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overview

Old and new ways to combat human influenza virus

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Abstract

Influenza viruses have been with mankind for at least 300 years, with epidemics occurring every few years and pandemics every few decades. They replicate extremely rapidly in the host therefore demanding a fast and effective antiviral response. Despite the availability of seasonal trivalent vaccines and antivirals, which are effective for most recipients, influenza remains serious disease. The reason for that is a grand capacity of the influenza virus to adapt to new environmental conditions and evolutionary pressure. Vaccination remains the main protective measure against influenza for general population. The first vaccine was administered in the 1940s and ever since the influenza vaccine has provided tremendous relief against influenza infections. However, time has revealed the ultimate limit of the vaccine and the call for its reinvention has now come. The purpose of this review is to give a brief but comprehensive overview of the currently used prophylactic and therapeutic approaches against influenza and the new most promising developments in this field.

INFECTION, STRUCTURE AND REPLICATION OF INFLUENZA VIRUS

Influenza viruses have been with mankind for at least 300 years with epidemics occurring every few years and pandemics every few decades. The name came from Italian expression »influenza di freddo« meaning »influence of the cold« because influenza typically occurs during winter months. The identification and first isolation of a causative agent of influenza was achieved in the laboratory of P.P. Laidlaw *(1)* by accidental infection of ferrets by infected laboratory stuff.

Influenza viruses cause disease among all age groups. The rates of infection are highest among children, but the rates of serious illness and death are highest among persons aged =65 years, children aged <2 years, and persons of any age who have medical conditions that place them at risk for complications from influenza (e.g. chronic medical conditions).

Influenza virus is shed into respiratory secretions, and then coughed or sneezed into the air, which spreads the virus from person to person. Typical incubation period for influenza is 1–4 days. Acute infection is characterized by an abrupt onset of symptoms that include fever, chills, cough, headache, myalgia, sore throat, malaise, anorexia and many other non-specific symptoms. However, influenza may exacerbate underlying medical conditions (pulmonary or cardiac disease).

None of the acute features are pathognomonic but the constellation of respiratory and systemic symptoms at a time when influenza activity

has already been confirmed in the locality is likely to lead to accurate diagnosis.

Influenza viruses are a negative-strand RNA viruses belonging to the family Orthomyxoviridae. There are three types of influenza viruses according to the structure of the nucleoprotein gene: A, B and C.

Influenza viruses of type A cause epidemics and pandemics. They infect multiple species (humans, pigs, horses, dogs, cats, various bird species, etc.) and are further divided into subtypes according to the composition of the hemagglutinin (HA) and neuraminidase (NA) genes. There are 16 HA subtypes (H1 to H16) and 9 NA subtypes (N1 to N9) that probably exist naturally in all 144 possible permutations. The majority of influenza A virus subtypes exist in various waterfowl, causing subclinical gut infection *(2)*.

Influenza B viruses cause epidemics, but not pandemics and infect humans only causing a respiratory infection similar to that caused by type A. The third type is type C which infects humans only and causes mild respiratory infection.

Type A and type B influenza viruses are subjects of this review.

Both influenza A and B viruses contain eight genome segments (total of 13,588 nt) of negative single-stranded RNA. The gene assignment is as follows: segment 1 codes for PB2, segment 2 for PB1, segment 3 for PA, segment 4 for HA, segment 5 for nucleoprotein (NP), segment 6 for NA, segment 7 for matrix proteins 1 and 2 (M_1 and M_2, respectively), and segment 8 for non-structural proteins 1 and 2 (NS_1 and NS_2, respectively). PB1, PB2 and PA proteins form a complex responsible for RNA synthesis, NP is a major structural protein which interacts with the genome and together they form a ribonucleoprotein (RNP), hemagglutinin (HA) is a surface glycoprotein which binds host cell receptor, neuraminidase (NA) is a second subtype specific transmembrane glycoprotein which enzymatically cleaves sialic acid from glycoproteins, matrix proteins M1 and M2 are structural proteins, M1 is placed on the inside of the of the lipid envelope, M2 is the integral membrane protein which acts as an ion channel protein, NS1 is non-structural protein which functions as a posttranscriptional regulator, NS2 is a structural protein which forms an association with M1.

Influenza virus attacks host epithelial cells of the respiratory tract by binding to sialic acid residues present on cell surface glycoproteins or glycolipids through the receptor-binding site in the distal tip of HA molecule *(3)*. Different influenza viruses have different specificities for sialic acid linked to galactose: á2, 3 linkages are specific for avian viruses, both á2,3 and á2,6 linkages are specific for swine viruses and á2, 6 is specific for human viruses.

The virus is then endocytosed and fuses with the endosomal membrane by conformational change in HA under low pH conditions. The uncoating of influenza virions in endosomes is dependent on the acidic pH of this compartment. M2 ion channel activity is essential for the uncoating process. The low pH activates ion channel of the virion associated M2 protein, which permits the flow of ions from the endosome to the virion interior disruption protein-protein interaction and frees RNP from the M1 protein, thereby averting the nucleocapsid (RNA, NP, PA, PB1 and PB2) into the cytoplasm. The nucleocapsids pass to the cell nucleus, where transcription into viral RNA and mRNA takes place. The translation of M1, NP, NS, PA, PB1 and PB2 proteins occurs on free ribosomes, whereas that of HA, NA and M2 proteins occurs on membrane-bound ribosomes.

Several hours after infection the newly synthesized viruses are released from the infected cells by the action of another major glycoprotein, neuraminidase (NA).

IMMUNITY TO INFLUENZA VIRUS

Influenza infection occurs in a seasonal pattern and leads to an extensive burden of disease. In healthy young adults, influenza can cause debilitating febrile illness lasting 1-2 weeks. In patients with pre-existing respiratory or cardiovacular disease, and in the frail elderly, influenza infection can be more serious.

