New technologies for the control of influenza

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General introduction

Influenza in humans is caused by viruses from one of three genera within the family of Orthomyxoviridae: influenza A virus, influenza B virus, and influenza C virus. While influenza B and C viruses are predominantly human pathogens that have sporadically been isolated from seals and swine, respectively [1, 2], influenza A viruses have been isolated from many species including humans, pigs, horses, mink, marine mammals, and a wide range of domestic and wild birds [3, 4]. The primary hosts of influenza A viruses are wild aquatic birds, namely ducks, geese and shorebirds among which a wide variety of influenza A viruses circulate, generally without causing disease. Influenza A viruses are classified on the basis of the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins expressed on the surface of virus particles. To date, influenza A viruses representing 16 HA and 9 NA subtypes have been detected in wild birds and poultry [5].

Disease burden

Whereas cases of influenza C virus infection are relatively rare, influenza A and B viruses cause annual epidemics during autumn and winter in temperate regions and circulate throughout the year in some tropical countries with one or two peaks during rainy seasons. Such “seasonal influenza” is characterized by sudden onset of fever, cough, headache, muscle and joint pain, sore throat and runny nose. Although most people recover within a week without requiring medical attention, influenza yearly results in about three to five million cases of severe illness and up to 500,000 deaths worldwide, particularly among the very young, elderly and chronically ill [6]. Furthermore, influenza A viruses occasionally cause major pandemics involving multitudes of these numbers of severe illness and death.

Several characteristics of influenza viruses are responsible for their continuous disease burden. First, influenza is primarily transmitted via air droplets which are produced by sneezing, coughing and even talking and spreads rapidly under favorable conditions. Second, influenza viruses evolve rapidly as a consequence of their segmented (negative sense) RNA genomes. Due to the inherent lack of proofreading capability of RNA polymerases, point mutations readily accumulate in the viral genome. When such mutations lead to aminoacid substitutions in the major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), the virus may escape from antibodies induced by vaccination against, or previous infection with, a closely related strain. This “antigenic drift” causes the regular occurrence of influenza epidemics. Furthermore, when two different influenza A viruses co-infect the same cell, progeny virions with new combinations of the RNA segments, so called “reassortants”, can be formed. When such a reassortant contains HA and NA segments derived from a bird or swine influenza virus
against which humans have no immunity, an event called “antigenic shift,” it may cause a pandemic, at least when it is easily transmitted from human to human.

**Pandemics**

During the past century, four pandemics have occurred of which the “Spanish flu” in 1918, was the most devastating, causing the death of approximately 50 million people [7, 8]. Whereas the subtype H1N1 virus that caused the Spanish flu appears to be an avianlike virus derived entirely from an unknown source [9, 10], the “Asian flu” (H2N2) and “Hong Kong flu” (H3N2) pandemics in 1957 and 1968 were the result of reassortment between human viruses and strains from the avian reservoir. The fourth pandemic, the “Russian flu” in 1977 was caused by the sudden re-emergence, presumably from a laboratory freezer, of the H1N1 subtype [11, 12] which had been circulating since 1918 until it disappeared with the emergence of the H2N2 pandemic strain in 1957. In turn, viral descendants of this “Asian flu” strain disappeared from human circulation with the appearance of the H3N2 “Hong Kong flu” in 1968. As a result, influenza A viruses of subtypes H1N1 and H3N2 currently circulate among humans undergoing gradual antigenic drift and causing annual epidemics. In addition, two antigenically and genetically distinct lineages of influenza B viruses, called “Victoria lineage” and “Yamagata lineage” co-circulate among humans (figure 1).

