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Influenza virus –
Protection and Adaptation

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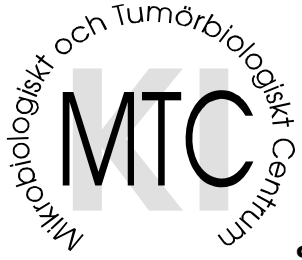

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Front page: Flying birds symbolises that influenza virus can travel fast with the help of wings and that many different variants of the virus may infect new hosts. Three big coloured circles symbolise the three great influenza pandemics occurring during the 20th century. Red blood cells symbolise sufferers and the diagnostic importance of erythrocytes.

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Summary

Influenza is an acute respiratory disease caused by influenza type A and B viruses. Human influenza viruses may infect up to 15% of the total population during the seasonal epidemics, causing many cases of severe illness. Each year, approximately 350 million doses of influenza vaccine are produced for protection of the risk groups against severe disease. The thesis focuses on the protection against influenza virus infection and disease (Paper I and II) as well as the analysis of the antigen variation found in primarily the hemagglutinin (HA) gene of the virus (Paper III and IV).

The time required for the production of influenza vaccines is 6-8 months. A more effective and rapid method of production is desirable, both for the annual epidemics and in case of an influenza pandemic. A DNA-based vaccine could be a useful alternative. In paper I, the immunological response in ferrets after intramuscular immunisation with a plasmid construct expressing chimeric influenza HA proteins was evaluated. Strain specific antibodies were elicited but none of the ferrets immunised with DNA or subunit vaccine were protected from infection when challenged with an influenza A/H3N2 virus homologous or heterologous to the vaccine. Considerable enhancement of the immune response induced by DNA immunization will be needed before the approach can be a realistic alternative for vaccination of humans.

Hemagglutination inhibition (HAI) is the standard method for determination of a protective antibody level against influenza. The method is not efficient for all virus subtypes and strains, and alternative methods suitable for large-scale examinations are desirable. In paper II, an *in situ* neutralisation test (NT) for the measurement of influenza antibodies was created and evaluated in two human cell-lines, human fibroblasts (HS27) cells and human salivary gland epithelial duct (HSG) cells, and in Madin-Darby canine kidney (MDCK) cells. The HS27 cell line gave stable results and was most suitable for antigen detection with enzyme-linked immunosorbent assay, and was chosen for the analysis of the humoral response after an influenza A infection in patients treated or not treated with the antiviral drug zanamivir. No titre differences between the groups could be verified at 28 days after onset. The NT using HS27 cells also revealed heterologous NT-titre rises after the influenza infection.

The antigenic drift occurring in the influenza viruses is mainly affecting the cell receptor binding glycoprotein, HA. It is important to identify antigenic changes in the HA occurring *in vitro* since they may be mistaken for drift occurring *in vivo*. In paper III, we characterised the variable region of the HA gene from nine recent human influenza A/H3N2 viruses after up to 11 passages in both HSG cells and MDCK cells. Ten amino acid alterations were identified in both MDCK and HSG propagated strains. All altered residues were either close to the receptor-binding site or within it. We found that alterations in the HA gene most likely represent an adaptation to growth *in vitro*.

To further study antigenic drift *in vitro* we have passaged a human influenza A virus in MDCK cells in the absence or presence of three polyclonal neutralising sera (Paper IV) and sequenced the HA gene from selected passages. One mutation causing the amino acid change I140M was found after 20 and retained after 28 passages in the presence of serum. Our analysis of the HA gene from different passages showed that during propagation *in vitro* in the presence of specific sera escape mutations in HA are not easily obtained and the study confirms the relative genetic stability of the HA gene of influenza A/H3N2 viruses in cell culture.

Papers included in the thesis

I. Ljungberg K., **Kolmskog C.**, Wahren B., van Amerongen G., Baars M., Osterhaus A., Linde A., Rimmelzwaan G. DNA vaccination of ferrets with chimeric influenza A virus hemagglutinin (H3) genes. 2002. Vaccine; 20:2045-2052.

II. **Mittelholzer C.M.**, Brokstad K.A., Pauksens K., Jonsson R., Brytting M., Linde A. Human cell-lines used in a micro neutralisation test for measuring influenza neutralising antibodies. 2006. Scandinavian Journal of Immunology. In press. Vol 63.

III. **Mittelholzer C.M.**, Brokstad K.A., Brytting M., Jonsson R., Linde A. Hemagglutinin variants of recent human influenza A/H3N2 viruses appearing after cell culture propagation. Submitted.

IV. **Mittelholzer C.M.**, Mittelholzer C., Winberg G., Brytting M., Linde A. Genetic stability of influenza A/H3N2 under antibody pressure *in vitro*. In manuscript.

Abbreviations

A	alanine
aa	amino acid
APC	antigen presenting cell
C	cysteine
CPE	cytopathogenic effect
cRNA	complementary RNA
CTL	cytotoxic T lymphocyte
D	aspartic acid
DC	dentritic cell
E	glutamic acid
ELISA	enzyme-linked immunosorbent assay
EMEM	culture medium
ER	endoplasmatic reticulum
F	phenylalanine
G	glycine
H	histidine
HA	hemagglutinin
HA assay	hemagglutination assay
HAI assay	hemagglutination inhibition assay
HSG	human salivary gland
I	isoleucine
IC_{50}	inhibiting concentration 50%
IFN	interferon
Ig	immunoglobulin
IL	interleukin
K	lysine
L	leucine
M	methionine
M1 and 2	matrix protein 1 and 2
MDCK	Madin-Darby canine kidney
MHC	major histocompatibility complex
mRNA	messenger RNA
N	asparagine
NA	neuraminidase
NK	natural killer
NP	nucleoprotein
NS1	non-structural 1
nt	nucleotide
NT	neutralisation test
P	proline
PB1/PB2/PA	polymerase proteins
PBS	phosphate buffered saline
Q	glutamine
R	arginine
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	rounds per minute

S	serine
SA	sialic acid
SA-α2,6-Gal	sialic acid-α2,6-galactose
SA-α2,3-Gal	sialic acid-α2,3-galactose
SD	standard deviation
T	threonine
$TCID_{50}$	tissue culture infectious dose 50%
TCR	T cell receptor
Th	T helper lymphocyte
TNF	tumor necrosis factor
UTR	non-coding region
V	valine
vRNA	viral RNA
W	tryptophan
Y	tyrosine

Table of Contents

1. Introduction

1.1. The Influenza Viruses and the Replication of Influenza Type A Virus

Influenza type A virus is among the most serious infectious threats for humans, due to the severity of the disease, the variability of the virus envelope proteins and its ability for a rapid and global spread. This has been clearly evident from the annual epidemics and several pandemic outbreaks, which have occurred at irregular and non-predictable intervals.

1.1.1. Classification, structure and nomenclature

Influenza viruses are members of the family *Orthomyxoviridae*. There are three types of influenza: A, B and C. Morphologic examination of primary influenza virus isolates revealed that they are often pleomorphic and after several passages *in vitro* spherical, with a diameter of 80 to 120 nm. The lipid envelope contains spikes, which are integral proteins that project out from the host-derived envelope (Fig. 1). Inside the influenza type A and B virions eight different RNA genome segments (type C = 7 genome segments) of negative polarity are found, coding for up to 11 different viral proteins (Table 1). To be infectious and reproductive, a single virion must contain each of the 7-8 unique gene segments. Influenza type A, B and C viruses can be distinguished by antigenic differences in their nucleo- (NP) and matrix- (M) proteins.

Influenza type A viruses are divided into subtypes based on the serological reactivity to the combination of surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). With the help of sequence analysis and verification by serological reactivity, 16 distinct HA and 9 distinct NA types have to date been recognised (46, 107). Aquatic birds are the natural reservoir of all influenza A viruses (210). All known subtypes of influenza type A are perpetuated in migrating waterfowl.

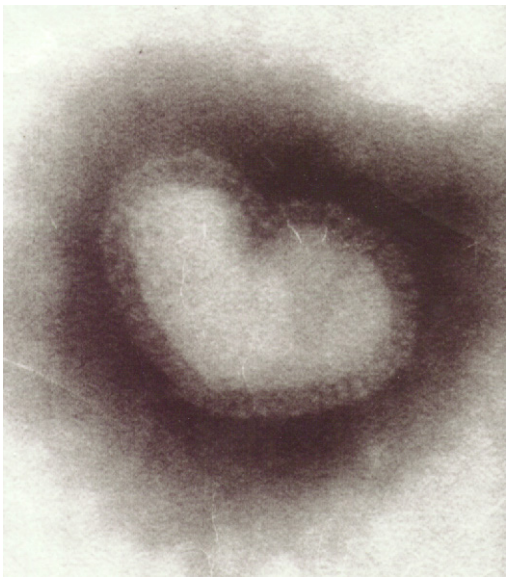


Figure 1. Electron Microscopy picture of an influenza virion (Kjell-Olof Hedlund SMI, Solna, Sweden).

Table 1. The influenza type A gene segments and the function of encoded proteins.

Segment	Length* of nucleotide	Encoded Polypeptide	Function
1	2341	Basic polymerase protein 2 (PB2)	Initiation of viral mRNA transcription through recognition and binding of the 5' cap-1 structures of host pre-mRNAs used to generate primers for viral transcription.
2	2341	Basic polymerase protein 1 (PB1)	Responsible for elongation of the primed nascent viral mRNA and elongation in template RNA and vRNA synthesis. It contains the conserved motifs characteristic of RNA-dependent RNA polymerases. It also contains sites for sequence-specific binding to conserved 5'- and 3'-terminal sequences of vRNA and cRNA molecules.
	86	PB1-frame 2 (PB1-F2)	May have a role in modulating the host response to influenza A virus by hastening the death of immune cells (20).
3	2233	Acidic polymerase protein (PA)	Thought to be involved in viral RNA replication. Strong suggestion has been made that PA is involved in the assembly of functional viral RNA polymerase complexes from their inactive intermediates(92).
4	1778	Hemagglutinin (HA)	Binding of virion to host cell receptor and fusion between the virion envelope and the membrane of the endosome
5	1565	Nucleoprotein (NP)	Binds to and encapsidates viral RNA to form coiled ribonucleoprotein (RNP) complex to which the three polymerase proteins associate
6	1413	Neuraminidase (NA)	Cleaves terminal sialic acid from glycoproteins or glycolipids to free progeny virions from host cell receptors
7	1027	Matrix protein 1 (M1)	Forms a shell surrounding the virion nucleocapsids underneath the virion envelope. Play an important role in initiating progeny virus assembly
		Matrix protein 2 (M2)	The membrane-spanning domain serves as a signal for transport to the cell surface. Act as a proton channel to control the pH of the Golgi during HA synthesis (22, 45, 187) and to allow acidification of the interior of the virion during virus uncoating
8	890	Non-structural protein 1 (NS1)	Regulates nuclear export of mRNA and inhibits pre-mRNA splicing. Probably inhibits IFN-mediated antiviral responses of the host (52).
		Nuclear export protein (NEP)	Provides M1 with a nuclear export signal that mediates the nuclear export of vRNP from the nucleus to the cytoplasm (139, 143).

*A/PR/8/34(H1N1)**;** Fields Virology, Evolution and Ecology of Influenza A viruses (210).

The reference influenza strains at WHO reference laboratories all acquire names according to a standard nomenclature. The designation of an influenza virus strain consists of type of influenza virus/abbreviation for animal species (if not human)/place or area of isolation/sequential number at the isolating laboratory/year of isolation (subtype), e.g. *Influenza A/Stockholm/12/05(H3N2)* (33).

1.1.2. Entry into host cells

The influenza infection starts when viral HA binds to the sialic acid (SA) residues on the host cell receptor. Although all influenza viruses recognize oligosaccharides containing a terminal SA, the specificity of the HA towards these molecules differs. On the epithelial cells of human trachea SA-α2,6-galactose (SA-α2,6-Gal) is predominant (27) and human influenza isolates bind preferentially to SA attached with α2,6-linkages. Pig tracheas contains sialyloligosaccharides reactive with both SA-α2,6-Gal and SA-α2,3-Gal specific lectins (81, 185). Most avian influenza viruses preferentially bind to SA-α2,3-Gal. The binding specificity of the HA is one of the determinants of the host specificity of the virus (167).

The virions enter the host cell through receptor-mediated endocytosis. The uncoating of virions in endosomes is dependent on the acidic pH of this compartment. The low pH in endosomes permits the flow of ions from the endosomes to the virus interior through the M2 ion channel. A post-translational cleavage step of the HA protein is necessary for virus infectivity (97, 110). Cleavage allows the molecule to assume a structure that can subsequently be triggered by the acidic pH of the endosomes to undergo the molecular rearrangements required for fusion between the virus membrane and the endosomal membrane (18). Protein-protein interactions are interrupted to free the ribonucleoproteins (RNPs) from the M1 protein (107). The RNPs are then released in the cytoplasm of the infected cell (Figure 3a) (75). Nuclear import of RNP complexes results from the interaction of NP with host cell importin α (205).