Infection of influenza virus is restricted to respiratory epithelial cells. Most symptoms and complications therefore involve the respiratory tract. However, systemic complications are sometimes observed, and induction of inflammatory cytokines has been indicated as a potential explanation for the systemic feature of influenza infection.

Immunity to influenza virus infection has been a research topic for more than 70 years *(4)*. A rapid innate cellular immune responses are induced that aim at controlling virus replication. The adaptive immune system forms the second line of defense against influenza virus infection. Serum IgA are produced rapidly after influenza virus infection and the presence of these antibodies (Abs) is indicative for a recent influenza virus infection *(5, 6)*. Serum Abs of the IgG subtype predominantly transudate into the respiratory tract and provide long-lived protection *(7)*. IgM Abs initiate complement-mediated neutralization of influenza virus and are a hallmark of primary infection *(8, 9)*. Influenza virus infection induces virus-specific Ab responses *(10, 11)*, especially for the two surface glycoproteins HA and NA, which correlates with protective immunity *(12)*. Anti-HA Abs bind to the trimeric globular head of the HA predominantly and inhibit virus attachment and entry in the host cell. Thus, they neutralize the infectivity of the virus and are primarily responsible for preventing infection. Furthermore, these Abs facilitate phagocytosis of virus particles by Fc receptor expressing cells. Also binding to HA expressed on infected cells mediates antibody-dependant cell cytotoxicity (ADCC). HA-specific Abs are a solid correlate of protection provided that they match the virus causing the infection *(13)*. Anti-NA Abs do not directly neutralize the virus but by inhibiting enzymatic activity prevent the release of viruses from infected cells *(14)* and limit virus spread. The M2 protein

is the third viral membrane protein. This protein is highly conserved among influenza viruses of different subtypes and immunity against M2 was first demonstrated in mice by using therapeutic monoclonal Abs *(15, 16)*. The protein itself is present at low concentration in infected cells and thus M2-specific Abs are raised after natural infection to a limited extent.

CTLs specific for core proteins (mainly NP, M1 and PA) are responsible for reducing viral spread and thereby for accelerating the recovery from influenza *(17, 18, 19, 20, 21, 22)*. These proteins are highly conserved and therefore CTL responses display a high degree of cross--reactivity, even between different subtypes of influenza A virus.

Abs directed at the HA and NA surface glycoproteins of the virus mediate protection that is long lived in the absence of antigenic drift or shift. This was evident in 1977 when an H1N1 virus that had been circulated in the early 1950s reappeared in human population. Significant disease was only seen in persons born after the H1N1 virus had stopped circulating in 1957. Because individuals born after 1957 were infected multiple times with H2N2 and/or H3N2 viruses that share internal protein antigens (e.g. nucleoprotein) with the H1N1 virus, it was clear that cell-mediated immunity to shared antigens played relatively small role in resistance *(23)*.

Influenza viruses replicate extremely rapidly in the host. Peak titers are achieved before a cell-mediated immune response can be generated de novo or form memory to restrict replication. Therefore the major goal of the currently licensed influenza vaccines is to induce, prior to infection, Abs that function to dampen virus replication. New insights of adaptive immune responses against influenza virus infection and new correlates of protection could be the basis for development of novel vaccines.

VACCINES AGAINST INFLUENZA

Vaccination remains the main protective measure against influenza for general population. The first vaccine was administered in the 1940s and ever since the influenza vaccine has provided tremendous relief against influenza infections. However, time has revealed the ultimate limit of the vaccine and the call for its reinvention has now come. The purpose of this review is to give a brief but comprehensive overview of the currently used prophylactic and therapeutic approaches against influenza and the new most promising developments in this field.

In spite of the availability of influenza virus vaccines, yearly epidemics occur affecting 10 to 20% of general population and as much as 30% of school age children. Gradual changes in the coding sequences of the surface proteins HA and NA cause changes in these antigens that accumulate over time and are positively selected by immune response in vaccinated or infected individuals. This process, called antigenic drift, gives rise to variants that can infect individuals immune to the parental strain and gives rise to periodic epidemics every 2 to 5 years. Influenza B viruses undergo antigenic drift less rapidly than influenza A viruses. On several occasions, a mismatch occurred between the vaccine components and the prevailing dominant strains, as seen in 1997/1998 and 2003/2004 *(24)*. In such scenarios, the vaccine does not confer the desired level of protection and the number of infections increases.

Additionally, different influenza A virus subtypes can be involved in a process called antigenic shift. Antigenic shift refers to the reassortment of viral gene segments between various influenza viruses of human or zoological origin. This leads to the emergence of new strains that have caused most influenza pandemics *(25)*. In the most recent pandemic, the 2009 H1N1 pandemic, the seasonal vaccine did not contain pandemic strain and a substantial number of infections and deaths occurred *(26)*. Antigenic shift is not factored into the design of the current vaccine because it is too difficult to predict when and how the shift will occur. Consequently, a monovalent vaccine containing only the H1N1 pandemic strain was rapidly produced for administration along with the 2009/2010 trivalent seasonal vaccine *(27)*.

Yearly vaccination is required because each seasonal vaccine elicits neutralizing Abs that are specific only for the vaccine strains and closely related isolates, but rarely for divergent strains.

LICENSED (SEASONAL) VACCINES

Composition of seasonal influenza vaccine

The time required to implement any changes in vaccine production and ensure the timely delivery of influenza vaccine is a major constraint on the choice of virus strains to be included in vaccine recommendations; hence the recommendations are based on an assessment of the future impact of circulating viruses and in particular of any emerging antigenic variants, before their full epidemiologic significance is known. The scientific evidence that forms the basis for a vaccine recommendation thus includes not only the antigenic and genetic characteristics of the viruses but also their prevalence, geographical distribution and the rate of spread.

