unit in Salisbury [20] or the USA [21] with partially attenuated viruses. Our newly established quarantine unit in London now has the capacity to immunise and subsequently to infect 100 young volunteers with influenza A/Caledonia 20/99 (H1N1), A/Panama/2007/99 (H3N2) or influenza B viruses [22].

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Cross-protection of influenza vaccine after mucosal delivery: a mouse model

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Influenza is still a serious threat to public health throughout the world. The introduction of an influenza A virus possessing a novel haemagglutinin (HA) into an immunologically naive human population has the potential to cause the next influenza pandemic. The current licensed influenza vaccine has limitations in its protective ability because the virus alters its surface glycoproteins, HA and neuraminidase (NA), thus escaping the presence of pre-existing immunity [1]. The recent emergence of avian H5 and H7 influenza subtypes into the human population has renewed the search for a vaccine strategy that induces broadly cross-reactive or heterosubtypic immunity (Het-I), with the potential to protect individuals against multiple subtypes of influenza A virus. We have compared mucosal versus traditional parenteral administration of inactivated influenza vaccine for the ability to induce Het-I in BALB/c mice and evaluated a modified *Escherichia coli* heat-labile enterotoxin adjuvant LT (R192G) [2] for augmentation of Het-I. Mice that received multiple intranasal (i.n.) immunizations of H3N2 vaccine in the presence of LT(R192G) were completely protected against lethal challenge with a highly pathogenic human H5N1 virus and had nasal and lung viral titres that were at least 2500-fold lower than those in control mice receiving LT(R192G) alone. In contrast, mice that received three vaccinations of H3N2 vaccine subcutaneously (s.c.) in the presence or absence of LT(R192G) or incomplete Freund's adjuvant were not protected against lethal challenge and had no significant reductions in tissue virus titres observed on day 5 post-

H5N1 virus challenge. To understand the immunologic bases of Het-I, we tested whether CD4⁺ and/or CD8⁺ T cells accounted for the cross-protection. Acute depletion of CD4⁺ T cells did not significantly reduce the strength of Het-I. However, by depleting CD8⁺ T cells in Het-I immune mice, the study demonstrated that this T cell subset aids in controlling virus levels in the lung and brain tissue, but is not vital for the host's survival following lethal virus challenge. These results were confirmed using 2-microglobulin (-/-) mice genetically deficient in functional CD8⁺ T cells, which were protected against lethal Het-I challenge. Het-I was further evaluated by using gene-targeted B-cell (IgH-6 -/-) deficient mice. IgH-6 (-/-) vaccinated mice were not protected by Het-I, suggesting that B cells were vital for protection of mice against Het-I challenge. Furthermore, mucosal – but not parenteral – vaccination induced subtype cross-reactive lung IgG, IgA and serum IgG anti-HA antibodies, suggesting the presence of a common cross-reactive epitope in the HA of H3 and H5 [3]. These results suggest a strategy of mucosal vaccination that stimulates cross-protection against multiple influenza subtypes, including viruses with pandemic potential.

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Influenza DNA vaccines

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DNA vaccines have been the subjects of much effort over the past decade due to their ability to induce broad-based immune responses and protection in various animal models of infectious and non-infectious diseases. In particular, influenza DNA vaccines have been well studied and some of the earliest work on DNA vaccines was conducted using influenza as a model system. This work included demonstration of haemagglutination inhibiting (HI) antibodies, CTL and protective efficacy in mice [1–6], protective efficacy in chickens [7,8], induction of HI antibodies and protective efficacy in ferrets [9,10], and induction of HI antibodies in non-human primates [9]. The potency of DNA vaccines encoding influenza virus antigens was demonstrated by the low amounts of DNA required to induce immunity, administered either by i.m. injection (4) or by the gene gun [3,11]. The breadth of the immune responses induced by influenza DNA vaccines included both humoral responses (i.e., antibodies) and cellular responses (i.e., CD4+ and CD8+ T cells). With regard to antibody responses, DNA encoding haemagglutinin (HA) from various influenza virus strains has been shown to induce high titres of HI antibodies in species ranging from mice to monkeys. With regard to cellular responses, DNA vaccines encoding nucleoprotein (NP) were shown to induce CD8+ CTL responses in mice leading to protective efficacy against a heterosubtypic virus challenge [1]. In addition, NP DNA elicited CD4+ helper T cells of the Th1 phenotype, i.e., secreting gamma interferon and IL-2 [12]. Interestingly, both CD8+ CTL and cytokine-secreting CD4+ T cells contributed to the overall protection in the mouse challenge model. Therefore, immune responses against conserved internal proteins may have an important role in limiting influenza disease. Furthermore, combined immunization with DNA encoding HA (which can generate neutralising antibodies) and DNA encoding NP and M1 (which can induce broad T cell responses) may provide a greater breadth of protection than can be obtained with conventional inactivated influenza vaccines and may be useful in humans against influenza.