![Figure 1. Influenza viruses circulating in the human population. Influenza A viruses with three different hemagglutinin subtypes (H1, H2 or H3) and two neuraminidase subtypes (N1 or N2) have been identified in humans, together with 2 lineages, “Victoria” and “Yamagata” of influenza B virus. Solid squares indicate the introduction of the pandemic H1N1, H2N2 and H3N2 strains in 1918, 1957 and 1968, respectively. In 1977, H1N1 viruses similar to those of 1950 were reintroduced. Broken lines indicate the absence of virus isolates and only indirect evidence for circulating strains based on serologic data (adapted from P. Palese, Nat. Med. 2004 Dec 10).](image-url)
Over the past decade, human infections with avian influenza viruses, particularly highly pathogenic viruses of subtype H5N1, have led to pandemic alerts [13]. To date, over 400 confirmed human cases have been reported to the WHO with a fatality rate of over 60% [14]. So far however, these viruses have failed to transmit efficiently from human-to-human and get established in the human population. This may, at least in part, be due to the specificity of the HA for its cellular receptor, which is a major determinant of host range. Human influenza viruses preferentially recognize sialyloligosaccharides containing SAα2,6Gal [15, 16], matched by mainly NeuAcα2,6Gal linkages on the epithelial cells of the human trachea [17]. By contrast, avian viruses preferentially recognize SA 2,3Gal sialic acids [15, 16], in accordance with the predominance of sialyloligosaccharides with SAα2,3Gal linkages on the epithelial cells of duck intestine.

Although viruses with avian-type receptor specificity can infect humans, for efficient human-to-human transmission, the HA derived from an avian virus must preferentially recognize the human receptor [18]. Several reports have documented mutants of H5N1 viruses that have some affinity for α2-6 linked sialic acids, but they still retained predominant avian-type specificity [19-21]. Nevertheless, recent avian H5N1 viruses exhibit an increased propensity for acquiring human receptor specificity [22] and continue to pose a significant pandemic threat.

*Swine flu*

It was however a new strain of H1N1 that emerged in March and April 2009 in Mexico and the United States and rapidly spread across the globe that caused the WHO to declare a pandemic on June 11th [23, 24]. Swine are the suggested source of this virus as it is a reassortant from several viruses circulating in this species [25-27]. A role of domestic pigs in the ecosystem of influenza A is not something new as descendant lineages of the 1918 H1N1 and the reassorted human H3N2 viruses are enzootic in pigs. Pigs are highly susceptible to both human and avian influenza viruses [28], most likely due to the fact that epithelial cells of their trachea contain both SAα2,6Gal and SAα2,3Gal linkages [29]. Domestic pigs have therefore been described as a hypothetical “mixing-vessel” mediating by reassortment the emergence of new influenza viruses with avian-like genes into the human population, although no evidence exist that the 1957 or 1968 pandemic viruses originated in pigs.

Although currently the severity of the swine-origin H1N1 influenza pandemic appears moderate, it can vary, depending on many factors, substantially from one country to another. To date, the majority of cases have been detected and investigated in comparatively well-off countries in some of which around 2% of cases have developed severe illness, often with very rapid progression to life-threatening pneumonia. How this virus will behave under conditions typically found in the developing world is unknown. Furthermore, the virus may become more deadly over time as seems to have been the
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case during the 1918 pandemic in which a relative mild spring wave was followed by more severe second and third waves in autumn and winter [30].