The World Health Organization (WHO) reviews the world epidemiological situation annually. The need for global surveillance of influenza viruses was recognized as early as in 1947 and led to the establishment of the WHO Global Influenza Surveillance Network (GISN). Since its inception the GISN has developed and now comprises 125 National Influenza Centers (NIC) in 96 countries together with 5 WHO Collaborating centers (CC), 4 Essential Regulatory Laboratories (ERL) in USA, UK, Japan and Australia and other ad-hoc groups. Together these laboratories process around 500 000 respiratory specimens per year to monitor influenza activity around the globe. Approximately 8000 of the viruses isolated by the NIC are shared with the CC for more extensive antigenic and genetic characterization *(28)*. The resulting information is collected and used on an

TABLE 1

Changing formulation of influenza virus vaccines.

Influenza season	H1N1	H3N2	B type
1998-99	A/Beijing/262/95	A/Sydney/5/97	B/Beijing/184/93
1999-00	A/Beijing/262/95	A/Sydney/5/97	B/Beijing/184/93
2000-01	A/New Caledonia/20/99	A/Moscow/10/99	B/Beijing/184/93
2001-02	A/New Caledonia/20/99	A/Moscow/10/99	B/Sichuan/379/99
2002-03	A/New Caledonia/20/99	A/Moscow/10/99	B/Hong Kong/330/2001
2003-04	A/New Caledonia/20/99	A/Moscow/10/99	B/Hong Kong/330/2001
2004-05	A/New Caledonia/20/99	A/Fujian/411/2002	B/Shanghai/361/2002
2005-06	A/New Caledonia/20/99	A/California/7/2004	B/Shanghai/361/2002
2006-07	A/New Caledonia/20/99	A/Wisconsin/67/2005	B/Malaysia/2506/2004
2007-08	A/Solomon Islands/3/2006	A/Wisconsin/67/2005	B/Malaysia/2506/2004
2008-09	A/Brisbane/59/2007	A/Brisbane/10/2007	B/Florida/4/2006
2009-10	A/Brisbane/59/2007	A/Brisbane/10/2007	B/Brisbane/60/2008
2010-11	A/California/7/2009	A/Perth/16/2009	B/Brisbane/60/2008
2011-12	A/California/7/2009	A/Perth/16/2009	B/Brisbane/60/2008
2012-13	A/California/7/2009	A/Victoria/361/2011	B/Wisconsin/1/2010

annual basis in recommending influenza seasonal vaccine candidate viruses, once for the northern hemisphere (in February) and once for the southern hemisphere (in October).

Over the past several years (from 1998-99 season to 2012-13 season, Table 1), the formulation had to be changed due to antigenic drift of the strains circulating in the human population. Only in some of the seasons (presented in Table 1) the epidemiological surveillance indicated that the composition should be identical to the previous season.

Only egg-isolated viruses are used as potential vaccine candidates. It was understood that the egg will »filter out« many potential human viral contaminants from the clinical specimen and would not introduce any further mammalian viral contaminants.

TABLE 2

Major similarities and differences between inactivated and live attenuated influenza vaccines.

	Characteristic	Inactivated vaccine	Live attenuated vaccine
Similarities	composition	H1N1 H2N2 B type	H1N1 H2N2 B type
	substrate for virus growth	eggs	eggs
	composition redesign	annually	annually
Differences	virus	inactivated	live attenuated
	side effects	rare	occasional
	route of administration	s.c. (injection)	i.n. (sprayer)
	age of vaccinees	≥6 months	2–59 years

Any virus that has a »cell» (e.g., MDCK) passage history is deemed unsuitable as a candidate vaccine virus because the cells used by surveillance laboratories are not qualified for human vaccine manufacture and there is a precedent that all viruses used in vaccine production, including cell-based production, have been passaged only in eggs.

It is now a common practice to use reassorted strains giving high yields of the recommended surface antigens. Upon identification of suitable egg-derived candidate vaccine viruses, high growth reassortants are developed by co-infection of eggs with the recommended egg-derived vaccine strain and the high growth parental strain A/Puerto Rico/8/1934 (sometimes referred as A/PR/8/34 or PR8).

Such reassorted viruses are then given free of charge to vaccine manufacturers which then produce a vaccine for an upcoming season.

The current seasonal influenza vaccine is produced both as an inactivated and a live attenuated virus vaccine. Major similarities and differences between these two types of influenza vaccine are depicted in Table 2.

Inactivated influenza vaccine (IIV)

By definition, inactivated influenza vaccine is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in fertilized hen's eggs, inactivated and treated so that the integrity of the virus particles has been disrupted without diminishing the antigenic pro-

perties of the hemagglutinin and neuraminidase antigens. The stated amount of hemagglutinin antigen for each strain present in the vaccine is 15 μg per dose, unless clinical evidence supports the use of different amount *(29)*. There are several forms of inactivated vaccines depending on the integrity of the virion: whole virion, split virion, surface antigen or surface antigen presented in virosomes.

The prevailing inactivation method used today for the preparation of influenza vaccines is treatment with chemicals like formalin or beta-propiolactone, optionally followed by a detergent disruption process called splitting.

Whole inactivated viruses that are not split have consistently proved to be better immunogens and confer a more efficient protective response *(30)*.

The vaccine is administered by subcutaneous injection, and vaccine protection is mediated largely through the stimulation of HA-specific neutralizing Abs. This immunity is short-lived and antigen specific, but lacks any mucosal or T cell component.

Clinical studies have established that two doses of currently formulated inactivated vaccine are required to elicit protective Ab titers in immunologically naïve individuals. In practical terms, it means that each winter previously unimmunized children should receive two doses of vaccine one month apart, whereas single vaccine dose can protect previously primed children and adults.