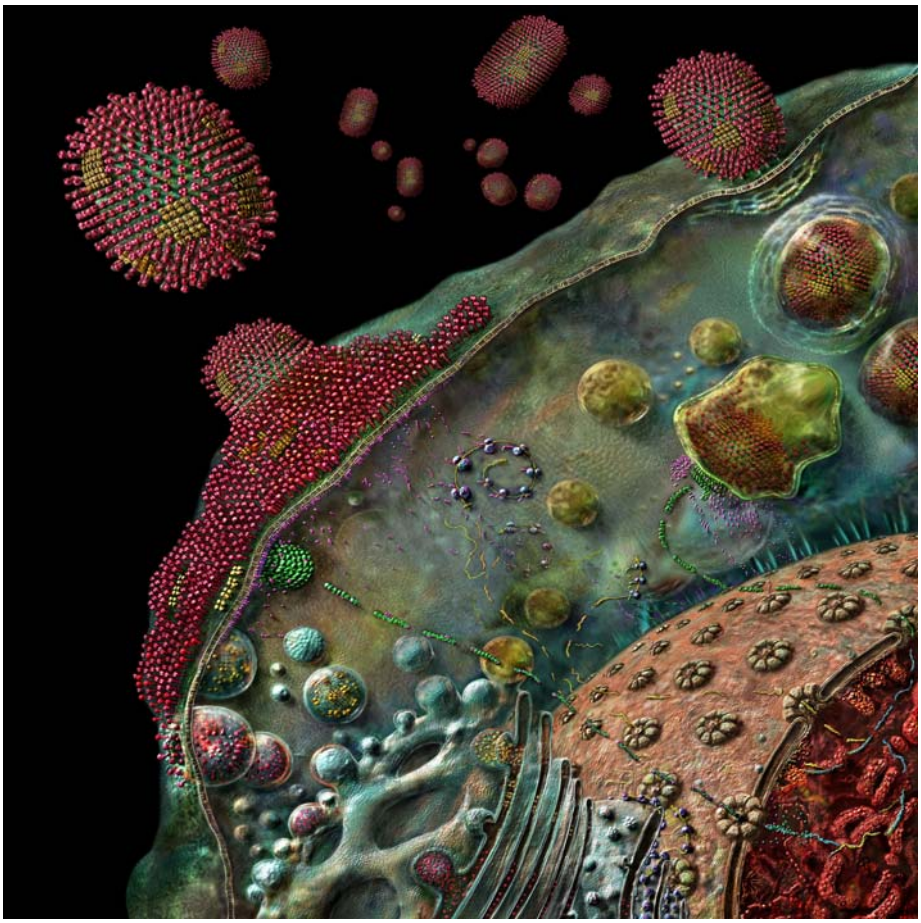
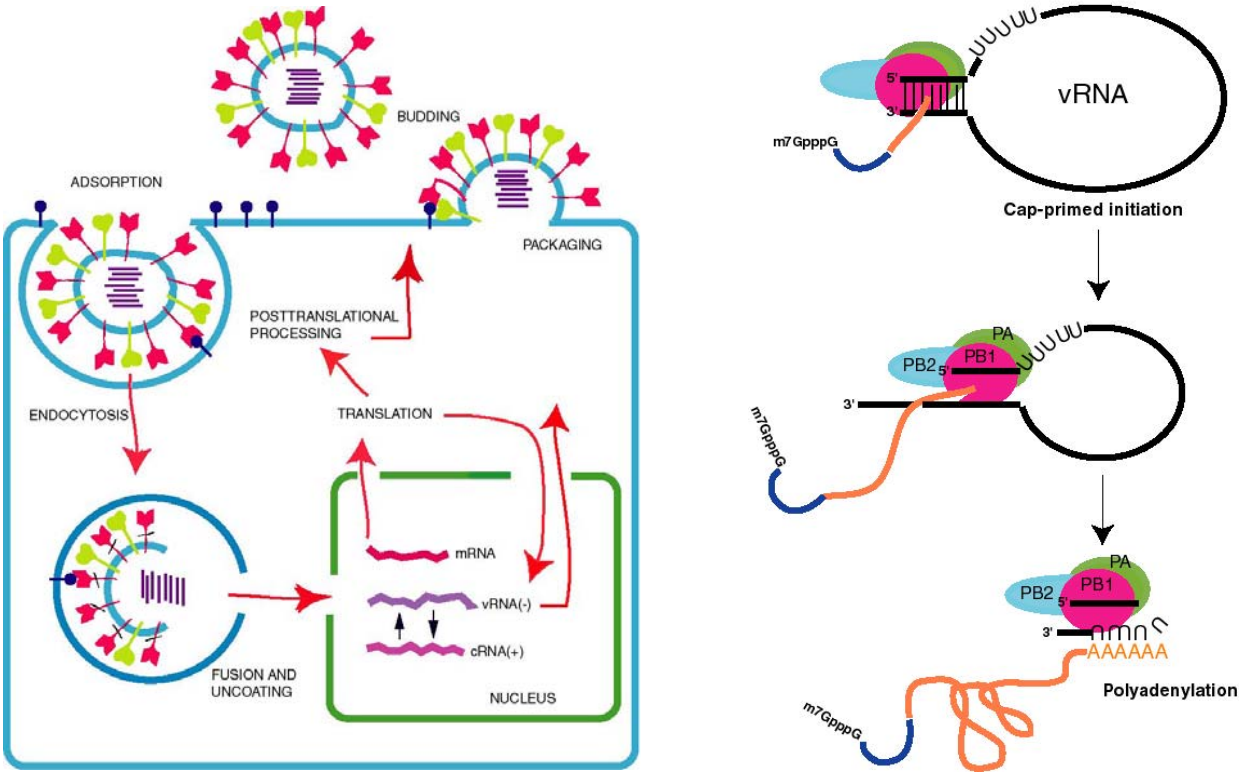


Figure 2. Replication of influenza virus (Russell Kightley Media).



a, **b,**

Figure 3a, Structured figure of the influenza virus replication **b,** Viral mRNA synthesis.

1.1.3. Viral mRNA and vRNA synthesis

In the nucleus of infected cells, the influenza viruses RNA (vRNA) are both transcribed to messenger RNA (mRNA) and copied to new vRNA. Influenza viral mRNA synthesis is primed by 5' capped fragments, which are removed from newly synthesised host cell RNA polymerase II transcripts by the PB2 polymarase ("cap-snatching"). After priming with 5' capped fragments, the elongation of the mRNA chains are mediated by the PB1 polymerase. The copying of the template continues up to a point at which a stretch of uridine residues is reached, 15 to 22 nucleotides before the 5' ends of the vRNAs (Figure 3b) (219). Polyadenylate residues are added to the mRNAs. Newly synthesised viral mRNAs are protected from degradation since the polymerase complex binds to the specific sequence 5'-AGCAAAAGCAGG-3' found in all mRNAs which is complementary to nucleotides 1 to 12 of the 5' end of each vRNA segment (175). All segments of influenza have an identical 12-nucleotide sequence at the 3' end and a 13-nucleotide sequence at the 5' end. These identical sequences and endings are necessary for activation of catalytic functions of the polymerase. Full-length amplification for molecular characterisation of influenza A virus has been designed based on these genomic regions (78). The change from mRNA synthesis to the synthesis of full-length template RNAs requires a switch by the polymerase to unprimed initiation. A recent hypothesis concerns the ability of NP to bind directly to PB1 and PB2, thereby altering the transcriptional function of the polymerase through direct protein-protein contacts (132, 155). Complementary RNAs (cRNA) are transcribed from all the vRNAs at equimolar amounts. Full-length anti-genomic cRNA serves as template for vRNA synthesis. During this synthesis the lack of proofreading allows for the introduction of replication errors at a rate of approximately 1.5×10^{-5} mutations per nucleotide per infectious cycle (150, 210).

1.1.4. Translation, assembly and budding

Translation of mRNAs by ribosomes occurs in the cytoplasm. Newly synthesised RNP components PA, PB1, PB2 and NP migrate into the nucleus where they assemble with newly synthesised vRNAs (56, 100). RNP nuclear export is thought to occur by the formation of an NEP-M1-RNP complex that interacts with a highly conserved host-cell nuclear export receptor (139, 143). During passage over the endoplasmic reticulum and the Golgi apparatus, the proteins are posttranslationally modified and folded. The surface glycoproteins and the M2 protein are transported to the cell surface. The final step of the replication cycle of influenza viruses involves the budding of the newly formed particles from the cellular membrane. Matrix protein 1 is the major factor in formation of the budding particle. This implies that M1 contains the structural information needed for self-assembly, interaction with cell membranes, and the budding process (55). The presence of the viral trans-membrane protein HA stimulates the binding of M1 to the cell membrane (43), and the interaction between the cytoplasmic tail of HA and M1 probably targets NEP-M1-RNP to the site for virus assembly (55). Influenza A virus RNA contains non-coding regions (UTRs) at both ends (5' and 3') of its coding region (107). Efficient packaging of the NA, HA, and NS segments also requires coding sequences immediately adjacent to the UTRs (48, 208). The two ends of any given vRNA may collaborate in forming specific structures to be recognized by the viral packaging machinery (113). Progeny viruses are released when NA cleaves sialic acids from cellular receptors.

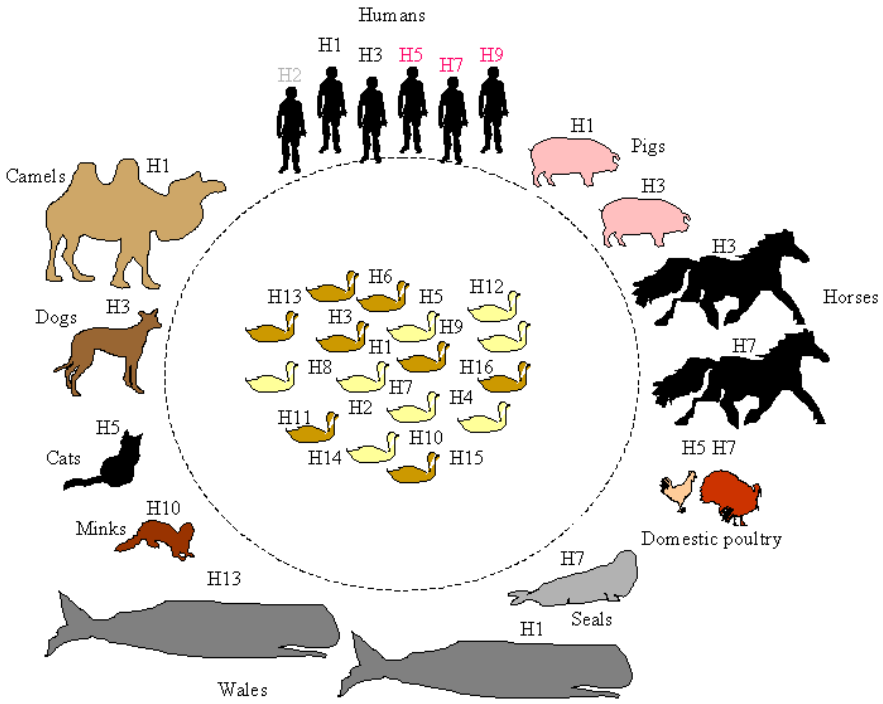


Figure 4. The avian influenza A virus reservoir and documented cross-species transmission.

1.1.5. Influenza infects many species

All different subtypes of influenza A have been isolated from birds, but not all avian strains are pathogenic, and the pathogenicity varies depending on bird species infected. Different

avian influenza viruses produce varying syndromes in birds, ranging from non-symptomatic to mild upper respiratory infections caused by low-pathogenic avian influenza (LPAI) to loss of egg production and rapidly fatal systemic disease caused by highly pathogenic avian influenza (HPAI) (40). Without exception, all of the HPAI viruses belong to the H5 or H7 subtype, for unknown reasons (79). The HPAI viruses can cause devastating lethal influenza outbreaks in domestic birds, whereas LPAI viruses rarely generates outbreaks of severe disease (210).

Influenza type A viruses from waterfowl have been able to cross host barriers and infect humans, domestic poultry, pigs, horses, seals, whales, minks, camels, cats and dogs (Figure 4) (30, 98, 106, 210, 220). Prediction of when an avian influenza virus will be transmitted to new host species is impossible. Humans can relatively easily be infected with swine influenza virus but the infection seldom results in severe disease or becomes epidemic in man (219).

Ferrets are susceptible hosts for all human influenza A and B viruses. This animal model has been important within influenza research ever since the first human influenza A virus was isolated in 1933. The influenza virus infection in newborn ferrets can be lethal, since they have low levels of immunoglobulins. In the adult ferrets influenza infections generally result in mild upper respiratory tract symptoms. The pathogenesis of the highly pathogenic avian influenza A/Hong Kong/97(H5N1) virus was evaluated in a ferret model. The results from these studies showed that ferrets can be used as a model system for the study of avian influenza virus in mammals (226). Because of their susceptibility, the human-like clinical response after infection and their high production of antibodies ferrets are often used for evaluation of new influenza vaccines. Ferrets are also used to produce post-infection antisera for hemagglutination inhibition (HAI) assays (219), necessary for the study of antigen drift.

1.2. Antigen changes of influenza virus

1.2.1. Antigen drift and epidemic influenza

The HA present in the two circulating human influenza subtypes (H1N1 and H3N2) together with the HA from influenza B virus change continuously. The viral RNA polymerase do not harbour proof-reading mechanisms resulting in point mutations at a rate of about 1.5×10^{-5} (150) during transcription. In the presence of selective pressure the virus will search for the variant most fit for replication (33). In humans, due to our longevity and frequent re-infections with influenza, the selective pressure produced by herd immunity is higher than in for example pigs and birds and the difference in pressure causes the difference in evolution rate in different species (19, 186). Neutralising antibodies directed against HA are usually protective against infection (75). It is therefore not surprising that this protein, which is projecting out from the virus lipid envelope, is under the highest selective immune pressure. The proteins inside the envelope do not play a part in the attachment to host cells and are of little importance in eliciting a neutralising antibody response. These proteins are therefore more conserved than the HA. However they still adapt both as a result of immune pressure and during adaptation to a new host species (191, 216). The viral proteins adapt to escape both recognition by neutralising antibodies (172) and cytotoxic T lymphocytes (10, 104, 144).