Determinants of virulence

Obviously, the course and outcome of an influenza virus infection is determined by both the host and virus. Pre-existing immunity of the host by vaccination or prior exposure to a related strain will mute the effects of infection, but in an immunologically naive host, virulence is mostly determined by the virus. Numerous studies have shown that influenza virus virulence in mammalian species is a polygenic trait, which may require a critical constellation of genes [31-34]. However, a single gene (or a mutation in a single gene) can also markedly affect virulence. Next to its role in receptor binding (and thus host range restriction), the HA protein promotes the release of the viral genome and replication machinery in the cytoplasm of the host cells by mediating fusion of the viral envelope with the endosomal membrane. It is synthesized as a precursor protein that is cleaved into 2 subunits (HA1 and HA2) by host cell proteases. HA cleavage is a prerequisite for fusion of the viral and endosomal membranes and, therefore, for viral infectivity [35]. Low pathogenic avian influenza viruses possess a single Arg residue at the cleavage site, recognized by extracellular, trypsinlike proteases. These proteases are thought to be secreted only by cells of the respiratory and intestinal tract and consequently limit infections to these organs. In contrast, highly pathogenic avian viruses possess multiple basic amino acids at the cleavage that are recognized by ubiquitous intracellular proteases that thus trigger systemic infection. The cleavability of the HA is therefore considered a major determinant of tissue tropism of avian viruses [36]. Whereas the HA protein initiates infection by mediating virus binding to sialic acid-containing host cell surface molecules, the NA protein promotes the release of newly synthesized particles from infected cells by removing sialic acid residues. Efficient virus replication thus requires the balanced actions of both proteins. Accordingly, mutations in NA have been described that match its substrate specificity (SAα2,6Gal or SAα2,3Gal sialic acids) with the receptor specificity of the HA protein [37, 38]. The efficiency of virus release is furthermore influenced by the length of the NA stalk, which separates the head region with the enzymatic center from the transmembrane and cytoplasmic domains. Typically, shortened stalks result in less efficient virus release since the active site in the head region cannot efficiently access its substrate [39, 40]. However, the sialidase activity of NA proteins with a short stalk is more resistant to low pH [41] and a deletion in the NA stalk is believed to extend the tissue tropism of the virus by allowing replication in the upper digestive tract [18]. But other genes also play a role in virulence. The NS gene of the 1918 virus, for example, was shown not to be optimal in mice when it replaced the NS gene of a mouse-adapted
strain [42], probably as a result of the high degree of species-specificity of the interferon antagonist activity encoded by this gene [43]. Furthermore, the disease symptoms experienced by pigs infected with a highly pathogenic H5N1 virus increased substantially by a single aminoacid substitution at position 92 of the NS1 protein [44]. Other examples of single mutations influencing the outcome of infection concern aminoacid changes at positions 627 of the PB2 protein and position 66 of the PB1-F2 proteins, which alter the ability of the virus to replicate and induce apoptosis, respectively [45-47]. Thus, reassortment events as well as specific mutations in a single gene may render a more pathogenic virus.

**Influenza Surveillance**

The most effective way to prevent the disease or severe outcomes from the illness is vaccination. Although influenza vaccines have been available and used for more than 60 years, influenza viruses, particularly of subtype A, continue to be a challenging target for vaccination. Whereas antibodies against viral proteins as NA and matrix protein 2 (M2) reduce disease severity, only antibodies directed against HA can effectively neutralize the viral infection. The difficulty arises not only from the existence of 16 different HA serotypes, but also from the two mechanisms that create antigenic diversity, antigenic drift and antigenic shift. Since vaccination is only effective when the vaccine viruses are well-matched with circulating viruses, the WHO Global Influenza Surveillance Network (GISN), a partnership of National Influenza Centers around the world, monitors the influenza viruses circulating in humans. Based on the antigenic, genetic, human serological, and epidemiological data collected, WHO annually recommends a vaccine composition that targets the three most representative strains of each subtype (H1N1, H3N2, B) in circulation. For the northern hemisphere, the recommendation is made in February, to allow for the vaccines to be manufactured and people to be vaccinated in time for the influenza season which typically peaks sometime between December and March. The process is similar for the southern hemisphere with the recommendation made in September. Effectively, vaccines strains have to be selected almost 1 year before the influenza season in which they will be used [48].