Live attenuated influenza vaccine (LAIV)

There are two types of LAIVs licensed for human use: one is produced in Russia, the second is produced in the USA.

In Russia, live cold-adapted influenza virus vaccine based on master strain for influenza live reassortant vaccine A/Leningrad/134/17/57 (H2N2) *(31)* has been used for more than 30 years and administered to tens of millions of children with protective efficacy and without evidence of deleterious side effects. In addition, there is no sign of the spread of virulent revertants, either within Russia or globally *(32)*.

A cold-adapted LAIV produced in USA was licensed in 2003 and is approved for persons aged from 2 to 59 years *(33)*. Its basic concept is the attenuation of two master strains A/Ann Arbor/6/60 and B/Ann Arbor/1/66 by repeated passage on chicken eggs under decreasing temperature conditions. The final cold-adapted variants replicate only in the mucus membrane of the upper respiratory tract where temperature does not exceed 32–33 °C. The genetic basis of cold-adaptation are mutations in a number of the viral genes that code for the internal parts of the virus particle *(34, 35)*.

Both LAIVs are adminstered intranasally by sprayer, thus having the advantage of inducing a mucosal immune response that closely mimics the response induced by natural influenza virus infection.

In 2005, WHO stated that live, attenuated vaccines based on genetic reassortment and formulated for nasal spray delivery appear to be safe and efficacious and represent a substantial technical development, particularly in view of possible future mass vaccination campaigns *(36)*.

How to choose: IIV or LAIV?

Overall comparison of similarities and differences between IIV and LAIV are presented in Table 2. A meta-analysis was performed on eighteen randomized comparative clinical trials involving a total of 5000 vaccinees of all ages *(37)*. LAIV (cold-adapted) induced significantly lower levels of serum hemagglutination inhibiting antibody and significantly greater levels of local IgA antibody than IIV. Yet, although they predominantly stimulate different antibody compartments, the two vaccines were similarly efficacious in preventing culture-positive influenza illness. Safety concerns about the use of LAIV in humans have been expressed because: (a) of a close anatomical connection between the respiratory epithelium and CNS and the risk of vaccine-induced neurological complications; (b) spontaneous genetic changes with unpredictable consequences; (c) gene reassortment with non-human influenza virus to yield a virus with pandemic potential *(37)*. The choice between the two vaccine types should be based on weighing the advantage of the attractive non-invasive mode of administration of LAIV, against serious concerns about the biological risks inherent to large scale use of infectious influenza virus, in particular the hazard of gene reassortment with non-human influenza virus strains.

Alternative substrates for the production of influenza vaccine

An idea to use substrates other than eggs for the production of influenza vaccine has arisen from two major reasons: (i) genetic modification of the virus growing in eggs compared to the originally isolated virus and (ii) shortage of egg supply.

Influenza vaccine production is dependent on the availability of embryonated eggs for virus growth. This is an extremely cumbersome system with many disadvantages with respect to selection of virus variants and the presence of adventitious viruses.

To address the limitations of egg-based vaccines, research has focused on the development of cell-culture vaccine production technology using mammalian cells.

The use of mammalian cells was proposed for the propagation of influenza virus either in MDCK (Madine Darby canine kidney cells) *(38, 39)*, Vero (African green monkey kidney cells) *(40)* or PER.C6 (fetal retinoblast immortalized upon transfection with E1 of adenovirus type 5) *(41)* cells. Research over many decades indicates that when a human influenza virus is adapted to grow in eggs, it undergoes phenotypic changes that might include changes to its antigenicity/immunogenicity. A virus isolated on cells does not undergo the type of selection

that occurs during initial passage in eggs and typically the HA of a cell isolated virus is structurally and antigenically identical to the virus found in clinical material. Primary isolation of type A (H3N2) virus in MDCK cells resulted in the virus with HA identical to that of the virus replicating in the infected individual, whereas similar isolation of virus in the embryonated eggs resulted in the selection of variants with amino acid substitutions in the globular head region of HA molecule *(42, 43)*. The same was shown for human diploid cells MRC-5, money kidney cells LLC-MK$_2$ and guinea pig kidney primary culture *(44, 45)*.

Many studies showed that influenza vaccine produced on the MDCK *(46, 47)* or Vero *(40, 48-50)* cell culture is comparable with respect to immunogenicity and safety to a conventional egg-derived vaccine.

However, none of the seasonal vaccines (inactivated or live) has already been approved for use in humans.

PANDEMIC INFLUENZA AND MOCK VACCINE

In recent years, global pandemic preparedness initiatives have stimulated the research and development of new influenza vaccines for the control of epidemic and pandemic influenza outbreaks.

In the last century pandemics took place three times: a »Spanish flu« from 1918 to 1919 caused by the H1N1 virus resulted in 20–50 million death cases, an »Asian flu« from 1957 to 1958 caused by the H2N2 virus resulted in 2 million deaths and a »Hong Kong flu« from 1968 to 1969 caused by the H3N2 virus resulted in 500 000 deaths.

As each pandemic virus became established, it rapidly replaced the previous subtype. However, in 1977, an H1N1 virus last seen in 1950 reappeared and its drifted descendents have co-circulated with H3N2 virus to this day. Increased worldwide surveillance has also noted the introduction into the human population of novel strains that did not become pandemic, probably because they were unable to spread person-to-person. These include the 1997 Hong Kong H5N1 virus and the 1999 H9N2 virus. Once the pandemic is over, viruses accumulate mutations in the HA under the positive selection pressure of neutralizing Abs and within 4 years a virus can evade previously acquired immunity, and cause new epidemics *(51)*.