Mutations introduced into the antigenic sites of the HA gene of circulating human influenza viruses may result in the selection of escape mutants (95), which can give the virus the possibility to evade the specific acquired immunity, causing *antigen drift*. The antibodies against the influenza HA protein are primarily directed to five defined antigenic sites (A-E) on the globular head of the protein (214). It has lately been suggested that these can be divided into several smaller sites (137). The accumulation of amino acid substitutions in the HA protein during evolution promote irreversible structural changes. Therefore antigenic changes in the H3 HA protein may not be limited (136) as earlier suggested (15).

The ability of the virus to change and cause antigen drift is the reason for the long-term persistence of a virus subtype in the human population. It is the explanation to why influenza virus causes annual epidemics. For many years, the continuing antigenic drift has been monitored by the hemagglutination inhibition (HAI) assay, using specific sera from ferrets immunised with various influenza A strains (33). Today the antigen drift can also be followed and visualized using phylogenetic trees based on primarily HA gene sequences from virus strains. However, the correlation between mutations and antigenic variation is not always obvious. It is still important to perform HAI to verify whether or not the observed mutations are of immunological relevance (178). By creating an antigenic map using the results from the HAI assay it has recently become possible to visualize and better understand the antigenic importance of the ongoing antigenic drift (178).

New antigenic variants due to drift occur more often for the A/H3 virus than for the A/H1 and the B virus (70). Antigenic drift often demands a change of the influenza vaccine composition when the emerging new influenza virus variants are not sufficiently recognised by the antibodies induced by the existing vaccine.

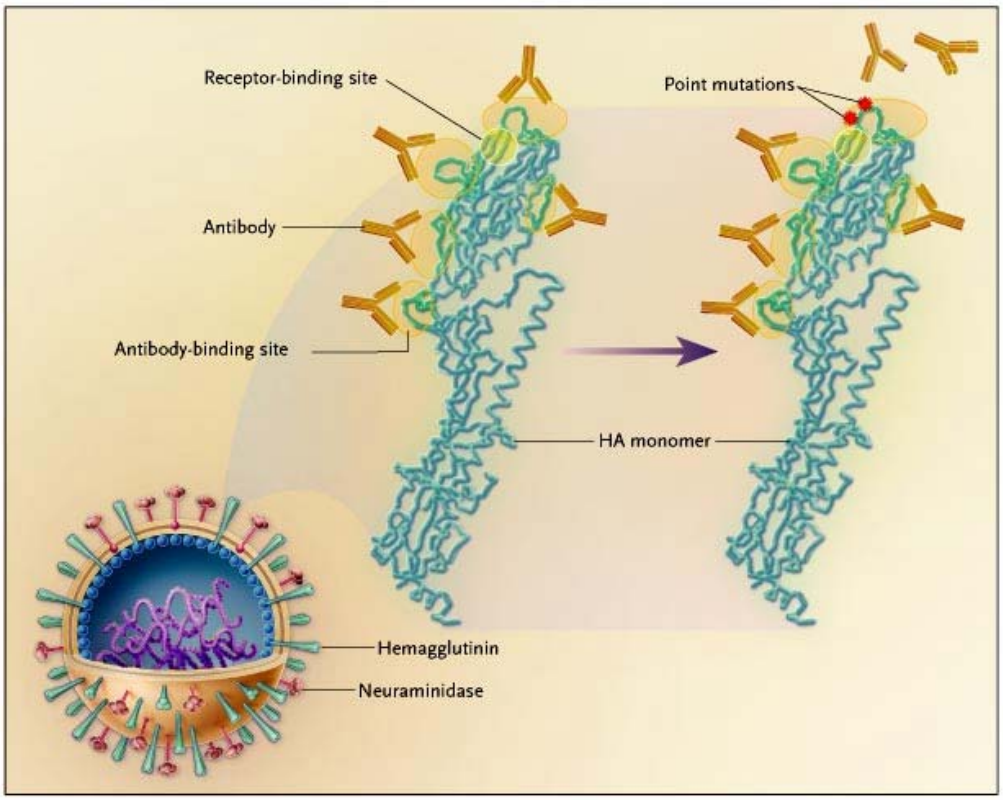
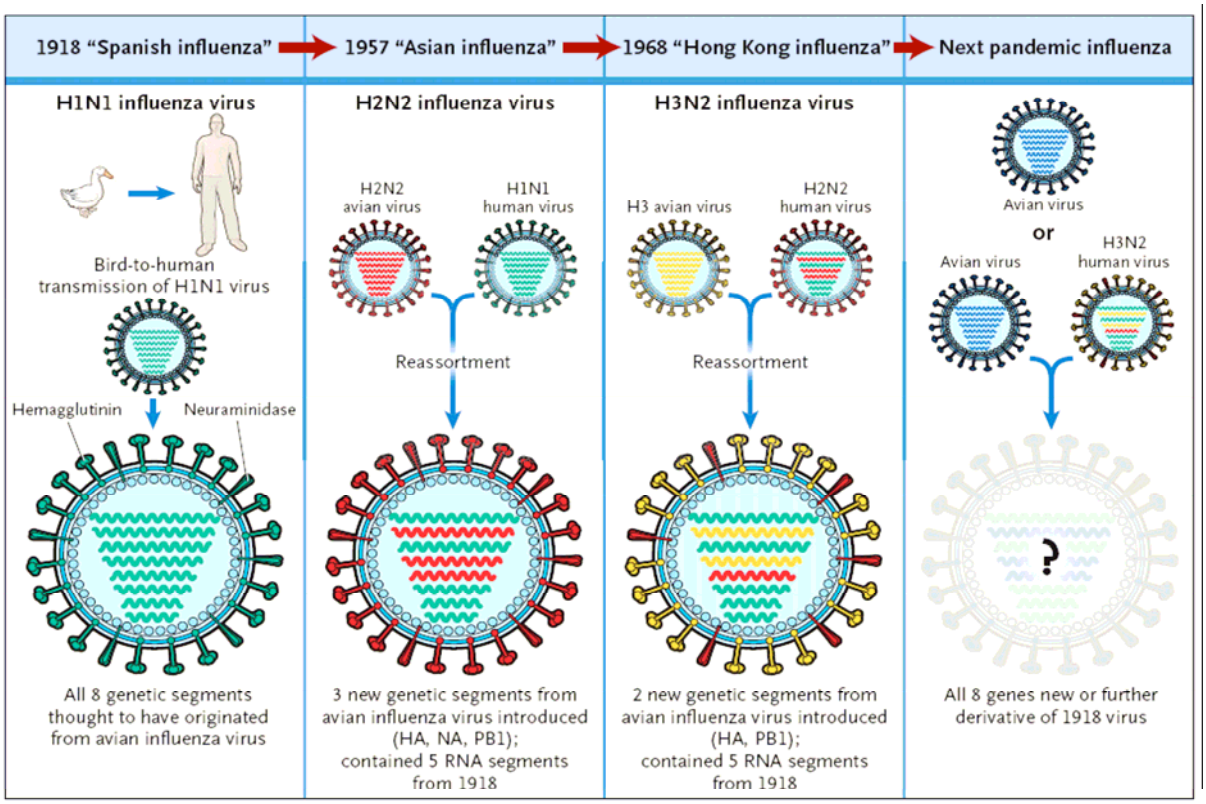


Figure 5. Antibody binding sites present on the tip of the HA proteins (196).

1.2.2. Antigen shift or direct adaptation resulting in pandemic influenza A strains

Pandemics may occur when a new subtype of influenza A virus crosses the host barrier between birds and man. This can happen through adaptation of an avian influenza virus to human cells, or through reassortment of gene segments (*antigen shift*) in a host cell infected simultaneously by two different subtypes of influenza A, one from man and one from e.g. a bird. At least the HA gene need to be exchanged for a antigen shift to emerge (82). Pigs are thought to be a common "mixing vessel".

Earlier it was believed that avian influenza viruses could not infect humans by direct transmission. However, in studies from the early 1990`s seroconversion in humans to influenza H5 and H9 strongly suggested direct transmission of these subtypes (64). In 1997, it was finally shown that avian influenza virus (A/H5N1) could be transferred directly from birds to humans (24). It thereby became obvious that not only pigs could function as mixing vessels, but that theoretically human cells could also be infected simultaneously by an avian and a human influenza strain. Lately, the huge ongoing outbreak of highly pathogenic avian influenza A/H5N1, starting in south-east Asia in 2003, has increased the probability of a reassortment event to occur (152). Since avian influenza strains are poorly adapted to circulation in the human host, the virus almost never has accomplished more than a few generations of serial transmissions (33, 66). However, direct adaptation by mutations in several of the viral genes of the avian virus and successive selection of the fittest variant for infecting humans could cause a new pandemic. It can be guessed that the adaptation process may take several years. The 1918 Spanish flu was not reassorted. It has recently been suggested that the adaptation process from an avian to a human influenza strain took around



17 years (191).

Figure 6. Three large influenza pandemics have occurred in the 20th century: the Spanish flu (1918), the Asian flu (1957), and the Hong Kong flu (1968).

The ability of direct adaptation of influenza viruses from the natural avian source to humans has induced a new research area. To locate the amino acid positions important for human adaptation creates a possibility to focus the molecular surveillance to specific genomic sites. The receptor-binding site located on the tip of the HA protein are highly conserved among avian influenza viruses, while those of human viruses display distinct variability. For human influenza A/H2N2 and H3N2 viruses the amino acid positions 226 and 228 in the HA gene are especially important for host range restriction (215), while HA positions 190 and 225 are important for human influenza A/H1N1 viruses (123). The amino acid change from Glu627 to Lys627 in the PB2 protein has been related to the host-cell tropism in humans (67, 176, 183). Recently, nine additional amino acid positions in the polymerase genes, PB2 positions 199, 475, 567, 702, PB1 position 375, and PA positions 55, 100, 382, 552, have been suggested to have important roles in human adaptation (191).

The pandemic threat posed by influenza A/H5N1 has led to improvements of the surveillance in the South East Asia. Both the research and the strategic planning of how to stop the virus in case it starts to spread effectively between humans are key issues to increase the pandemic preparedness (117, 206).

The Spanish flu, has been estimated to have killed approximately 50 million people according to modern calculations (85). The origin and virulence of this highly pathogenic influenza A/H1N1 virus has been discussed and carefully analysed. Sequencing all gene segments from Spanish flu victims (158, 159, 190, 191) and reconstructing the virus by reverse genetics is today possible (199). This has shed light on the reasons for the high pathogenicity of the virus. Today, it is proposed that the Spanish influenza virus was entirely an avian-like virus adapting to the human host (191). It is argued whether or not the Spanish flu like the Asian and Hong Kong flu emerged in Eastern Asia and thereafter spread around the world. It was proposed that the Spanish flu may have originated in France in 1916 (145). On the basis of amino acid replacement rates in human influenza polymerase genes from the 1918 virus, it has been suggested that the humanisation of an avian strain started as early as in 1900 (191).

During an avian influenza A/H5N1 outbreak in Hong Kong 1997, eighteen people were infected by the influenza A/H5N1 virus, and six of them died. Before that outbreak it was not generally believed that avian influenza strains would transmit directly to man and cause severe disease in humans (24, 80, 184). In December 2003 human cases of HPAI A/H5N1 were again reported in South East Asia (184). Since then 70-80 people have died from the HPAI A/H5N1 virus. Because of the pandemic threat that it constitutes it has become necessary for Influenza Centres worldwide to be capable of rapidly identifying subtypes of influenza virus (23). At the end of February 2003 a highly pathogenic avian influenza outbreak caused by HPAI A/H7N7 occurred in The Netherlands (42). The same virus was detected, causing mainly conjunctivitis, in 86 humans handling the birds (47). The ongoing influenza A/H5N1 outbreak reached Europe in 2005 (1) and Africa in 2006. The virus has recently caused fatalities in several European countries, including Sweden. The virus has been verified in primarily wild birds, but has also spread to poultry, cats and a few persons in Turkey.

1.3. Influenza Symptoms, Pathogenesis and Immunity in Humans

1.3.1. Symptoms

Human influenza viruses replicate almost exclusively in epithelial cells of the respiratory tract (124, 219). These cells rapidly die by cell membrane disruption caused by budding virions. Respiratory droplets (aerosols, <10μl) very efficiently transmit influenza virus from person to person. Clearly, crowding of people and global transport favours a rapid spread of influenza virus. The incubation period is short, one to four days, and the onset of illness is usually abrupt. Clinical symptoms normally persist for three to four days, even though the cough and malaise may persist for one to two additional weeks. Influenza infection can cause severe disease and complications, especially in people belonging to the high-risk groups (Table 2). It is important to emphasize that influenza virus can also cause sub-clinical infections, as well as mild illness (75).

Table 2. Clinical influenza and influenza in risk patients (29, 75)

Common clinical symptoms	Complications	Risk patients (29)
• High fever >39°C • Malaise • Headache • Dry cough • Lassitude • Nasal congestion • Anorexia	• Viral pneumonia • Secondary bacterial pneumonia, commonly caused by *Streptococcus pneumoniae* *Some rare complications:* • Encephalitis • Meningitis • Acute myositis (more common for influenza B) • Acute renal failure	• People with chronic cardiovascular or pulmonary disease including asthma • People age 65 or older • People with renal dysfunction, haemoglobinopathy (not included in Iceland, Norway and Sweden) • People with chronic metabolic disorders including diabetes (not included in Iceland and Norway) • Children (6 months–8 years old) on long-term aspirin therapy due to an increased risk of Reye's syndrome (not included in Austria, Denmark, Germany, Greece, Iceland, Norway, Portugal, Spain, Sweden and UK) • Immunosuppressed patients • Pregnant women in second or third trimester (only recommended in USA, Belgium and Switzerland)

1.3.2. Pathogenesis

It is still poorly understood why certain influenza strains are highly pathogenic. Both viral factors and host factors may determine virulence. During an influenza infection the macrophages mediate lysis of infected cells and secrete interleukin (IL)-1, IL-6, tumor necrosis factor (TNF) α, and IL-12 (84, 91, 188). They are infected by the virus, and can carry it to different places in the body without replicating it. Endogenous pyrogens such as IL-1, IL-6 and TNF-α enter the circulatory system and stimulate production of prostaglandin E2 in the hypothalamus. This endogenous mediator induces the febrile response as well as the constitutional flu-like symptoms (17).