**Current influenza vaccines**

Annually, almost 300 million doses of influenza vaccine are produced [49]. Although intranasally administered live attenuated vaccines are available in some countries [50], the vast majority of influenza vaccines are intramuscularly administered preparations containing the HA and NA proteins of the three recommended strains that are purified, to various extends, from chemically inactivated viruses grown in the allantoic cavity of embryonated chicken eggs. For the H1N1 and H3N2 components of these trivalent
inactivated vaccines, high egg-growth reassortant viruses are used to improve yields. Such reassortants are derived by coinfecting embryonated hens’ eggs with the circulating strain and the egg-adapted A/Puerto Rico/8/34 (PR8) strain, and subsequent screening of the progeny for viruses that have the haemagglutinin (HA) and neuraminidase (NA) segments of the first, and at least those internal segments of the second that confer a high growth phenotype in eggs. This seed virus is subsequently used for large-scale vaccine production in eggs (figure 2). In addition to increased growth, the reassortant strains are attenuated for humans, which provides an extra level of safety in the manufacturing process [51]. Reassortants also form the basis of live attenuated vaccines. In these strains, the HA and NA genes of the target viruses are incorporated into the backbone of master strains that are attenuated by cold adaptation. Cold adaptation introduces temperature sensitivity, which limits the replication to the upper respiratory tract. The primary mechanism of inactivated influenza vaccines is the production of antibodies that target the HA and inhibit infection by preventing attachment of the virus to host cells. Vaccination with inactivated vaccines prevents laboratory-confirmed influenza in 70-90% of adolescents and adults (14-60 years of age) [52]. In contrast to inactivated vaccines, live attenuated vaccines are thought to stimulate both humoral and cellular immuneresponses [53]. These latter may be targeted toward more conserved viral proteins resulting in an advantage when a mismatch occurs between the vaccine and the circulating strain. However, the two studies that directly compared the efficacy of live attenuated vaccines

Figure 2. Traditional method of influenza vaccine production. First, a vaccine seed virus is generated by co-infection of eggs with the clinical strain against which the vaccine should offer protection and a backbone strain that grows well in eggs. Next, the progeny virions are screened for reassortants that combine the HA and NA proteins of the clinical strain with a high growth phenotype in eggs. Antibodies against the HA and NA of the backbone strain are used to aid the selection process. The seed virus is subsequently inoculated into the allantoic cavity of chicken eggs at large scale, after which the virus is harvested, inactivated and the HA and NA proteins are purified.
and inactivated vaccines to date found no difference, and the inactivated vaccine to be more efficacious, respectively [54, 55]. Factors such as how well the vaccine strains match the circulating strains and age of the vaccinees may influence the results of such comparisons and a definitive answer will require further head-to-head trials [56].

**Limitations of current influenza vaccines**

Despite the annual recommendations for vaccine strain composition by the WHO, antigenic mismatches between the vaccine virus strain and the circulating strain occur that negatively influence vaccine effectiveness [57]. Between 1997 and 2007, there were five occurrences of mismatch, and 11 occurrences of partial mismatch across the three vaccines strains in Europe and the USA [58]. Factors causing such mismatches include: (1) appearance of drifted viruses after the recommendation has been made; (2) inability to isolate a seed virus in egg for vaccine manufacture, or the absence of a high-growth reassortant seed; (3) co-circulation of different viruses from the same subtype. Furthermore, although influenza illness affects people of all ages, adults over 65 years of age account for approximately 90% of all influenza-related mortality [59]. Vaccination programs currently recommend that older adults (USA: ≥50 years of age [60]; Austria, Germany, Hungary, Russia: ≥60 years of age; most other European countries: ≥65 years of age [61]) should be vaccinated against influenza, as well as people who live with, or care for older adults [60]. However, current influenza vaccines are less effective among older adults than among younger adults [52, 60, 62-64] and prevent laboratory-confirmed influenza in only 30-40% of people over 65 years of age [52]. This is caused by changes that occur in the immune system with advancing age resulting in a reduced immune response and reduced capacity to produce antibodies [65, 66].

**Treatment**

Currently, two classes of drugs are available for the treatment and/or prophylaxis of influenza infections: the adamantanes or M2 inhibitors, and the neuraminidase (NA) inhibitors. However, the adamantanes (amantadine and rimantadine) are associated with several toxicities, particularly of the central nervous system, rapid emergence of drug-resistant strains, and are not active against influenza B viruses [67, 68]. Compared to the adamantanes, the two licensed NA inhibitors zanamivir (Relenza) and oseltamivir (Tamiflu) are associated with little toxicity and are less prone to selecting for resistant influenza viruses [69, 70]. Nevertheless, emergence of resistance after oseltamivir treatment has been reported for both seasonal and avian influenza strains [71-75] and recently for the pandemic swine-origin H1N1 strain [76, 77]. Moreover, during the 2007-2008 influenza season, oseltamivir resistance among H1N1 viruses increased significantly worldwide, apparently unrelated to oseltamivir use [78] and oseltamivir-resistant H1N1
viruses are now circulating on all major continents [79, 80]. Although to date, these viruses are usually susceptible to zanamivir, the increasing use of zanamivir monotherapy due to the increasing resistance to oseltamivir may well lead to the development of zanamivir resistance [81]. Furthermore, the use of zanamivir is limited to patients who can actively use an inhaled drug, which excludes young children, impaired older adults, or patients with underlying airway disease [82]. There is thus an urgent need for the development of new strategies to prevent and treat influenza infections.