H5N1 viruses

Influenza infection in waterfowl tends to be asymptomatic, and the viruses are in ecologic stasis with the hosts *(2)*. In contrast, influenza virus A infections in humans elicit an immune response that provides selective pressure and drives the virus to evolve. In 1997, a highly pathogenic avian H5N1 influenza virus was isolated from 18 humans of whom 6 died, demonstrating direct transmission of avian influenza viruses to humans (52–55). A cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO in the 2003–2012 period (by May, 2012) is 603 cases and 356 deaths (59%) *(56)*.

Since then, a new pandemic threat has arisen due to avian influenza viruses, such as H5N1 strains, which are widespread in poultry and migratory birds and occasionally infect men.

2009 »Swine flu«

Swine influenza was first recognized as a disease entity during the 1918 »Spanish flu« pandemic. Data *(57)* support the hypothesis that the 1918 pandemic influenza virus was able to infect and replicate in swine, causing a mild respiratory disease, and that the virus was likely introduced into the pig population during the 1918 pandemic resulting in the current lineage of the classical H1N1 swine influenza viruses. Due to a strong resemblance of the clinical signs to the human influenza disease, a clinical name of »hog flu« was given by J.S. Koen to this new disease of pigs *(58)*.

In March 2009, a swine origin influenza A (2009 H1N1) virus was introduced in the human population and quickly spread from North America to multiple continents.

Interestingly, the 1918 swine influenza virus may still be circulating in swine *(59)*.

Swine has been proposed as an intermediate host (a »mixing vessel«) in the indirect transmission of influenza A viruses from an avian reservoir to humans, based on the unique distribution in pigs of α2,3- and α2,6-linked sialic acid moieties that are considered to be avian- and human-specific receptors for influenza A viruses, respectively. The HA gene of the 2009 H1N1 strain has been present in classical swine H1N1 viruses that have circulated in pigs at least since their discovery by Shope *(58)* in the 1930s. In contrast, the HA of human H1N1 influenza viruses circulating from 1918 to 1957 and from 1977 to the present drifted progressively away from the 1918 virus HA *(60, 61)*. The 2009 pandemic H1N1 virus caused greater morbidity and mortality in children and young adults while people over 60 years of age showed a higher prevalence of cross-reactive H1N1 antibodies, suggesting that they were previously exposed to an influenza virus or vaccine that was antigenically related to the pandemic H1N1 virus. There are several studies which provide a mechanistic understanding of the nature of serological cross-protection in people over 60 years of age during the 2009 H1N1 pandemic. Krause *et al. (62)* showed that naturally occurring human monoclonal antibodies raised to 1918 influenza virus are able to bind and inhibit the 2009 H1N1 virus in vitro. O'Donnell CD *et al. (63)* studied in ferrets cross-protection H1N1 viruses from 1934, 1947 and 1950 against pandemic H1N1 2009 virus. They concluded that the loss of protective efficacy occurred between 1947 and 1950 and is associated with the additional glycosylation site.

The efficacy of seasonal live attenuated influenza vaccine (2008-2009 season) against virus replication and transmission of a 2009 H1N1 virus in ferrets was studied *in (64)*. Results showed that immunization with seasonal LAIV or H1N1 virus infection provides some cross-protection against the 2009 H1N1 virus, but had no significant effect on the transmission efficiency of the 2009 H1N1 virus.

Development of anti-pandemic vaccines

The match between vaccine virus strains and epidemic virus strains is generally good because the evolution of human influenza viruses is continuously monitored through careful, global virological surveillance, and vaccine strains are updated based on these data. For pandemic influenza there is no such basis upon which to predict the antigenic characteristics of the virus which will cross the species barrier and cause the pandemic.

The high case-fatality rates caused by outbreaks of H5N1 in 2004 highlighted the huge shortfall in global influenza vaccine production capacity in the event of a pandemic. Initiatives have been undertaken to raise the preparedness to the level which will decrease morbidity and mortality in the case of pandemic. A major initiative launched by the WHO to meet the Global Pandemic Influenza Action Plan objective to increase vaccine supply involved the transfer of influenza vaccine production technology to developing countries *(65)*.

Since pandemic influenza can occur at any time of year and may spread rapidly through the world, vaccines will form the main prophylactic measure against pandemic influenza. Speed in vaccine development is vital and there are guidelines which provide the basis for a fast track licensing procedure for pandemic vaccines within the EU and USA *(66, 67)*.

In order to ensure that, when the time comes, a vaccine can be produced, tested and shown to be safe, immunogenic and protective, WHO has asked vaccine manufacturers to start making vaccine based on strains that may be related to an eventual pandemic strain, naming such vaccines as »mock vaccines«.

The investigational H5 and H9 inactivated »mock« vaccines have been shown to be less immunogenic than interpandemic influenza vaccines. The amount of HA required in pandemic vaccines to elicit a serum Ab response of a magnitude similar to that of the licensed interpandemic influenza vaccine is likely to exceed the 15 μg present in the current inactivated virus vaccine *(68, 69)*. Such a low Ab response is explained by the naïve immune system to avian influenza viruses which has not been circulated in human population so far. Two-dose regimen or the addition of an effective adjuvant have been proposed in order to improve the response to such vaccines *(70–73)*.

In contrast, clinical trials of pandemic A/H1N1 vaccines have shown a single vaccine dose to be sufficient to induce adequate levels of seroprotection *(74–77)*.

ALTERNATIVE INFLUENZA VACCINES STILL IN DEVELOPMENT

Despite the global use of the seasonal vaccine (either IIV or LAIV), these vaccines have major restraints: (i) they must be administered yearly to population due to the high variability of HA and NA, (ii) they cannot be manufactured until the circulating viral strains have been identified and (iii) the efficacy rate goes as low as 59% in 18–64 year-old population, while falling to 35 % in those over 65 years of age *(78)*. Therefore the need is highlighted for novel vaccination forms (in particular universal, pan-influenza vaccine) and formulations to circumvent these limitations.