For naturally occurring avian influenza A viruses, the most important determinant of pathogenicity is the cleavage site structure of the HA. Analysis of the HA1-HA2 junction

regions in influenza viruses with different pathogenicity revealed the presence of a stretch of basic residues in the HA of pathogenic strains (13). Influenza viruses lacking the multibasic cleavage sites failed to replicate in tissue culture in the absence of trypsin (14). The demand of the enzyme trypsin helps to restrict multi-cycle replication of influenza to the upper respiratory tract (179), while highly pathogenic influenza viruses contain a cleavage site that is recognised by more ubiquitous expressed host cell proteases. As a result, these viruses can spread throughout the lungs and in some cases throughout the body. Interestingly, the Spanish influenza HA protein from 1918 is also cleaved into its active form in the absence of trypsin (199). However, the 1918 virus HA does not have a multibasic cleavage site. Instead, its own NA protein is involved in cleavage of HA by a new mechanism that is not yet understood (169).

Pathogenicity is increased when the NS1 protein down-regulates components of the interferon cascade (50-52, 88). High cleavability of the HA glycoprotein has an essential role for infectivity, and the pathogenicity of avian influenza correlates directly with increased cleavability. Mutations at the HA cleavage site made the avian influenza A/Hong Kong/97(H5N1) virus less virulent when tested in mice (67). In pig lung epithelial cells, the NS gene of H5N1 viruses has been shown to confer resistance to the antiviral effects of interferons (IFNs) and TNF-α (173). Using reverse genetics (68) the function of the HA protein from the Spanish flu virus from 1918 has been studied, and evidence of increased pathogenicity have been identified (101). The importance of the polymerase genes for increased pathogenesis has earlier been indicated since the gene coding for the PB1 protein was present together with the HA gene in the reassorted strains causing the Asian flu (1957) and the Hong Kong flu (1968). Lately, the importance has also been indicated by the association of a lysine residue at 627 in PB2 in both H5N1 and H7N7 avian influenza viruses infecting humans (47, 67). In mice the presence of lysine at position 627 has been shown to lead to more aggressive viral replication, resulting in high mortality (176).

Obviously other genes contribute to influenza pathogenesis. The role of the viral NA and PB1 and host factors will require further studies (79, 225). Increased understanding of the molecular determinants of influenza A virus pathogenicity, transmission, and host range should continue to be a major research objective (46).

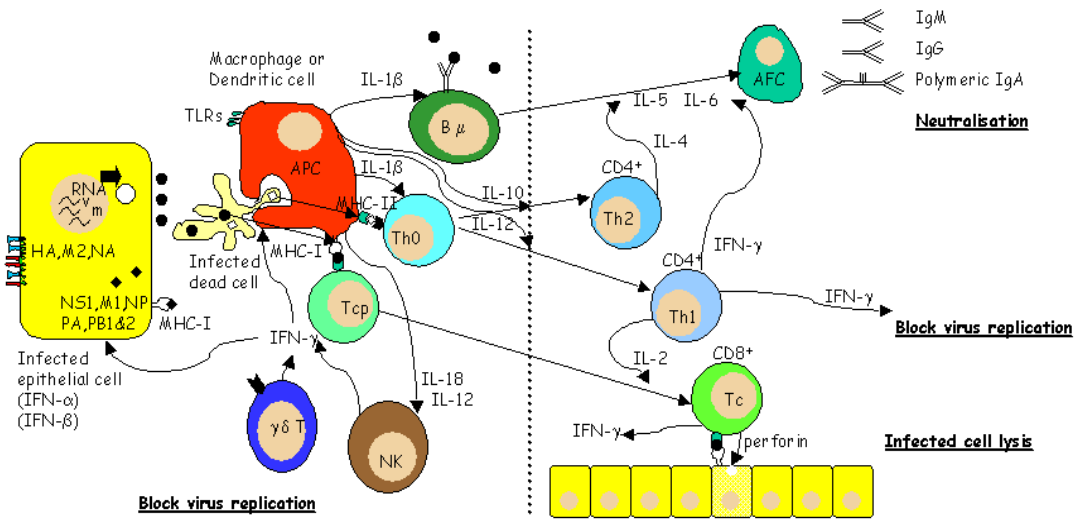


Figure 7. Immune responses induced by influenza virus infection. Modified from (188)

1.3.3. Innate immune mechanisms

The influenza viruses are often detected and destroyed within a few hours by the innate immune mechanism (4, 188). Components of the "first line of defence" are the mucus, secretory IgA, and natural killer (NK) cells. Fever and complement as well as the macrophages, IFN α, β and other cytokines belong to "the second line of defence". Interleukin-6 and IFN-α reach their peak by day two after infection and play a major role in symptom formation (73). IFN-α also plays an important role in limiting the spread of influenza virus infection since it helps to inhibit replication. IL-12, in synergy with TNF-α, can elicit the IFN-γ production by NK cells. IL-1β, secreted by antigen presenting cells (APCs), macrophages and dendritic cells, is one of the most important cytokines in bridging the innate and adaptive immune systems (125). IFN-γ is also released by activated T-cells. IFN-γ can activate important pathways associated with direct antiviral functions (11). NK cells are activated with the help of their main activating receptor (NKp46), and may recognise HA on influenza infected cells and lyse them (122). Nitrous oxide (NO) is induced by cytokines shortly after the infection with influenza virus and inhibits viral replication (161). NO production also has immunopathological consequences (5). To enhance the innate immune response various constituents of the pathogens can be used. For example cholera toxin prior to infection activated macrophages and NK cells, when tested in BALB/c mice, resulting in a non-specific replication reduction of the viruses in the respiratory tract (125).

1.3.4. The adaptive immune response against influenza

Antigen presenting cells (APCs) are essential in the induction of the adaptive immune responses (6). Through endocytosis of the antigens the APCs acquire peptides that can bind to MHC (class I or II) molecules and be presented on the surface. Primarily neutralising antibodies are strain specific IgM, IgG and IgA antibodies directed against HA, but in normal individuals antibodies against NA, NP and M proteins are also produced during an influenza A infection (75). Antibodies to the HA and NA are associated with resistance to infection and illness whereas antibodies to the internal M1 and NP proteins are not. HA antibodies can neutralise the infectivity by preventing virions from binding to the host cell receptors. NA antibodies help to restrict the infection to the respiratory tract (219). This may be a reason why children, lacking a sufficient immune response against NA, more easily may contract an influenza virus viremia. Secretory immunoglobulin A (S-IgA) and IgM are the major neutralising antibodies directed against mucosal pathogens. These antibodies are detected on day 5 after infection and reach a plateau at around day 11. The first year after infection the antibody level successively diminishes (86, 87). Mucosal IgA work to prevent pathogen entry and can function intracellularly to inhibit replication of virus (29). S-IgA antibodies are involved primarily in the prevention of influenza in the upper respiratory tract, whereas serum IgG antibodies predominate in the prevention of lethal influenza pneumonia (157, 188). Immunity against influenza virus gradually increases after several infections. It is known that one infection generates antibodies that react only with a limited number of antigenic sites on HA and that several infections generate a broader range of specificities (205).

1.3.5. The cellular immune response

The cellular immune response to influenza virus infections is important for clearing virus-infected cells and cellular immunity can prevent influenza-associated complications (29, 89). This has been well defined in the murine model but less well in humans (89). However, it is notable that findings of antigen recognition and cytotoxic T lymphocytes (CTL) contributes to recovery in the mouse parallel the limited data in man (195).

In humans the pre-infection level of virus-specific CTL is associated with accelerated clearance of the virus from the respiratory tract (129). Virus specific T cell responses are detectable between day 3 and 6 in humans and return to baseline levels by day 28 (36). CTLs appear in the blood of infected or vaccinated individuals on days 6 to 14 and disappear by day 21 (44). After primary infection, naïve T cells expand and differentiate into cytotoxic effector cells that are able to eliminate virus-infected cells. After viral clearance, the effector T cell pool contracts and a virus-specific memory T cell pool persists that can undergo rapid reactivation after reinfection. Memory cells primed by respiratory viruses persist both in secondary lymphoid organs and the lungs.

The cytotoxic T lymphocyte response is cross-reactive for serologically different influenza A viruses (222). Antigen presenting cells migrate from the site of infection to the local lymph nodes, where the antigen is presented to activate naïve influenza specific T-cells. In the lymphoid organs the cellular elements of the immune system, the precursors of the $CD8^+$ cytotoxic T lymphocytes (CTL), the $CD4^+$ helper T-cells, the antigen presenting dendritic cells and the B lymphocytes that give rise to the antibody-producing plasma cells, can be brought into proximity (35). In mice operating CD4+ T-cells can eliminate virulent influenza viruses with low virulence, but more virulent variants demand a $CD8^+$ response (160). Passively transferred influenza specific $CD4^+$ T cells are not able to clear an ongoing influenza infection by themselves, but require functional B cells to accomplish this clearance (194). In mice the development of both a $CD8^+$ and a $CD4^+$ T cell response is dependent on the concentration of the presented antigen. The results suggest that the inoculum dose and replication rate of a pathogen entering the respiratory tract may regulate the strength of the adaptive immune response and the subsequent outcome of infection (112). The number of $CD8^+$ T cell epitopes on the influenza viral proteins is much more restricted than the number of B cell or $CD4^+$ T cell epitopes (219). Two significant $CD8^+$ restricted epitopes are situated on the NP protein and on one of the polymerase proteins, PA (35).

Cellular immune responses after influenza infection provide cross-reactive protection between serologically distinct influenza virus subtypes (182). Despite this the design of an influenza vaccine that induces a protective response against all influenza A subtypes has so far not been possible. Subunit vaccines, containing purified glycoproteins, generally induce $CD4^+$ T-cell responses but not $CD8^+$ CTLs (203). A CTL response that eliminates infected cells before new virions are produced is sufficient for clearance and full protection, but CTLs are not necessary to prevent infection if a vaccinated individual obtains a sufficient level of neutralising antibodies after vaccination (182). After influenza immunisation both the antibody and cell-mediated responses of the elderly are significantly decreased as compared to that in the younger age groups (126). To improve the cell-mediated response after vaccination still constitutes a challenge. Different available vaccine models have been used to obtain increased knowledge in man. Mouse experiments may provide clues, but mice are not humans. Thus, what is possible to achieve in mice might not be directly transferable and possible to accomplish in humans.

1.4. Influenza vaccines, Adjuvants and Antiviral Treatment

1.4.1. Influenza vaccines

Individuals belonging to the risk groups (Table 2) are at high risk of contracting a more severe disease and complications following influenza infection. Therefore more active measures have to be taken in order to prevent them from catching the illness (142).

The viral strains to be used in the vaccines are grown in embryonated eggs or in tissue culture. Antigen from three strains are today included in the yearly vaccines, one A/H3, one A/H1, and one influenza B strain. The vaccines currently in use are whole-virus, split or subunit vaccines. Whole-virus and split vaccines contain all influenza proteins, while the subunit vaccines only contain the two glycoproteins HA and NA, since protective immunity is associated with neutralising antibodies against these two surface glycoproteins. Annual vaccination of people at risk of severe disease (Table 2) is implemented in the whole of the Western World. Vaccine strains for the coming season have to be decided upon at least half a year before use, since it takes more than six months to produce, control and distribute the vaccine in sufficient amounts. About every tenth year, the epidemic influenza strain has drifted further during the time between the decision on vaccine strains and the arrival of the epidemic strain, creating a mismatch in the vaccine. This is one reason why more broadly reactive vaccines and a more rapid production are desirable. The effectiveness of the vaccines that are currently available for influenza depends primarily on the antigenic "match" between the circulating viruses and the strains included in the vaccine (148).

The efficacy of the inactivated vaccines may be influenced by a range of different factors, including age, health status and use of concurrent medications, prior vaccination and prevaccination antibody titres (65). In general 70-80% of the healthy individuals between 10 and 65 years of age obtain a protective immune response after vaccination. In children <9 years and in adults >65, the immunogenicity and efficacy of the vaccines may be reduced (163, 197). Even though the vaccine efficacy may protect against symptomatic disease only in 30-40% of the elderly, vaccination may be up to 80% effective in preventing death caused by influenza in this age group (151). It is known that aging affects the cellular and humoral immunity. The effect of repeated vaccination against influenza varies depending on the antigenic distance between the vaccines from year to year (177). Annual immunisation of the elderly is important to maintain as high a level of antibodies as possible (53). Although the production of antibodies is reduced in the elderly the pool of memory CTLs is maintained (12). The antibody level in serum following administration of an influenza vaccine are, however, significantly higher than before two weeks after vaccination in the elderly (59). The immunity induced by inactivated vaccines normally decreases with time. It is well established that effective homotypic immunity to natural challenge rarely exceeds 8-12 months (94, 219). Therefore re-immunisation is required annually even if the antigenic difference in circulating virus between two seasons does not require an update of the vaccine strains.