**New vaccine options**

Several approaches are being explored to develop vaccines that provide both higher and broader protection against homologous and heterologous strains. These include adjuvanted and virosomal vaccines, high-dose vaccines, vaccines based on foreign expressed HA, MVA- and adeno-based recombinant viral vector vaccines, the use of live attenuated vaccines in combination with inactivated vaccines, DNA vaccines, the use of different modes of delivery, and alternative (less variable) antigens (reviewed in [56, 83]).

One of the most advanced alternative approaches concerns the substrate on which vaccines are produced. The current production on embryonated chicken eggs can take up to 9 months and requires meticulous planning. Using cell culture systems instead of eggs will add substantial flexibility to the influenza vaccine production process as it is easier to manage an inventory of frozen cells than flocks of chickens. Although the end user will probably not notice any difference between the egg-grown and cell culture-grown vaccines, the increased speed of production of the latter will allow for better responses to antigenic drift and the sudden emergence of pandemic strains. Cell types that are being explored as substrate for the production on influenza vaccines include Madin-Darby canine kidney (MDCK) cells, Vero cells and human immortalized PER.C6 cells [56].

Of these three, MDCK cells are the standard for surveillance of circulating strains and clinical diagnosis on which annual update of the influenza vaccine relies. However, in recent years, growth of clinically representative influenza isolates, particularly those of the H3N2 subtype has become increasingly difficult in MDCK cells due to changes in their receptor binding properties that have occurred during their continued circulation in the human host [84]. MDCK cells overexpressing α-2,6-sialyltransferase have been shown to improve primary isolation and growth of these viruses compared to conventional MDCK cells [85-87]. PER.C6 cells express both SAα2,6Gal and SAα2,3Gal on the surface and support the replication of influenza virus [88]. However it has not been investigated whether the abundance of SA on PER.C6 cells limits the growth of recent influenza strains. **Chapter 2** describes the generation of a PER.C6 cell line stably overexpressing ST6Gal1, PER.C6-α2,6ST and compares the susceptibility to infection and ability to support growth
of recent human influenza strains with that of the parental cells. As discussed, for the H1N1 and H3N2 components of trivalent vaccines high egg-growth reassortants are used. The traditional method of co-infecting eggs with the target and backbone strains is lengthy due to the randomness of the reassortment process and subsequent need to screen progeny viruses for the desired genotype. Furthermore, the method requires that all strains are capable of egg growth. This has to lead to cumbersome, and sometimes unsuccessful, searches for isolates capable of egg growth that matched with circulating strains. A recent example is the H3N2 influenza A/Fujian/411/02 virus that was predicted to be the dominant strain in the 2003-2004 season. Since the A/Fujian/411/02-like viruses did not replicate in eggs, it was difficult to produce a matched vaccine [89, 90]. In fact, such a vaccine strain could not be produced in time for the seasonal vaccine production cycle.

The plasmid-based “reverse genetics” technology allows for the generation of influenza viruses entirely from cloned viral cDNA [91, 92]. By removing the random aspect of the reassortment process, reverse genetics makes it possible to create high-growth vaccine seed strains at unprecedented speed (figure 3) and even to incorporate desirable safety and growth characteristics [56, 93-96]. Successful recovery of recombinant viruses using plasmid-based reverse genetics relies on the transfection efficiency of the cells used, and efficient amplification of the recombinant virus. Because of these technical requirements, and because of regulatory requirements for a well-characterized cell bank, the number of cell lines that can be used for the generation of vaccine seed strains is limited. In chapter 3, the suitability of PER.C6 cells for the recovery of influenza vaccine seed strains by reverse genetics is assessed. The generation of vaccine seed strains on cell culture has been inhibited by concerns about the potential carryover of adventitious agents from clinical specimens. Egg propagation is thought of as a filter for such agents. Since the use of reverse genetics for