PAN-INFLUENZA VACCINE BASED ON CONSERVED HA EPITOPE(S)

New strategies to design the influenza vaccine rely on selective inducing of broadly neutralizing antibodies that are specific for highly conserved viral epitopes – a pan-influenza vaccine – providing universal strain coverage.

Using whole virus or large viral proteins in the vaccine provides B cells with a wide variety of binding sites. This dilutes the desired broadly protective antibody response with non-neutralizing specificities, as well as specificities that are confined to strains within the same subtype. However, routine vaccination can produce powerful, heterosubtypic antibodies that provide protection against novel strains of influenza virus (reviewed in *79*). Manipulating the vaccination regimen or prime/boost combination can further enhance the quality and quantity of the antibody response *(79)*.

The humoral response to influenza comprises both newly activated naïve cells and recalled memory B cells. Many influenza epitopes shift each year, therefore newly activated cells will be specific for influenza antigens unique to the latest variant, whereas recalled memory cells would be specific for epitopes that have not changed. In 2008 it was demonstrated that individuals born in 1915 or earlier still had circulating memory B cells to the 1918 influenza pandemic strain *(80)* indicating that the B cell memory compartment is extremely long lived. The very same antibodies isolated against the 1918 pandemic strain cross react to a conserved epitope on the 2009 pandemic H1N1 strain *(62)*.

Furthermore, mAbs from human IgM^+ memory B cells from the library constructed from B cells of patients immunized with the seasonal vaccine were heterosubtypic in that they had neutralizing activity against antigenically diverse H1, H2, H5, H6, H8 and H9 influenza subtype (group 1) and the Ab CR6261 was able to confer prophylactic protection and therapeutic efficacy in mice challenged with H5N1 or H1N1 viruses *(81)*.

The latest and most exciting discovery was of a neutralizing Ab that recognizes a stalk epitope conserved by virtually all influenza A strains that are infectious to humans *(82)*. Corti *et al. (82)* screened 104 000 peripheral blood plasma cells from eight donors infected

and/or vaccinated for Abs that recognize all HA subtypes. They were able to isolate four plasma cells which produce the same Ab named FI6 and bind to all 16 HA subtypes, neutralize infection and protect mice and ferrets from lethal infection. Crystal structure of FI6 Ab bound to H1 or H3 HA proteins revealed that it binds to a stalk domain which connects the head to the viral membrane and is important for the fusion of the viral and host cell membranes so that the pathogen can invade human cell. The immune system usually does not have a strong immune response to the partially hidden stalk domain. A globular head domain that binds to cellular receptors during viral entry contains the major antigenic sites targeted by the immune system. However, there are other modes of Ab action as mentioned above.

The key to a universal vaccine is to induce these Abs in a more dominant fashion (reviewed in *79*).

Besides the conserved HA stalk region, other studies have emerged to show that the conserved extracellular domain of the viral M2 protein (M2e) is able to elicit cross-reactive Abs that can confer protection in mice challenged with H5N1 or H1N1 viruses *(83, 84)*.

TARGETED DESIGN OF A LAIV

The advent of techniques to engineer site-specific changes in the genomes of negative-strand RNA viruses *(85-89)* has made it possible to consider new vaccine approaches. Specifically, it is now possible to tailor-make strains with unique properties that lead to attenuation.

Research has been focused on: (i) modification of the promoter regions of a specific viral gene, (ii) modification of the amino acid composition of a specific protein or (iii) modification of the nucleotide composition of a specific gene creating non-optimal triplet codon composition.

Garcia-Sastre A *et al. (90)* showed that the NS1 protein of influenza viruses has IFN-antagonist activity. Changing the NS1 protein can result in an altered virulence characteristic. Truncated NS1 proteins are responsible for increased attenuation of both influenza A and influenza B viruses in mice *(91)*. Genetically modified viruses expressing N-terminal 99 to 126 aa of the NS1 protein possess intermediate IFN-antagonist activity in mice. Such genetically engineered viruses may have optimal phenotypic characteristics for stimulating a robust immune response in humans, while at the same time being safely attenuated because they cannot completely overcome the IFN response of the host *(91–93)*. It was later shown that specific viral protein expression by NS1--truncated viruses is known to be decreased in infected cells. Since the HA protein is the major immunogenic component in influenza virus vaccine, Maamary J *et al. (94)* created the influenza virus carrying G3A C8U »superpromoter« mutations in the HA genomic RNA segment. This strategy retained the attenuation properties of the recombinant virus but enhanced the expression level of the HA protein in infected cells, and the mice immunized with such virus demonstrated enhanced protection from wild-type virus challenge compared to the mice vaccinated with the virus without the »superpromoter« *(94)*.

Other authors have been working on different influenza genes, modifying either non-translated or translated regions. Introduction of silent mutations in conserved regions of the NP protein was proven to be a promising strategy, allowing attenuation of the master strain but leaving the antigenicity of the gene product unaltered *(95)*.

Mutations in putative zinc finger (CCHH) of the M1 protein reduce virulence and such mutated attenuated virus protected mice from lethal challenge with wt influenza virus *(96)*. The M1 protein was also a target of the study of Liu T *et al.* (97) who made a double mutation in the nuclear signal *(98, 99)* of the M1 protein (R101S – R105S), which resulted in attenuated properties of this virus and suggested its potential in development of live attenuated influenza virus vaccines.

Polymerase complex has been shown to be a potential target to develop a novel attenuation strategy either by introducing mutations in a highly conserved protein--protein interaction of the viral polymerase subunits PA and PB1 (100) or by mutating the promoter region *(101)*.