The lead-time between influenza vaccine strain selection and the supply of the finished product is 4-8 months, which is shorter than that for any other human vaccine. This is, however, too long in case a new pandemic emerges or a mismatch is found between the produced vaccine and the circulating epidemic strains (31, 207). Different techniques have been developed to increase the speed and the flexibility of influenza vaccine production. MDCK or Vero cells can be used instead of embryonated chicken eggs to produce equally

effective influenza vaccine (16, 146). The cell-cultivated vaccine is an important alternative in case a pandemic avian influenza starts spreading, since no eggs will then be usable.

1.4.2. Alternative vaccine strategies

The generation of negative-sense RNA virus from cloned cDNA through genetic modification of influenza viruses by reverse genetics enables rapid production of reassortant viruses where the included gene segments can be specifically chosen (141). Reverse genetic systems are also an important tool for learning more about the functions of viral proteins and their contributions to viral pathogenicity. The ability to design a 'master' vaccine with multiple attenuating mutations in the genes encoding the internal proteins in combination with genes encoding the influenza virus surface proteins could be a big advantage for future vaccine production (140).

Other types of vaccines are also in use or have been tested. Live virus vaccines that infect the upper respiratory tract with "attenuated" viruses of reduced pathogenicity have proven to be immunogenic and protective (94). These types of vaccines have been registered in the US and induce a more broadly cross-reactive response. DNA vaccines encoding influenza virus antigens can induce immunity in animals like mice and monkeys, and are easily produced (200). In genetic immunisation the desired influenza gene is inserted into a plasmid that is delivered to host cells, which express the encoded protein (162). The most effective routes and methods for DNA immunisation include gene gun delivery (bombardment with particles coated with DNA), intramuscular injection, and intradermal delivery (209). DNA vaccines can express not only the surface proteins of influenza, but also interior proteins, thereby giving rise to both humoral and cellular immunity in mice (166, 202). Today, DNA vaccines may be useful in priming the immune system for subsequent boosting by either natural infection or conventional vaccination, but not for definitive immunisation. However, better knowledge concerning the immunology and cell transfection mechanisms will further enhance the possibilities for improved immunogenicity by DNA vaccines.

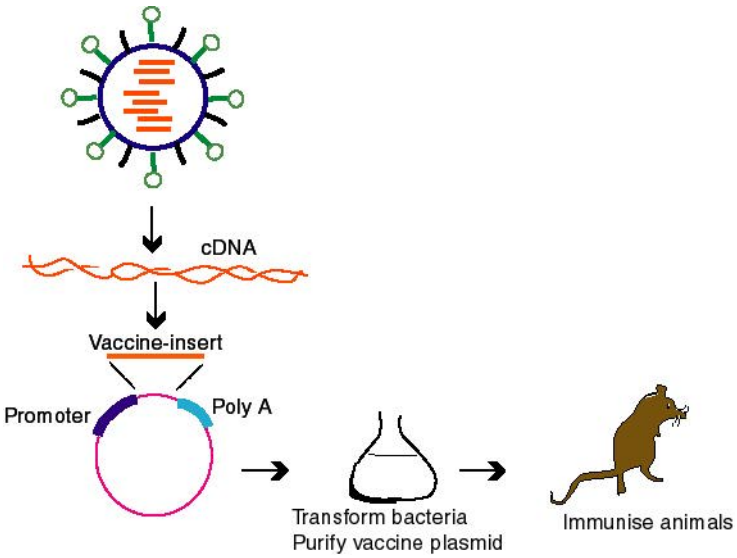


Figure 8. DNA vaccination

1.4.3. Vaccine immunogenicity and adjuvants

Immunogenicity refers to the ability of a vaccine to induce an immune response (antibody- and/or cell-mediated immunity) in a vaccinated individual (65). In primed adults, one dose of inactivated influenza vaccine will induce protective antibody levels in the majority of the vaccinated individuals. In contrast, two to three times as much vaccine is required to reach the same antibody titres in unprimed individuals. The main focus is on the improvement of the immunogenicity of the existing vaccines (93). It has lead to the development of new adjuvants and antigen delivery systems. The first commonly used adjuvant was alum, which was tested in vaccines already in 1930. Different new adjuvant formulas are already approved and others are undergoing clinical evaluation.

Examples of new adjuvants are immune stimulating complexes (ISCOMs) and adjuvant MF59, an oil in water emulsion, which induce increased humoral and cellular response after vaccination (162). Bacterial toxins have been used as mucosal adjuvants in humans, but toxicity and allergenicity of the toxins have caused problems. To resolve these, various derivatives have been suggested (189). However less toxic and less allergenic adjuvants often lead to a lower immunogenicity effect. Unmethylated CpG motifs present in bacterial DNA rapidly trigger an innate immune response characterised by the activation of Immunoglobulin (Ig)- and cytokine-secreting cells. Synthetic oligonucleotides (ODNs) containing CpG motifs mimic this activity, triggering monocytes to proliferate, secrete and/or differentiate. Two structurally distinct classes of CpG motifs that activate human monocytes have been identified (99). Type 1 IFN has been found to be an unexpectedly powerful adjuvant (156). Type-1 cytokines are important for the generation of a protective immune response and the use of them as adjuvants together with influenza vaccine might open up new possibilities.

Table 3. Different types of adjuvants (174).

Mineral salts	Aluminium hydroxide and aluminium or Calcium phosphate.
Oil emulsions and surfactant	MF59 (micro fluidised detergent stabilised oil in water emulsion), QS21 (purified saponin), montanides (stabilised water in oil emulsion).
Particulate	Virosomes (unilamellar liposomal vehicles with influenza antigens), ISCOMS (structured complex of saponins and lipids), PLG (poly-lactic-co-glycolic acid), Chitosan.
Microbial (natural and synthetic) derivatives	CpG ODN, 6-O-Acyl derivatives of N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) analogues, bacterial (mutant) toxins (Colera Toxin, Escherichia coli heat-labile enterotoxin).
Endogenous human immunomodulators	Human granulocyte macrophage stimulating factor (HgM-CSF) and interleukins (IL-12, IL-2).

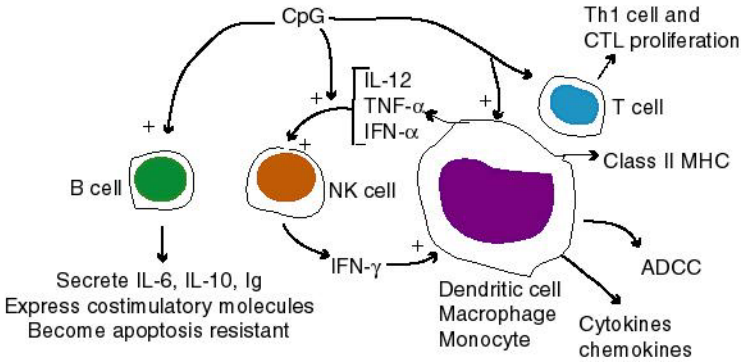


Figure 9. CpG activation of dendritic cells, monocytes and macrophages increases the immunological response after DNA vaccination (105).

1.4.4. Antiviral Treatment

Antivirals against influenza constitute means to shorten the duration of influenza disease and may be used as prophylactics during interpandemic periods. An influenza pandemic could spread very rapidly, and the vaccines will most likely not be ready in time for a global vaccination. Efficient antivirals against influenza virus may be used as prophylactics to limit the spread of infection in the early pandemic phase and for treatment.

The first influenza drug was developed when amantadine, a drug used in Parkinson's disease patients, was shown to have an anti-influenza effect. Amantadine and rimantadine are two influenza antivirals that inhibit virus replication by blocking the acid-activated ion channel, formed by the integral viral membrane protein M2 (219). Influenza B lacks the M2 protein and cannot be treated with these drugs.

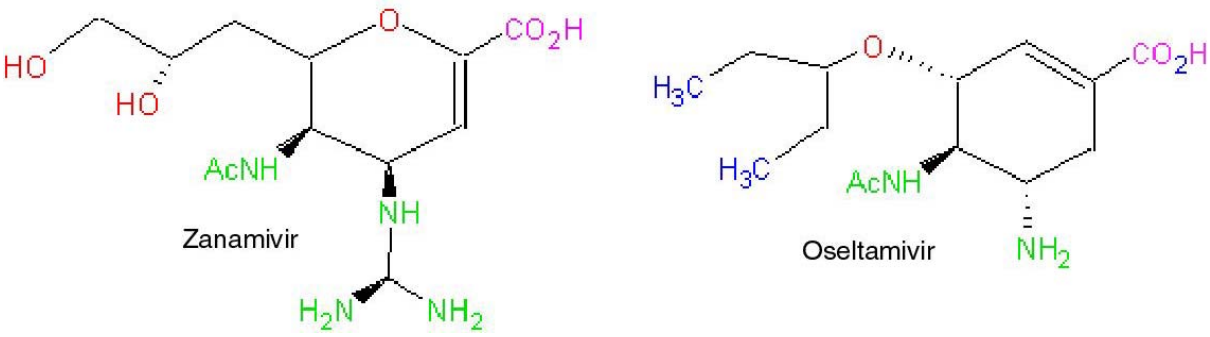


Figure 10. Approved neuraminidase inhibitors for use against influenza virus type A and B.

After the crystal structure of the HA and NA glycoproteins of influenza were determined, computer-assisted design was used to design the first NA inhibitor, zanamivir, which is effective against both influenza type A and B (72, 74, 75, 217, 218). Shortly thereafter the second an orally administered NA inhibitor, oseltamivir, was designed (2, 41, 76). NA inhibitors prevent the release of progeny virions from the infected cells by competitive binding to the enzyme active site on the NA protein. They are effective *in vivo* when applied at the site of infection. The NA inhibitors inhibit the enzyme activity of all nine subtypes of NA *in vitro* (61). The replication of the virus is interrupted and this enables 1-3 days earlier recovery in patients. Treatment of patients must start as soon as possible (within 48 hours), otherwise the drug will have no effect since the immune response thereafter eliminates the virus as efficiently (127). In immuno-compromised patients the drug can be effective during an extended time. Different NA-inhibitors bind differently to the active site and therefore resistance against one drug does not necessarily mean resistance against all known NA inhibitors. When NA inhibitors have been prophylactically tested they are around 90% effective in protecting against illness (212) and can help to protect the frail elderly against infection (153).

1.4.5. Antiviral resistance

A single mutation at any of 1-5 sites within the trans-membrane region of M2, can lead to resistance. The variants are fully cross-resistant to both M2 inhibitors (71). The side effects and rapid emergence of resistance are reasons why amantadine and rimantadine have not been widely used.

Resistance to zanamivir has been observed only in an immunocompromised host to date (60). During or after oseltamivir treatment, <1% of immunocompetent adults and ~8–18% of H3N2-infected children have shed resistant variants (71, 96). Zanamivir resistant mutants often have amino acid changes at positions 119, 292 or 294 in the NA protein (62, 96). These mutants have been shown to have a 100-1000-fold lower sensitivity to zanamivir in plaque-forming assays. Oseltamivir and zanamivir have different chemical structures, which interact differently with the enzyme active site giving variable cross-resistance patterns (213).

There has not been any human to-human transmission of NA resistant viruses identified to date. If the functional balance between HA and NA is disturbed by mutations, in either gene segment coding for the proteins, compensating mutations can arise (204). Mutations in both HA and NA contribute to resistance *in vitro* but it is not clear if HA mutations contribute to resistance *in vivo* (128, 193). When studied in animal models, the viruses that have neuraminidase mutations have been able to spread (77), but the mutated viruses generally have reduced infectiousness and virulence (127, 221, 223). Oseltamivir treated children are able to shed virus even after five days of treatment (96). During the ongoing influenza A/H5N1 outbreak drug resistant viruses have been collected (111). In Japan approximately 5% of the infected individuals, during the 2003-2004 season, received oseltamivir treatment, and resistant viruses were collected from approximately 0.4% of the strains (3). Further, oseltamivir resistant variants often retain full susceptibility to zanamivir (213). Therefore, the strategy for the treatment of influenza A (H5N1) virus infection should include additional antiviral agents (34).

1.5. Diagnostic Methods

1.5.1. Virus isolation

Influenza virus can be isolated in cell culture or in embryonated eggs. It normally takes between one to three days to obtain detectable viral growth. Most avian influenza viruses grow readily in embryonated eggs while some human and porcine viruses grow poorly in eggs. The most widely used cell line for isolation is Madin-Darby canine kidney (MDCK) tissue cultures. Isolation of human influenza viruses in eggs selects variants with amino acid substitutions that cluster around the receptor binding site of the HA molecule (83, 165). Lately, it has also been observed that after repeated passage in MDCK cells a virus more suited for replication in these cells can be selected (57, 63, 130, 134, 164). Changes due to propagation in cell lines or eggs are important to identify and should always be considered in the influenza surveillance.

When MDCK cells are used for isolation of influenza virus the inoculation medium needs to contain trypsin to facilitate cleavage of the HA to HA1 and HA2. After infection, the cell monolayer is studied under the microscope at regular intervals, as infection of cells gives a visible cytopathogenic effect (CPE): cells become more refractile and rounded and eventually loose cells can be seen in the growth medium (224). The presence of influenza virus as a cause of the CPE must be verified. Techniques commonly used for this purpose are hemagglutination (HA), antigen detection *in situ*, most frequently performed with immunofluorescense (IF), enzyme-linked immunoadsorbent assay (ELISA) or polymerase chain reaction (PCR). Neutralisation assays with known antibodies can also be used for

verification, but are often regarded to laborious for this purpose. Apart from HA, the mentioned methods can also be used for direct detection of influenza infection in patient materials, and all except PCR can be reversed and used for detections of antibodies.