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**Figure 3.** Vaccine seed strain generation using reverse genetics. Sets of plasmids encoding the viral RNA segments (vRNAs) of HA and NA of a clinical strain and the other 6 segments of a backbone strain, together with plasmids encoding the viral replication machinery (PB1, PB2, PA and NP) are transfected into eukaryotic cells. In cells that received all plasmids, new copies of the vRNAs and viral proteins are generated leading to the formation of infectious viruses with the desired constellation which can subsequently be amplified in eggs.
the generation of vaccine seed strains eliminates this risk, seeds strains can be generated for vaccine production on cell culture. Since for many human influenza isolates, propagation in eggs selects for mutations [97] some of which give rise to a mismatch in antigenicity between the egg-grown virus and the naturally circulating virus [98], such an approach may yield vaccines that are better matched with the circulating strains. However, since current manufacturing capacity is largely based on eggs, an ideal seed virus should replicate in both cell culture and eggs. In chapter 4 we assess the possibility of using reverse genetics to generate a vaccine seed capable of growth in both eggs and cell culture with the antigenic properties of a virus that itself did not grow in eggs.

New treatment options

A number of alternative antiviral drugs are being investigated, including Intravenous zanamivir for patients hospitalized with severe influenza and for those in whom neither oral nor inhaled routes are an option; Peramivir, a neuraminidase inhibitor that is being developed in intravenous and intramuscular formulations; Long-acting inhaled neuraminidase inhibitors based on enhanced potency of dimeric derivatives of zanamivir; Fludase (DAS181): A sialidase fusion construct that cleaves the sialic acid receptors that influenza viruses use for attachment, removing influenza receptors from the airway epithelium and preventing infection of lung cells; Cyanovirin-N: A hemagglutinin inhibitor that may block viral entry; Short interfering RNAs; and T-705: A substituted pyrazine compound that is active against neuraminidase-inhibitor–resistant and amantadine-resistant viruses and that probably inhibits the RNA polymerase. Other promising antiviral avenues under investigation include signal transduction inhibitors and interferon inducers (reviewed in [67]).

A different approach for the prophylaxis and treatment of influenza concerns the use of passive immunization [99]. The potential for passive immunization against influenza virus has been evident since the 1918 pandemic where transfusion of human convalescent sera was associated with a reduction in mortality [99]. Recently, the benefits of treatment with convalescent plasma in instances of H5N1 influenza have also been reported [100, 101]. Monoclonal antibodies (mAb) are attractive biologic drugs due to their exquisite specificity and well understood mechanisms of action [102] and in recent years considerable effort has been directed to the generation of neutralizing mAbs against H5N1 viruses [103-106]. Some of these antibodies have broad H5N1 cross-clade reactivity [104, 105], or cross-subtype reactivity to H1 viruses [105], and are effective in suppressing H5N1 virus disease mice [104]. Recently, human monoclonal antibodies able to neutralize an unprecedented spectrum of influenza virus subtypes have been derived, and some to these antibodies provide protection in experimentally infected mice [107, 108]. Instead of blocking viral attachment to the host cell by binding to the highly variable receptor binding site on the globular head of the HA, these antibodies
target conserved domains in the membrane-proximal stem of the HA and neutralize the virus by inhibiting membrane fusion and uncoating of the virus [107, 109]. These antibodies are particularly attractive candidates for mAb-based immunotherapy due to broadly neutralizing activity.

In chapter 5, the prophylactic and therapeutic efficacies of mAb CR6261, representative of this novel class of antibodies, is compared to those of the leading antiviral oseltamivir in lethal mouse models for H1N1 and H5N1 influenza. In chapter 6, the efficacy of mAb CR6261 is assessed in a highly stringent ferret model of H5N1.

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