A VACCINE BASED ON M2 PEPTIDE

The M2 protein of influenza viruses forms a proton channel in the virion and intracellular membrane and functions to release influenza virus genes from endosomes. The M2 protein Abs protect mice from subsequent challenge *(102, 103)*. This viral protein is highly conserved and Abs to M2 protein are cross-protective between different subtype virus infections, although the level of production is low *(104)*. A recombinant protein comprising the TLR5 ligand flagellin fused to four tandem copies of the ectodomain of the conserved influenza matrix protein M2 was expressed in *E.coli* and purified *(105)*. Mice immunized with 4xM2e in aqueous buffer, without adjuvants or other formulation additives, developed potent M2-specific Ab response that was quantitatively and qualitatively superior to those observed with M2 peptide delivered in alum. Also, 4xM2e protected mice from a lethal challenge with influenza A virus.

A COMBINED VACCINE TO NP, M1 AND M2

A broad CTL repertoire was detected in the human memory CTL specific for the NP, NA, HA, M1, NS1, and M2 viral proteins, the NP being the strongest internal antigen and a major target for $CD8^+$ cells *(106)*.

Based on this fact together with the knowledge that the NP and M proteins are the most conserved influenza virus proteins, Pleguezuelos *et al. (107)* designed a vaccine that consisted of equimolar mixture of four synthetic polypeptides for NP (two polypeptides), M1 and M2. These polypeptides represent potentially immunoreactive conserved regions identified in silico and immunological

analyses in ferrets *(108)*. The Phase I clinical study showed that the potential vaccine formulation is safe and CD8+ T-cell response was induced compared to pre-vaccination level. However, no significant Ab response was developed. The authors of this vaccine suggest that cellular immune response may control and mitigate infection and illness during natural infection.

AN INTERFERING VACCINE

Preventive and therapeutic measures have improved since the time of the 1918 pandemic, but new approaches are needed. An interfering vaccine is one of them. The interfering vaccine comprises non-infectious, defective interfering (DI) influenza A virus.

In the course of replication, most viruses make defective interfering (DI) viruses, which are virus particles composed of a normal set of viral proteins encapsidating a deleted version of the viral genome. Because they lack essential genetic information, DI viruses are replication deficient. Replication of the defective genome is achieved by the presence in the same cell of genetically compatible infectious genome (helper virus), usually from the virus that has generated the DI genome and which provides the missing function(s) in trans. DI RNAs can arise from deletions in any segment, but originate most often from the three largest genomic RNAs *(109)*.

Noble S *et al. (110)* used a vaccine which was UV-irradiated to render it non-infectious but the vaccine retained its interfering activity. Such interfering vaccine was able to act prophylactically, preventing influenza in mice and also converting an otherwise lethal infection into one that is avirulent and immunizing if interfering vaccine was applied shortly before, during or shortly after the infection took place. Similar results were obtained from studies in ferrets by *(111)*.

Dimmock N J *et al. (112)* went further and showed that the particular defective influenza virus RNA of 244 nt incorporated into virion which, although non-infectious, delivers the RNA to those cells of the respiratory tract that are naturally targeted by infectious influenza virus. Those DI viruses protected mice against strong challenge of homotype virus H1N1 but also against other subtypes which were tested, H2N2, H3N2 and H3N8, when administered up to 1 week before infection. There was also a clear therapeutical benefit when the 244 virus was administered 24 to 48 h after a lethal challenge. The mechanism by which DI viruses prevent disease in treated animals is not fully understood. However, it seems that the mechanism is far more complex than solely a competition for replication with the challenge virus *(113)*.

DNA VACCINE

DNA vaccine has many advantages over current inactivated and live attenuated influenza vaccines: high surge capacity, stability and safety. Influenza DNA vaccine is based upon the use of a direct inoculation of purified plasmid DNA encoding various influenza viral genes which are constantly expressed and presented to the immune system via intracytosolic antigen presentation pathways and activate both cell-mediated and antibody responses.

Several studies have demonstrated that inoculation of plasmid DNA encoding HA of the influenza virus, via gene gun or intramuscular injection, elicited specific immune responses and provided protection against the influenza virus in mice, ferrets and chickens *(114–119)*. The findings of *(120)* demonstrate comparable localization of antibody forming cells in response to challenge in mice vaccinated with either HA DNA or live virus.

However, to achieve such a good response they had to administer the vaccine twice in amounts of as much as 100 µg per animal.

Additionally, some authors have shown that the immunostimulatory sequence within the plasmid backbone may stimulate the innate immune system which creates a cytokine milieu that favors the generation of a Th1-biased immune response.

A plasmid construct carrying M2e-HSP70c sequence was shown to be immunogenic in mice and was able both to confer protection against lethal challenge of H1N1 virus and reduce viral load in lungs of infected mice *(121)*.

Co-expression of HA and either RIG-I, IL-6 or IFNá6 demonstrated that DNA vaccine potency may be augmented by the incorporation of the immunoregulatory proteins (*122-124*, respectively). Influenza B type virus DNA vaccination is consistent with those obtained from the influenza A vaccination *(125)*.

While HA and NA-expressing DNA induced a high level of specific antibody response, M1-, NP-, or NS1-expressing DNA given to mice failed to provide protection against lethal viral challenge, although M1- and NP-DNA did induce detectable antibody response *(126)*. This was also shown earlier by Murphy and Clemens *(127)* by passive transfer of anti-M1 and anti-NP mAbs which failed to protect mice from challenge by a wild-type influenza virus.

Although progress has been impressive, with protection shown against influenza challenge following DNA vaccination, using DNA will be more appropriate for prophylaxis of animal influenza, at least until the light is shed on some of the unknowns.

ANTIVIRAL AGENTS (ANTIVIRALS) FOR THE TREATMENT OF INFLUENZA INFECTIONS

Although annual vaccination is the primary strategy for preventing complications of influenza virus infections, antiviral medications with activity against influenza viruses can be effective for the chemoprophylaxis and treatment of influenza. Four influenza antiviral agents

are available: amantadine, rimantadine, zanamivir and oseltamivir.