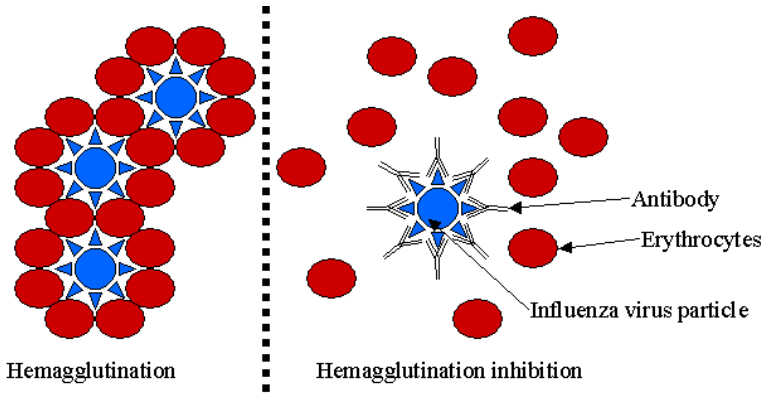


Figure 11.

1.5.2. Hemagglutination and hemagglutination inhibition

Hemagglutination (HA) occurs when sialic acid residues, similar to the cellular receptors, on erythrocytes bind to the receptor-binding site present on the tip of the viral HA proteins (Figure 11). By adding HA recognising antibodies before adding erythrocytes in the assay this binding can be inhibited, hemagglutination inhibition (HAI). When performing HA assays the cell culture supernatant from CPE affected cultures or the allantois from infected eggs are titrated in PBS using V/U bottomed 96 well plates. For agglutination 1% suspension of erythrocytes, normally from guinea pig or turkey, is used. The erythrocytes attach to the HA, and give a visible pattern in the wells. When performing HAI assays sera from infected or vaccinated patients or animals are normally titrated in the plates, to which a known amount of influenza virus, four HA units, and erythrocytes are added.

1.5.3. Antigen detection

IF or other *in situ* methods using labelled antibodies for microscopic detection of infected cells is often used for direct detection of viral antigens in patient samples, or after cell cultivation. The infected cells are fixed on glass slides and incubated with monoclonal antibodies, reacting with certain influenza proteins, followed by a labelled antiserum to the monoclonal. The cells are subsequently viewed and infection verified in a fluorescence microscope. Depending on the specificity of the monoclonal antibodies the method can be used for subtyping of the virus, or characterisation of other viral antigens. For large-scale antigen detection ELISA-methods are used. Infected material is titrated in antibody-coated ELISA plates and incubated before monoclonal antibodies, reacting with specific antigens are added. A 2nd, enzyme labelled antibody reacting with the monoclonal is added and if the addition of the substrate generates a reaction the presence of antigen is verified.

For rapid detection of respiratory viruses, commercial tests have been developed. These tests react with e.g. influenza virus antigen and generate a positive reaction indicating infection in only a few minutes. Rapid diagnosis of respiratory tract infection is important for focused and timely therapeutic intervention, for hygiene measures and for identification of new outbreaks (8).

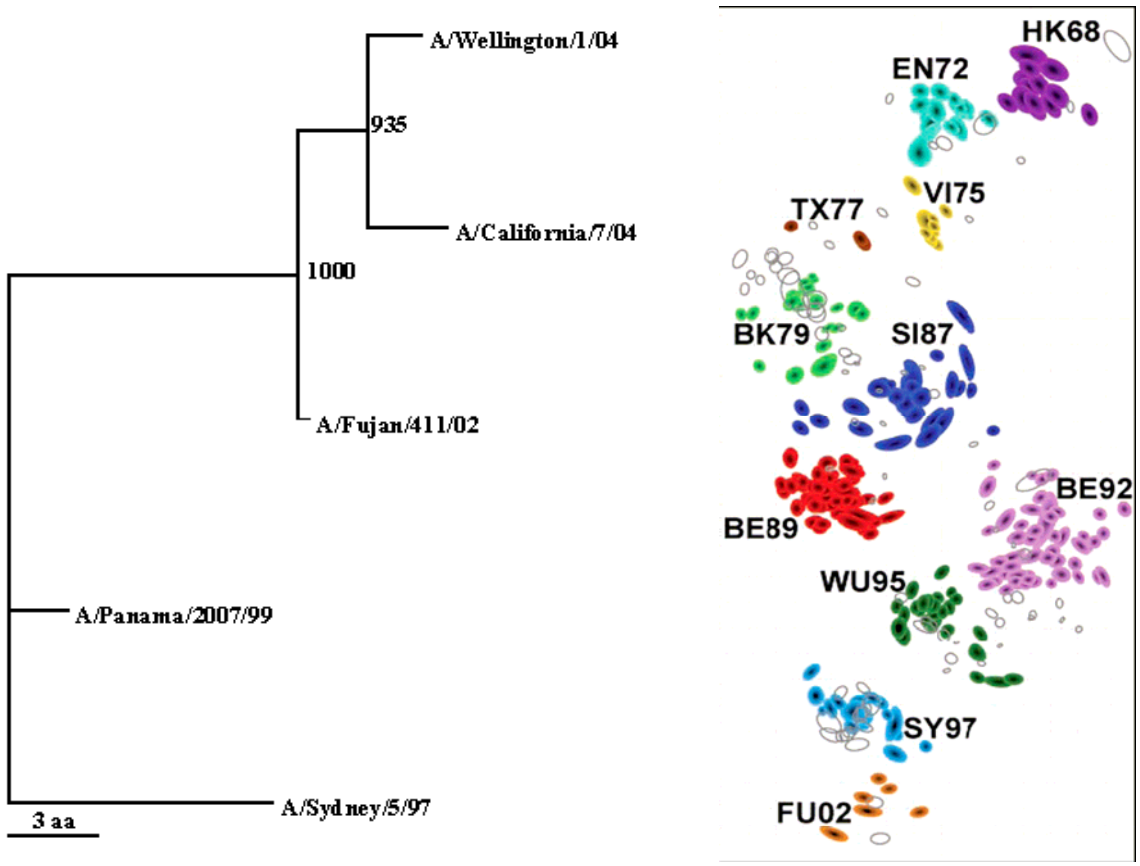
1.5.4. Genome detection

Genome amplification by PCR or other techniques is a powerful technique for the identification of influenza virus genomes. Since the genome is single-stranded RNA, a complementary DNA (cDNA) must be synthesised after extraction prior to the PCR reaction. Reverse transcriptase (RT) is a polymerase used to synthesise such cDNA. For the sensitivities and specificities of RT-PCR based methods the choice of primer sequences is the most crucial parameter.

1.5.5. Molecular and antigenic characterisation of influenza viruses

The HAI assay is the gold standard for antigenic characterisation of influenza viruses. The binding of strain specific antibodies to the viral HA proteins inhibits the hemagglutination (Figure 11). For antigenic characterisation homologous ferret sera against the analysed influenza strains and/or different vaccine strains are commonly used. The ferrets are used for production of homologous serum since they are easily infected with human influenza strains and produce high titres of neutralising antibodies after infection. If the antibodies are homologous to the virus added, high HAI-titres are obtained. The loss of antibody response against a specific hemagglutinin protein is seen when the titre is lowered. For surveillance purposes influenza virus isolates from many different countries are annually screened against ferret type sera. In this way antigenically related or not related strains from the same season can be found. If a strain is closely antigenically related to a known vaccine strain, like the A/Calefornia/7/2004(H3N2), it is A/Calefornia/7/2004(H3N2)-like.

The HAI-titres are mostly presented in HAI-tables and these tables can be used for interpretation of the antigenic relationship between different influenza strains. HAI titres can also be used to create distance matrixes, illustrated by antigenic maps (178). Many kinds of erythrocytes may be used for HA, and the optimal erythrocyte may vary with the subtype and even the strain of the virus. Unfortunately HAI assays using turkey or chicken blood have proven to be relatively inefficient in agglutinating avian strains (181). Many types of erythrocytes may have to be tried out to find the best fitted for an emerging influenza virus.



a, **b,**

Figure 12. a, Phylogenetic aa tree, with bootstrap values, illustrating the genetic distance between the HA1 regions of recent influenza A/H3N2 vaccine strains; **b,** Antigen map of influenza A/H3N2 virus from influenza A/Hong Kong/68 to A/Fujian/02. Strain color represents the antigenic cluster to which the strain belongs. Each cluster is named after de first vaccine strain in the cluster, with the two letters referring to the location of isolation (Hong Kong, England, Victoria, Texas, Bangkok, Sichuan, Beijing, Wuhan, Sydney, and Fujian) and the two digits referring the year of isolation. The spacing between grid lines is 1 unit of antigenic distance, corresponding to a twofold dilution of antiserum in the HAI assay (178).

The entire influenza genome sequence can provide indirect knowledge about transmission, virulence and evolution of influenza A viruses. Specific primers make typing and subtyping of influenza possible (28). Many different subtype-specific PCRs are in use today. To verify and to characterise the amplified gene segment sequencing is often performed. The vRNA is first extracted from influenza isolates. Since influenza virus has a negative stranded RNA genome a reverse transcriptase PCR (RT-PCR) step is demanded to create cDNA. Using a universial primer set for the full-length amplification of all influenza A viruses (78), cDNA of all eight segments of the genome may be produced. Based on which influenza protein that is of interest, segment-specific primers can be used in the PCR to amplify full-length cDNA. The primers for full-length sequencing of for example the influenza segment that codes for the HA (consisting of 1778 bases) are shown in Figure 12. The readable sequence obtained from each successful sequencing reaction is between 500 to 800 bases. Approximately three isolates can be sequenced during one run (2,5h) and thereafter compilation as well as analysis of the sequence can start.

The full-length sequencing of the gene segments has created a number of possibilities. It is for example possible to study single mutations in the genes indicating antiviral resistance or loss of activity. From cloned cDNAs whole new influenza virus particles can be generated, a

technique known as reverse genetics (141). This method has recently been used to generate an influenza virus containing all eight gene segments of the 1918 Spanish influenza pandemic virus (199).

By phylogenetic analysis of the genomic mutations the evolution of influenza isolates, as well as their relation to each other can be illustrated. Most closely followed is the antigenic drift of the HA protein. Using phylogeny programs the sequence of the variable HA1 region from different isolates can be given as a distance and many sequences can be compared to engender a distance matrix. A phylogenetic tree based on the distance matrix can be created. In this way it is possible to genetically relate the HA1 sequences and decide if the circulating stains have drifted genetically from the vaccine strains. Even though a distance is seen in the phylogenetic tree it may not correlate to changes in antigenicity, which is depending on where in the HA1 the nucleotide or amino acid has been changed, and if that position is important for neutralisation by herd immunity.

1.5.6. Antibody detection

The Hemagglutination inhibition (HAI) assay, neutralisation tests (NT) and antibody ELISA:s are often used for the serological diagnosis of influenza infection (Couch and Kasel, 1995). For characterisation or detection of the viruses, known antisera are used in the various assays. For serology, the principle is the same as for antigen assays, but a known antigen is used for binding of serum antibodies. The whole repertoire of Ig classes (IgG, IgM, IgA) are detected in the HAI and NT assays, and both acute and convalescent samples have to be analysed to verify a recent infection, since the basis for diagnosis is a significant titre rise. Different dilutions of the sera are examined, and the results are expressed as titres: the inverted value of the last serum dilution giving a positive result. A more than two-fold increase in HAI or NT titres is regarded to be outside the intra assay variation, and is accepted as a significant titre rise. These assays are frequently used for analysis of the subtype-specific humoral immune response to influenza virus after infection or vaccination at specialised laboratories. In large diagnostic routines, ELISA methods for antibody detection are the rule. With ELISA, the Ig class, or even the subclass specificity of the serum antibodies that have bound to the known antigen can be identified. This is achieved by the Ig specificity of the 2^{nd}, labelled antibody used to react with the serum antibodies that have bound to the known antigen. Presence of specific IgM is normally a sign of an ongoing infection. However, influenza infections are often re-infections, inducing low levels or no IgM, and therefore the IgM tests for influenza are often insensitive. Also with ELISA, significant titre increases between acute and convalescence sera have to be used as the marker for recent influenza infection. This makes serology a slow method, and it is not relevant as guidance for the acute handling of the infected patient.

2. Aims of the present study

- To evaluate if previously described DNA constructs (116) are immunogenic in ferrets and may protect them against challenge with influenza A/H3N2 virus. (Paper I)

- To evaluate an *in situ* micro-neutralisation assay for influenza virus using human cell lines. In addition, to use the assay for comparison of neutralising titres in zanamivir-treated and untreated patients with diagnosed clinical influenza A, and investigate if the drug affected the humoral response after an influenza A infection. (Paper II)

- To map the occurrence of HA variants after *in vitro* passage of human influenza A/H3N2 viruses and to determine whether the use of a human cell line decreases the genesis of variants. (Paper III)

- To study the genetic stability of influenza A/H3N2 viruses in cell culture, and to increase the knowledge on antigenic the drift by passage of one human influenza A/H3N2 virus in cell culture in the absence or presence of three polyclonal neutralising sera. (Paper IV)

3. Paper I – IV in brief

3.1. Paper I

Aim: To evaluate if previously described DNA constructs (116) are immunogenic in ferrets and may protect them against challenge with influenza A/H3N2 virus.