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Regulatory issues for novel influenza vaccines: inputs from WHO

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International biological standardization at WHO is a constitutional responsibility which is mandated by 192 Member States through the World Health Assembly to resolve regulatory issues on safety and quality aspects of vaccines. The products of international biological standardization activities are written guidance, reference preparations and consensus on regulatory issues.

Internationally agreed technical specifications that may be of value in the development of regulatory guidelines for production and quality control of vaccines are defined in the WHO recommendations. WHO recommendations for production and control of inactivated influenza vaccine [1] were revised in 2003 in order to reflect significant new developments in methods of influenza vaccine production: increased development of mammalian cell lines for vaccine production; experience in use of adjuvants; rapid development of reverse genetics technologies for generation of vaccine viruses.

There has also been considerable effort in pandemic planning to ensure that safe and effective vaccines can be quickly produced in response to a pandemic emergency. There are, however, a number of issues for production and control of a pandemic vaccine: issues related to reverse genetics for seed production from a highly pathogenic virus, such as genetically modified virus seed and intellectual property right (IPR); initial lack of reagents for potency assays; use of adjuvants; requirement of high levels of biological containment for production; and vaccine liability [2,3].

Currently WHO is advised to negotiate with IPR holders of reverse genetics technologies to facilitate equitable international access. It is also recommended that efforts are made to standardise serology tests, such as virus neutralization test and neuraminidase content of influenza vaccines.

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Regulatory issues for development of new influenza vaccines

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The development of new vaccines takes many years. First the “proof of concept” is established during a laboratory phase. This is usually accompanied by publication in peer-reviewed journals. Subsequent phases involve the difficult transition from research laboratory to licensed vaccine. Although these phases differ in detail from country to country, they follow well-established principles:

- design and establishment of manufacturing capability;
- demonstration of quality and pre-clinical safety;
- approval and conduct of clinical trials;
- application for and approval of product licence.

Within the European Union (EU), a novel influenza vaccine will be evaluated and if satisfactory, it will be licensed by a centralised procedure, resulting in a licence for all EU Member States. In the United States of America, the Food and Drug Administration (FDA) coordinates the licensing process, which depends on prior approval of an Investigational New Drug (IND) application. After licensing, a vaccine is subject to evaluation by independent lot release, inspection of production facility and post market surveillance to monitor safety and efficacy of the vaccine. To assist in vaccine development, there are national and international guidelines for production, safety, quality and efficacy issues. Regulatory agencies are responsive to new vaccine developments and new guidelines are developed as required.

One of the key issues to evaluate for new influenza vaccines is clinical efficacy. In the EU, it is possible to use surrogates of efficacy (e.g., immunogenicity) for licensing, provided they are adequately validated. However for vaccines capable of developing broad spectrum and long-lasting immunity, immunological surrogates may not be well established at the time of licence evaluation. Another difficult aspect of licensing novel influenza vaccines is the need to update vaccine strains. Conventional influenza vaccines are updated on an annual basis because they stimulate humoral immunity, which lasts for

a relatively short time (about one year) and the immunity is relatively strain specific. Any new influenza vaccine will be expected to comply with these arrangements and convincing efficacy data will be needed for any departure to be contemplated.

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