Amantadine and rimantadine are related adamantanes which inhibit virus replication during the early stage of infection by blocking the ion channel formed by the M2 protein *(128)*. These drugs are active only against influenza viruses of type A and the resistance level is extremely high. Viral resistance to adamantanes can emerge rapidly during treatment because a single point mutation at positions 26, 27, 30 31 or 34 of the M2 protein can confer cross resistance to both amantadine and rimantadine *(129, 130)*. By the year 2006, resistant H3N2 viruses predominated in all regions of the world *(131–133)*. Center for Disease Control (CDC) also reported in 2006 that 193 (92 %) of 209 influenza A (H3N2) viruses isolated from patients in 26 states of the USA demonstrated a change at amino acid 31 in the M2 gene that confers resistance to adamantanes *(134)*.

Zanamivir and oseltamivir are chemically related antiviral drugs. They are analogues of the viral N-acetyl neuraminidase and act as neuraminidase inhibitors *(135)*. They have activity against both influenza A and B viruses shortening clinical disease by 1.5–2.5 days but have to be administered within 2 days of infection *(136)*.

It was shown earlier that resistance to zanamivir and oseltamivir can be induced in influenza A and B viruses in vitro *(137–144)*, but induction of resistance required multiple passages in cell culture. By contrast, resistance to amantadine and rimantadine *in vitro* could be induced with fewer passages in cell culture *(145, 146)*.

Despite low levels of oseltamivir use in most countries, the resistance of virus to oseltamivir emerged in the 2007–2008 season in Europe *(147)* and has now spread from Europe to the rest of the world *(148)*. The resistance is caused by an amino acid substitution H275Y in the NA and is also associated with the D354G substitution *(28)*.

During the December 2008-January 2009 period, a total of 30 countries from all WHO regions reported oseltamivir resistance for 1291 of 1362 (=94 %) A(H1N1) viruses analyzed *(149)*.

CONCLUSION

Influenza remains serious disease despite the availability of seasonal trivalent vaccines and antivirals which are effective for most recipients. Although these modalities of medical intervention are helpful, new approaches are being developed (Figure 1). Major improvements, based on recombinant DNA techniques, promise to change the landscape of vaccinology against influenza and many other infectious diseases. However, it is unlikely that a universal vaccine protecting against all influenza viruses, and yet devoid of all unwanted characteristics, will be invented in spite of so many potentially new vaccine forms. The major reason for that is an enormous potential of this virus to mutate and evolve under selective pressure such as immune response. Only a very few of the new prophylactic and therapeutic approaches will reach the goal of being approved and licensed for use in human population. Others will be held somewhere along that way.

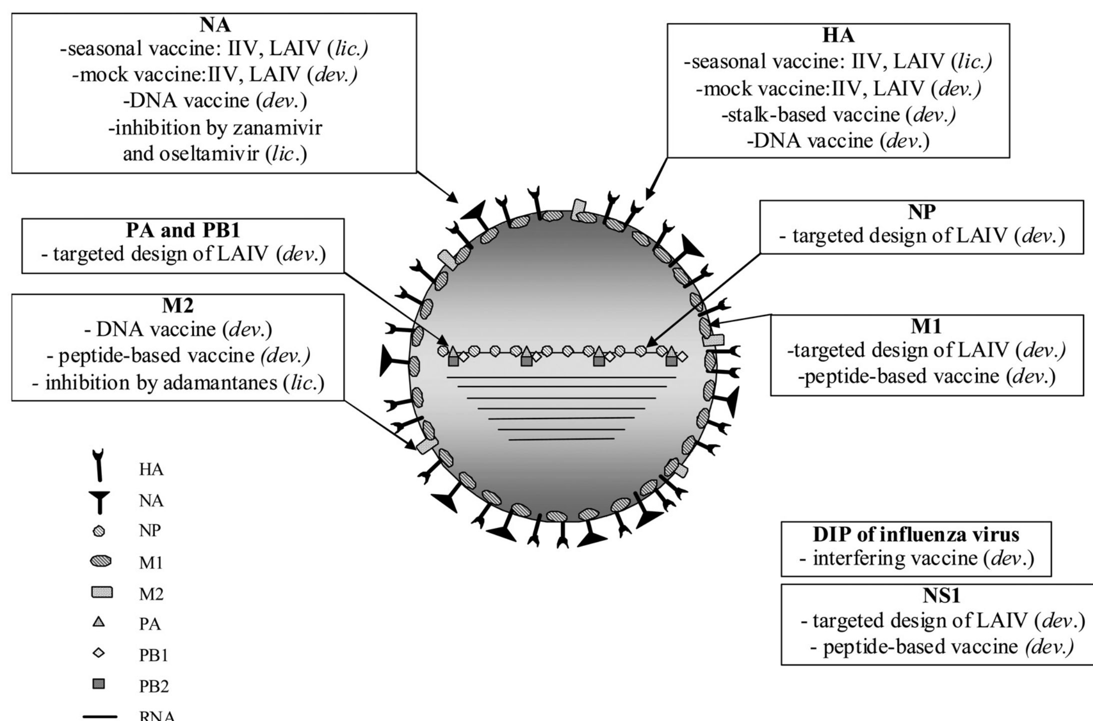

Figure 1. *Scheme of the influenza virus and targets of licensed* (lic.) *vaccines and antivirals and those in development* (dev.).

Even if a more perfect way to combat human influenza will be found, each one of us should be aware of the simple influenza paradox saying that even a highly effective vaccine for a widespread disease will only provide limited benefits at population level if the vaccine is grossly underutilized. On the other hand, modest effective vaccine might still provide considerable benefits at population level if widely used for the target population *(150)*. This concept, which was developed for influenza, is widely applicable to other infectious diseases preventable by vaccination.

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