3.1.1. Constructs, immunisation and methods

Vaccine construct: Two different influenza A/H3 constructs [pKCMV HA A/Stockholm(St)/7/97 and pKCMV HA A/NL/18/94] were made. Two different influenza A/H3 virus strains, the human strain A/St/7/97 and the ferret adapted strain A/NL/18/94, were grown in MDCK cells and vRNA was extracted. The gene fragment encoding the variable part of the HA gene (1048bp) was amplified by RT-PCR. Amplicons of the HA1 region from the two strains were used in the produced constructs, pKCMV HA A/St/7/97 and pKCMV HA A/NL/18/94. These amplicons was inserted into a basic construct encoding the influenza A/Stockholm/6/96 (H3N2) gene by homologous recombination cloning. Homologous recombination can occur when overlapping sequences in the PCR fragment recombine with homologous sequences of the vector handle. Plasmid containing clones were selected after co-transformation into *Escherichia coli* XL1 blue. An earlier made construct encoding the NP gene was included in one vaccination group in order to increase the cellular immune response. The purified DNA plasmid vaccine was tested by expression *in vitro* in a human cell line, HEK293 before immunisations.

Immunisation and challenge: In total six groups containing four ferrets were used in the study.
Group 1 naïve control ferrets,
Group 2 received the 1998 human sub-unit vaccine (containing HA and NA from the A/Sydney/5/97-like (H3N2), A/Beijing/262/95-like (H1N1) and B/Beijing/184/93-like viruses),
Group 3 received the empty vector,
Group 4 received pK-CMV-HA A/St/7/97 (A/Sydney/5/97-like),
Group 5 received pK-CMV-HA A/Netherlands (NL)/18/94 (A/Shangdong/9/93-like), and
Group 6 received pK-CMV-HA A/NL/18/94 plus pBK-CMV-NP. The NP gene came from influenza A/PR/8/34 (H1N1).

Four immunisations, each with 100μg plasmid, were given intramuscularly with an interval of 2 weeks. The plasmid expressing NP was added to group 6 in the attempt to improve the cellular immune response and protective immunity. Seven weeks after the last immunisation the ferrets were challenged by intra-tracheal administration with influenza A/NL/18/94(H3N2) virus. ELISA and HAI assays were used to evaluate the humoral response after vaccination. Antibodies to the influenza A/Nanchang/933/95 was analysed in the ELISA and antibodies to A/NL/18/94, A/St/7/97 and A/Sydney/5/97 was analysed in the HAI assay. The cellular response to influenza A/Nanchang/933/95 was analysed by a lymphocyte proliferation assay.

3.1.2. Results

Vaccine and immune responses after vaccination: Homologous recombination allows for a rapid production of chimeric influenza A virus H3 genes that can be expressed from plasmids. A specific antibody response was induced in the ferrets after DNA vaccination with the constructs. Three out of four ferrets vaccinated with pK-CMV-HA A/St/7/97 responded with higher IgG titres than the ferrets vaccinated with the sub-unit vaccine. However, in the sub-unit group all four ferrets responded to vaccination. The plasmid pK-CMV-HA A/NL/18/94 induced relatively low antibody-titres in five of the eight vaccinated ferrets (group 5 and 6). Nine out of twelve of the DNA immunised animals responded, as compared to the 4/4 responding ferrets immunised with the human subunit vaccine. Possibly, a too low dose of DNA vaccine was used. The time intervals chosen between the immunisations may also explain the response differences. Varying responses after immunisation with different plasmids were observed with both ELISA and HAI, and may be explained by differences in the level of antigen expression of different HA antigens between the plasmids. Ferrets have only been used in a small number of DNA immunisation studies and the knowledge concerning optimal immunisation strategies is therefore limited.

Challenge: Even though antibodies were obtained in nine of twelve ferrets after vaccination with the chimeric constructs, infection could not be prevented when the animals were challenged. The sub-unit vaccinated ferrets were also not protected when challenged. Four out of twelve ferrets immunised with plasmid exhibited somewhat lower virus titres in the nose swabs than the unvaccinated control ferrets. However, the differences were not significant. The addition of the NP gene plasmid in the vaccine did not contribute to improved protective immunity against infection. No proliferative T cell response was detected in the ferrets after immunisation, in contrast to previous observations in mice (203).

3.2. Paper II

Aim: To evaluate an *in situ* micro-neutralisation assay for influenza virus using human cell lines. In addition, to use the assay for comparison of neutralising titres in zanamivir-treated and untreated patients with diagnosed clinical influenza A, and investigate if the drug affected the humoral response after an influenza A infection.

3.2.1. Materials and methods

Cell lines and virus: Human foreskin fibroblast (HS27) cells, human salivary gland (HSG) duct cells, and MDCK cells were evaluated in the NT. Three different influenza A viruses, A/Stockholm/5/99(H3N2) (A/St/H3N2), A/Umeå/1/98(H1N1) (A/Um/H1N1), and A/New Caledonia/20/99(H1N1) (A/NC/H1N1; vaccine strain propagated in MDCK cells), were used for the NT.

Neutralisation test: An NT using MDCK cells was first established and optimised in 96-well micro titre plates. The neutralisation of virus was verified by measurement of antigen expression with *in situ* ELISA. The cells were fixed in the wells with acetone and washed before monoclonal antibodies directed against influenza A nucleoprotein were added.

The same assay protocol was then tested using HS27 cells or HSG cells. The trypsin concentration was lowered and the propagation time extended when using human cell-lines.

Serum from 34 influenza A infected, zanamivir-treated or zanamivir-untreated patients were diluted two-fold from 1:16 to 1:8192 and incubated in micro-titre plates with 15 $TCID_{50}$ per well of homogolous or heterologous influenza A virus for three days. We further analysed the specific neutralising antibody titres of serum samples from six patients, four vaccinated with the split influenza vaccine (season 2002/2003) one to two weeks before bleeding and two were unvaccinated controls.

Analysis of sialic acids: The sialic acid linkages on the surface of the cells used were examined by incubations with linkage specific lectins and enzymatic fluorescence staining (DIG Glycan Differentiation Kit, Roche). The staining was analysed under a microscope and the reactivity with α-(2,3) and α-(2,6), respectively, was visually evaluated.

3.2.2. Results

Evaluation of the neutralisation test: Results could be obtained within 24h and infectivity was easily measured using the optical density (OD) obtained in the *in situ* ELISA. However, the MDCK cells produced a high and varied background level in the ELISA, which gave a high inter-assay variation. The HS27 and HSG monolayers gave a low and reproducible background (OD < 0.1). Both the HS27 and the HSG cells were sensitive to the trypsin concentration used for MDCK cells (3 μg/mL), which was lowered.

The influenza viruses did not reach the same titres in the human cells as in the MDCK cells. The use of a larger dose of virus in the NT when using the HSG cell line resulted in lowered antibody titres. When comparing the neutralising antibody titres measured in HSG cells with

the one measured in MDCK cells, almost ten times lower titres were seen for HSG cells. The difference in titres was less when using HS27, and by lowering the infectivity dose from 100 $TCID_{50}$ to 15 $TCID_{50}$ per well the difference in titres was equalised. The dose 15 $TCID_{50}$ using HS27 did not result in any virus negative control wells, and was chosen for the clinical study.

Sialic acid linkages: Unexpectedly the HS27 and HSG cells showed presence of both SAα2,6Gal and SAα2,3Gal, which indicated that both linkage types occur on human continuous cell lines.

The clinical study: The HS27 NT was used for evaluation of the antibody response after the zanamivir treatment. In total, 27 out of 34 the patients with influenza infection were serologically identified with the NT, while 23 out of 34 were identified with HAI performed earlier by Mäkelä et al (121). When comparing the antibody titres after infection it was shown that the production of neutralising antibodies was not affected by zanamivir treatment of influenza-infected patients.

3.3. Paper III

Aim: To map the occurrence of HA variants after *in vitro* passage of human influenza A/H3N2 viruses and to determine whether the use of a human cell line decreases the genesis of variants.

3.3.1. Materials and methods

Infection of cell monolayers: Nine different influenza A/H3N2 viruses; 5 nasopharyngeal aspirates [NPAs; A/Stockholm(St)/26/02, A/St/27/02, A/St/15/04, A/St/17/04, and A/St/12/05], and 4 primary isolates (A/St/19/98, A/St/1/99, A/St/25/02, and A/St/16/04) were examined. Isolation of the NPAs was performed both in the MDCK cell line and in the human salivary gland (HSG) duct cell line. Infection of the monolayer was verified with IF staining using A/H1 and A/H3 specific monoclonal antibodies. The infectivity titres ($TCID_{50}$ per mL) were measured by titration of the virus before and after up to 11 passages in HSG and MDCK cells.

Genetic characterisation: The variable region of the hemagglutinin genes (HA1) from the isolates was extracted, amplified and sequenced from altogether 42 materials (NPAs, after two passages and after 8-11 passages in MDCK cells or HSG cells). All sequences were compiled, aligned, and phylogenetically analysed using PHYLIPS 3.36 and MEGA3. In the phylogenetic analysis using PHYLIPS 3.36 we added in Swedish influenza HA sequences, from strains isolated the years 1997 to 2005, as well as the reference vaccine strains from the years 1987 to 2004 for comparison. In the phylogenetic analysis using MEGA3 we only added in reference vaccine strains from the years 1999 to 2004. All sequences obtained were also compared with the human influenza A/H3N2 sequences in the influenza sequence database (ISD) (119) for which cultivation information and passage numbers had been deposited, in total 445 sequences.

HAI assay: The HAI assays were carried out using five ferret reference sera (A/Panama/2007/99, A/Wyoming/3/03, A/Wellington/1/04, A/Shantou/1219/04, and A/California/7/04). Four HA units of the influenza antigens, from four analysed viruses after 2 and 11 passages in MDCK cells and control antigen [A/Panama/2007/99(MDCK-grown); A/Wellington/1/04(egg-grown) and A/California/7/04(egg-grown)], were added to the diluted antibodies.

3.3.2. Results

After 8-11 *in vitro* passages the infectious titres for HSG propagated viruses were between 1 and 10^5 $TCID_{50}$ per mL cell medium compared to titres between 10^1 and above 10^9 $TCID_{50}$ per mL for viruses propagated in MDCK cells, which clearly showed that MDCK cells are more efficient than HSG for propagation of recent human influenza A/H3 viruses. However, one influenza isolate gave higher titres in HSG than in MDCK cells.

After two passages in the two cell lines all sequences were unchanged compared to the NPA sequences. Alterations occurred after several passages and were either close to the receptor-binding site (positions 145, 196, 218 or 222), or within it (positions 138 or 226). Alterations

in the HA1 region of the HA gene were regularly found in viruses growing to infectious titres $>10^3$ $TCID_{50}$ per mL. Five out of the six aa residues were within the antigenic sites, A, B or D. Irrespective of in which cell line the propagation occurred, the influenza A/St/26/02 isolate obtained a V to I alteration at position 226. This change was also present after passage of A/St/25/02 in MDCK cells, and of A/St/27/02 in HSG cells. The change V226I was found *in vivo* after 2002. By investigating the passage history of isolates present in the ISD together with sequences from the National Influenza Centre in Sweden we have found that I226 has been present as a possible *in vitro* selected mutant from 1999 until 2004.

A N145K alteration was found in two isolates from 2004 after propagation in MDCK cells. This adaptation has earlier been found after propagation in embryonated hens eggs, and has been shown (49, 57) to be an antigenically important cluster difference between the A/Sichuan/87 and A/Beijing/89, as well as between the A/Beijing 92 and the A/Wuhan/95 clusters (178). The alteration required an update of the vaccine strain. We further found that S145 is today present in primary viruses present in nasopharyngeal aspirates. It has not been found in influenza viruses since the 1970-ties.

Our evolutionary phylogenetic analysis revealed that three out of the seven MDCK adapted influenza viruses obtained alterations that gave them a “negative” genetic evolutionary development. The HAI analyses that could be performed indicated a change in antigenic response of the four tested strains after cell adaptation.

3.4. Paper IV

Aim: To study the genetic stability of influenza A/H3N2 viruses in cell culture, and to increase the knowledge on antigenic the drift by passage of one human influenza A/H3N2 virus in cell culture in the absence or presence of three polyclonal neutralising sera.

In the two influenza seasons, 2003-2004 and 2004-2005, there have been partial mismatches in the vaccine due to the present, rapid evolution occurring in the H3 virus. A major public health goal is to refine the prediction techniques used to select vaccine strains.

3.4.1. Materials and methods

Virus, sera, and antigenic characterisation: The influenza A/Umeå/2/00 virus, from season 1999-2000, passaged 5 times in MDCK cells (p5), was used in the immune escape study. Two polyclonal convalescent sera, A and B, selected from persons with recent influenza A infections in 1999, and one pooled goat serum, C, containing anti-influenza A/H3 sera from season 1994-1995 until season 1999-2000 (anti-A/Sydney/5/97, anti-A/Wuhan/359/95, anti-A/Nanchang/933/95, anti-A/Johannesburg/33/94, and anti-A/Shangdong/9/93 sera; provided by WHO) were used to excert immune pressure in the immune escape study. All sera were characterised concerning influenza strain reactivity using HAI and NT. Different reference goat sera from 1972 until 1999 (anti-A/England/42/72, anti-A/Victoria/3/75, anti-A/Texas/1/77, anti-A/Bangkok/1/79, anti-A/Leningrad/360/86, anti-A/Sichuan/2/87, anti-A/Shanghai/11/87, anti-A/Shanghai/96/89, anti-A/Beijing/353/89, anti-A/Beijing/32/92, anti-A/Shangdong/9/93, anti-A/Johannesburg/33/94, anti-A/Nanchang/933/95, anti-A/Wuhan/359/95, anti-A/Sydney/5/97, anti-A/Panama/1001/99) provided by WHO were used for the HAI assays.

Immune escape study. The influenza A/Umeå/2/00(p5) virus was passaged in MDCK cells, 28 times in the presence or in the absence of an increasing amount of antiserum A or 32 times in the presence or in the absence of an increasing amount of antiserum B and C. We also combined the three different sera; first we performed 10 passages with only C, then 10 passages with both C and A, and finally twelve passages with C and A and B (32C+22A+12B). Each serum was added in concentrations that gave neutralisation between 0.5 and 0.03**.** Sequencing of the HA gene from A/Umeå/2/00 was performed after two and five passages. Cloning was performed after five passages to study the presence of pre-existing variants in the start material before onset of the immune escape study. From all crude supernatants from the last passage as well as from the original sample influenza virus RNA was extracted, amplified, and sequenced. The PCR products were also cloned into the pGEM®-T Easy plasmid vector, and the plasmid was transformed into *Escherichia coli* XL1 blue. All sequences from the different clones were aligned and analysed (BioEdit, MEGA3).

3.4.2. Results

The antibodies present in serum A were directed mainly against the recent influenza strains, with HAI titres of 400, compared to 100 or less for strains circulating more than six years before the serum was drawn. The HAI titres of the two additional sera were higher than for serum A, and the neutralising titres for the polyclonal sera A, B, and C against influenza

A/Umeå/2/00 were 256, 2048, and 32000, respectively. Since the amount of antibodies present in the suspension was kept just below the neutralising concentration, the virus still grew in the presence of antibodies. The virus titres in the passaged materials were not measured.

When analysing the directly sequenced HA genes after propagation in the presence of serum A one preserved amino acid alteration, I140M, was identified after 20 and 28 passages in MDCK cells. No aa alterations were found in the cloned HA1 parts of the HA genes after propagation with or without serum B or C+A+B. Point mutations were verified with cloning of the virus propagated in the presence of serum B. The clone consensus sequences of the virus were, however, identical with that of the original A/Umeå/2/00 virus.

Since in more a recent study we found that after several *in vitro* passages a more cell culture fit variant was selected (Paper III), the presence of cell adaptation of the isolate used in the immune escape study was retrospectively analysed by comparing the HA1 sequence from the A/Umeå/2/00(p2) with the HA1 sequence from A/Umeå/2/00(p5). We found an adaptation from K to I in aa position 229. The HA1 from A/Umeå/2/00(p5) was cloned and the sequences of the analysed four clones were not identical. One clone was identical to the sequence from the direct sequencing of the same material. The three other clones had sequences that differed from the total A/Umeå/2/00(p5) sequence in one or two aa positions.

4. Discussion

4.1. Protection

The most effective way to combat influenza virus infection and disease is to reduce transmission of the virus. This can best be accomplished through vaccination. However, the genetic variation of influenza viruses gives the virus the ability to gradually evade host immunity (115) and in addition the effect of a given vaccine against a homologous strain lasts for less than a year. Rapid production, improved immunogenicity and efficacy of human influenza virus vaccines is necessary to be prepared for sudden antigenic drift in epidemic strains and reassortment or adaptation of new influenza viruses to the human host.

Vaccines: The inactivated vaccines stimulate better systemic responses while the live attenuated vaccines are stimulating the local immune system (29). In the elderly the combined use of inactivated influenza vaccines and live attenuated influenza vaccines are giving a better protection against influenza infection than the use of inactivated influenza vaccines alone **(149, 180, 197, 198)**. Children, particularly seronegative children that are considered naïve, have more frequent serum antibody responses than adults after live attenuated influenza vaccinations (9, 29).

DNA vaccines could be an alternative strategy for influenza vaccination since they are rapidly produced and can induce a cellular response. DNA vaccines also serve as a potentially safer alternative to immunisation with certain live virus vaccines (21). In mice low doses of DNA administered by intra-muscular injection provided protection against influenza (201). DNA immunization can elicit a reasonable level of immunogenicity in several different animal models (38, 102, 103, 118, 120), and HA DNA plasmid vaccine has recently also been shown to induce hemagglutinin inhibiting antibody response in humans (39). Intra-muscular DNA vaccination of ferrets induced a response against the specific proteins encoded by the vaccine. However, no protective immunity was induced with the dose (100μg) of DNA chosen in our study (**Paper I**), and the chosen dosage may have been too low. A higher dose of DNA (1-2mg) has been shown to induce protective immunity in ferrets (37). Possibly a more effective route of administration should have been chosen. Delivery of DNA-coated gold beads by gene gun to the epidermis have been shown to be much more efficient than intramuscular delivery of DNA in aqueous solution (211). The immune response after vaccination can be further enhanced by the addition of other synthetic immunostimulants (adjuvants). We tried to increase immunogenicity by adding a plasmid containing the NP gene to the preparations, but protection against challenge was not obtained. Together with DNA vaccines CpG motifs or modifications to the vector have increased the immunogenicity (200), and it is likely that this could have improved the results. However, we clearly showed that the DNA plasmid given a new variable region by the use of homologous recombination was immunogenic in ferrets. Considerable enhancement of the immune response to DNA immunization and extensive safety characterization will be needed before DNA-based approaches can become a truly promising approach for humans (93).

Antivirals: There are currently four available antiviral drugs against influenza licensed for human use, amantadine (M2-inhibitor), rimantadine (M2-inhibitor), oseltamivir (Tamiflu; NA-inhibitor) and zanamivir (Relenza; NA-inhibitor). Development of resistant strains is under continuous surveillance. The commonly used NA enzyme inhibition assay measures the

reduction in enzymatic activity (the level of resistance) due to mutations occurring in the NA gene. The assay is unable to give valuable information about possible compensatory mutations in the HA gene. Since also plaque reduction assays in MDCK cells have been shown to produce misleading data, it would be of great value to be able to use human epithelial cells for cell-based analysis of resistance to NA inhibitors (192). In an attempt to find a human cell line for the cultivation and analysis of the functional balance between HA and NA in human influenza viruses we have analysed two different human cell lines (**Paper II and III**). We have tested the HS27 cell line for evaluation of antiviral resistance by exchanging antiserum for different dilutions of antivirals. In preliminary experiments with oseltamivir we found no difference in inhibiting concentration 50% (IC_{50}) for the drug related to the cell line used.

4.2. Surveillance

The isolation and characterisation of influenza viruses circulating worldwide are essential for the surveillance of influenza viruses. To monitor epidemic influenza strains worldwide, the WHO established a surveillance network across the world in 1948. Currently, 111 national centres in 83 countries collect and screen about 175 000 samples each year (109). The tracking and identification, through antigenic typing and sequencing, of influenza strains allows for accurate evaluation of the strains to be used in the annually produced vaccine (54). A rapid, standardised method that could be used for characterisation of any virus strain is indeed desirable.

Characterisation of the antibody response: The HAI assay is most commonly used to screen influenza virus isolates to determine strain variations/drift. Virus isolates with a few amino acid changes over antigenic sites are not always discriminated with the HAI. The effect of neglecting this fine antigenic drift and its impact on the production of an effective vaccine is poorly understood (7). The neutralisation test (NT) may be preferable in this task since it is more sensitive (32, 58). Antibody detection to avian influenza A/H5N1 viruses with high NT titres and low HAI activity have for example been reported (168). The reason why the NT has been less frequently used is because it is more laborious, and therefore better ELISA-format NT:s are sought (32). Our evaluation of an *in situ* micro-neutralisation test for influenza virus (**Paper II**) showed that the NT using human fibroblast cell-lines was more sensitive than the HAI assay. The NT method may be a more sensitive assay for detecting low but specific titres in vaccine studies. The immune responses to influenza viruses in infected adults are already primed due to earlier infections and vaccinations, and compared to the HAI assay it may be possible to measure heterologous titre raises with the NT. HAI assays are are relatively insensitive for the detection of human antibody responses to avian haemagglutinin, even in the presence of high titres of neutralising antibody after confirmed infection or vaccination. Therefore, the NT may be better suited for analysing the immune response to avian influenza strains.

Phenotypic and genetic changes in HA: Isolation and propagation of human influenza virus *in vitro* may cause selection of antigenic variants (90, 134, 165, 170, 171) (**Paper III**). Developing optimal survival, replication and transmission strategies are the driving force behind the Darwinian evolution of viruses (33). A change in the replication environment generates a need for adaptation. To identify amino acid alterations due to any type of *in vitro* propagation may be important for selection of strains for vaccine production and for

understanding the effect of large-scale cultivation of the vaccine strains in different systems. Introduced alterations should always be considered in the influenza surveillance.

Genetic characterisation: Differences in the genome sequence of different influenza viruses can be illustrated in a phylogenetic tree. The sequences may reveal variations in HA which were impossible to verify with HAI. With sequencing the positions of the changes can be identified and the antigenic importance evaluated (69, 114, 178).

Antigenic drift of influenza virus is closely followed from year to year, and correct selection of the vaccine strains depends on the prediction of which cluster of virus strains is likely to dominate transmission the following year (178). The evolution of human influenza A/H3N2 virus is faster than influenza A/H1N1 and B viruses (70). The higher evolution rate in influenza A/H3N2 viruses has required 21 changes in the vaccine component over 33 years (from 1972 until today), in contrast to 10 changes to the B component and 7 changes to the A/H1N1 component. The rapid evolution occurring in the A/H3N2 virus has led to partial mismatches in the vaccine component during the past two influenza seasons. There is therefore a need to refine the tools used to decide and predict which strain will give a vaccine that is matched to the coming epidemic strain.

The evolution of influenza virus is mainly driven by two parameters: the mutation rate and the herd immunity. During replication of the viral RNAs, the lack of proofreading by the polymerase allows for replication errors. The virus is striving for increased fitness, and mutations giving replicative advantages will be selected very rapidly. Host selection pressure varies between species (186). Monoclonal antibodies have been used to induce escape mutants (108, 138), but in the presence of polyclonal antibodies escape mutants have not been easily obtained (26, 133) (**Paper IV**). In vitro prediction of evolution by viral propagation in the presence of influenza-specific polyclonal sera probably demands that the virus used is a quasispecies containing the emerging strain. Our study confirms the relative genetic stability of the HA gene of influenza A/H3N2 viruses in cell culture and contributes to the increasing evidence that evolution of human influenza viruses in the human population is mainly driven by the varying immune responses present. The immunity lowered with time and the young naïve children are also important factors, which enable the virus to continue to circulate.

5. Conclusions

Paper I:

After immunisation of ferrets with DNA vaccine constructs expressing chimeric influenza HA proteins a specific antibody response was induced but did not protect against challenge.

The concept of homologous recombination was proven efficient but higher doses of the DNA vaccine, another mode of delivery and adjuvant are probably needed to obtain a protective response after DNA vaccinations.

Paper II:

A micro neutralisation test with antigen ELISA for evaluation of the growth inhibition was successfully established. The neutralising titres obtained using HS27 cells were found to be similar to the ones using the canine MDCK cell line. The assay using HS27 was found to be very sensitive, stabile, and should allow for automatisation and large-scale studies. The test may be a valuable analytic tool for vaccine studies and for the analysis of the human response after infection with influenza strains, which have a low agglutinating capacity, preventing the use of the HAI assay.

Paper III:

Propagation of influenza viruses *in vitro* to high titres rapidly allows for introduction of genetic alterations in antigenically important HA amino acid residues.

Cell adaptation variants can be identical to the variants circulating, and possibly the variant is present in the quasispecies of the patient material. Analysis of *in vitro* selections may reveal positions and alterations that possibly are undergoing positive Darwinian selection.

Surveillance of cell adaptations of human influenza A/H3N2 virus may become necessary since antigenically important aa alterations, similar to those earlier found after propagation in embryonated hens eggs, were regularly found in the HA gene after propagation in MDCK cells.

Paper IV:

Propagation of an influenza virus in the presence of antiserum with antibodies directed against several antigen binding sites seem to limit the ability to escape, and creates a “dead end” for the virus, where it is unable to adapt.

To obtain enough variants allowing for antibody escape we suggest that the virus must replicate many times in the presence of a varied immune pressure. The selection of variants with increased fitness may then take place.

6. Final remarks

This thesis has focused on the protection against influenza virus infection and disease as well as the analysis of the antigen variation in the HA gene of human influenza A/H3N2 viruses.

As our understanding of viral pathogenesis and human immunology deepens and the ability to manipulate the influenza virus and modulate the immune system expand, new hypothesis for efficient vaccine approaches and more effective ways to monitor the viruses are born. We have examined a rapidly produced DNA vaccine as alternative for today's influenza vaccines. DNA vaccines aid in speeding up the production step and could possibly be used for rapid correction of mismatches in the vaccine composition. When there is a good antigenic match between the strains included in the vaccine and the strains in circulation, the protective efficacy is often 70–95% in healthy young adults (131). The protective levels can be much lower in immune compromised and naïve individuals. So far, DNA vaccines have shown promising results in animal models, but will await further progress in administration techniques, the development of new adjuvants, and increased knowledge concerning immunogenicity before they can be used as efficient vaccines for humans.

To be able to confirm small response differences after vaccination and to improve the methods used for the testing of antiviral resistance, we established a neutralisation method in a human cell-line. Influenza viruses are affected by changes in the environment and we have analysed the effect of isolating and propagating the human influenza A/H3N2 viruses in two different cell-lines. Random mutations in the genome of influenza viruses occur during replication and mutations increasing the virus "fitness" will be selected. *In vivo*, the influenza virus has to struggle against a strong selective immune response; while *in vitro* the environment is different and partial immunity is lost. The change supports the selection of new variants. By propagating an isolated influenza virus in the presence of polyclonal antibodies we tried to shed light on what is required for the immune escape through antigenic drift.

Regardless of all new inventions there are still a number of unanswered questions. Finding out what makes an influenza virus transmissible in humans and animals, why some cells are infected in a susceptible host and others not, and what are the specific interactions between viruses and different immune cells, are among key issues to find new ways to prevent and treat influenza.

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