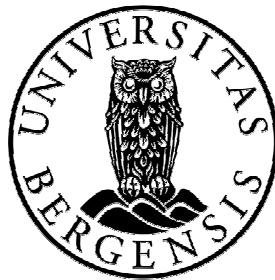


# **The effect of influenza virus vaccine formulation**

*- a potential for increased vaccine efficacy*

**Arnt-Ove Hovden**



Thesis for the degree Philosophiae Doctor (PhD)  
at the University of Bergen

October 2005

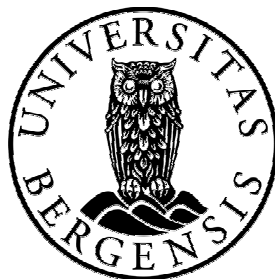
*To the many mice sacrificed for the advance of medical science*

# **The effect of influenza virus vaccine formulation**

*- a potential for increased vaccine efficacy*

**Arnt-Ove Hovden**

Thesis for the degree Philosophiae Doctor (PhD)



The Influenza Centre,

The Gade Institute, Faculty of Medicine

University of Bergen

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Bergen, October, 2005



Arnt-Ove Hovden



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## Summary

Influenza remains one of the most serious viral diseases in the world, causing substantial morbidity and is responsible for at least one million deaths annually. A vast influenza virus reservoir exists in birds to which man are immunological naïve. At irregular intervals novel influenza viruses cause pandemics with potential overwhelming consequences for health care systems and society at large. The most devastating pandemic in living memory was the 'Spanish flu' in 1918-19, claiming the lives of over 40 million people. The most efficient way of saving lives are by prophylactic use of influenza vaccines and vaccination also reduces the number of people suffering from influenza and its associated medical problems. However, due to the nature of influenza virus with a rapid accumulation of mutations changing the antigenic signature of the virus, the vaccine strains must be updated annually. The antigenic composition and timing of an antigenic shift of influenza A viruses, which can result in a pandemic, cannot be predicted and as a result is it not possible to produce a vaccine against the next pandemic strain in advance. This requires research to establish strategies for the most efficient way of using the vaccine that unquestionably will be in limited supply.

Inactivated vaccines can be made in several different formulations, based on how the inactivated virus is treated. Whole virus vaccine was widely used until the 1970s, but the less reactogenic split and subunit vaccines are the most commonly used vaccine formulations today. Nonetheless, whole virus vaccine is the most immunogenic of the three formulations and may be needed in a pandemic situation to ensure a good vaccine efficacy. We have tested the resulting immunity after vaccination with split and whole virus vaccine in a mouse model. Several important results were found. We used a whole virus vaccine produced at modern production facilities that was more immunogenic in immunological naïve mice than split virus vaccine. In a pandemic situation, we will not have any immunological memory directed against the novel influenza A virus, thus the increased immunogenicity in a naïve animal is important. Furthermore, we have confirmed the importance of pre-existing serum IgG in preventing viral shedding in the upper respiratory tract, lending evidence to an on-going debate of how the upper respiratory tract is protected against viral infection.

We found that the vaccine formulation also influences which T-helper response elicited. Split virus vaccine induced a low IgG2a/IgG1 ratio and a cytokine profile associated by a T-helper 2 response, which is characterised by a humoral immune response. Whole virus vaccine on the other hand, resulted in a T-helper 1 response as demonstrated by a predominance of the IgG2a subclass and type 1 cytokines, indicative of a more cell-mediated immune response. Interestingly, whole virus vaccine also induced higher concentrations of cytokines that stimulates an IgA response, which is a more cross-reactive antibody. Provided it has an acceptable reactogenicity profile in man, whole virus vaccine may become the preferred human influenza vaccine formulation, both for the next pandemic and possibly also for the annual influenza outbreaks.

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## List of papers

- Paper I:** Hovden, A.-O., Cox, R. J., Haaheim, L. R. (2005) Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. *Scand J Immunol* 62:36-43
- Paper II:** Hovden, A.-O., Cox, R. J., Madhun, A., Haaheim, L. R. (2005) Two doses of parenterally administered split influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral shedding upon challenge in mice. *Scand J Immunol* 62:342-352
- Paper III:** Hovden, A.-O., Brokstad, K., Szyszko, E., Cox, R. J., Madhun, A., Haaheim, L. R. (2005) The formulation of an influenza vaccine influences the T-helper response –analysis of the cytokine response profile. Manuscript for a short communication.
- Appendix** Supplementary data (H7N1 whole virus vaccine)

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## Abbreviations

<b>ADCC</b>	Antibody dependent cell-mediated cytotoxicity
<b>ASC</b>	Antibody secreting cell
<b>BM2</b>	Influenza B matrix 2 protein
<b>ca</b>	Cold adapted
<b>CD</b>	Clusters of differentiation
<b>CHMP</b>	Committee for medicinal products for human use
<b>CM2</b>	Influenza C matrix 2 protein
<b>CTL</b>	Cytotoxic T lymphocyte
<b>dIgA</b>	Dimeric IgA
<b>dsRNA</b>	Double stranded ribonucleic acid
<b>DTH</b>	Delayed type hypersensitivity
<b>eIF2<math>\alpha</math></b>	Eukaryotic translation initiation factor $\alpha$
<b>ELISA</b>	Enzyme linked immuno sorbent assay
<b>ELISPOT</b>	Enzyme linked immuno spot
<b>ER</b>	Endoplasmatic reticulum
<b>FADD</b>	Fas associated death domain
<b>FcR</b>	Fc receptor
<b>GM-CSF</b>	Granulocyte macrophage-colony stimulating factor
<b>GP</b>	General practitioner
<b>HA</b>	Haemagglutinin
<b>HEF</b>	Haemagglutinin-esterase fusion protein (Influenza C)
<b>HI</b>	Haemagglutination inhibition
<b>HIV</b>	Human immunodeficiency virus
<b>IFN</b>	Interferon
<b>Ig</b>	Immunoglobulin
<b>IL</b>	Interleukin
<b>ILI</b>	Influenza like illness
<b>ISRE</b>	Interferon stimulated response element
<b>M1</b>	Matrix protein 1
<b>M2</b>	Matrix protein 2
<b>MHC</b>	Major histocompatibility complex
<b>mRNA</b>	Messenger RNA
<b>NA</b>	Neuraminidase
<b>NB</b>	Influenza B NA –frame 2

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<b>NEP</b>	Nuclear export protein
<b>NK</b>	Natural killer cell
<b>NLS</b>	Nuclear localisation signal
<b>NMR</b>	Nuclear magnetic resonance
<b>NP</b>	Nucleoprotein
<b>NS1</b>	Non structural protein 1
<b>NS2</b>	Non structural protein 2 (renamed NEP)
<b>nt</b>	Nucleotide
<b>PA</b>	Protein acidic
<b>PB1</b>	Protein basic 1
<b>PB1-F2</b>	Protein basic 1 – frame 2
<b>PB2</b>	Protein basic 2
<b>pIgR</b>	Poly immunoglobulin receptor
<b>PKR</b>	Protein kinase R
<b>RNP</b>	Ribonucleoprotein
<b>SA<math>\alpha</math>-(2,3)Gal</b>	Sialic acid $\alpha$ 2,3 Galactose
<b>SA<math>\alpha</math>-(2,6)Gal</b>	Sialic acid $\alpha$ 2,6 Galactose
<b>SC</b>	Secretory component
<b>S-IgA</b>	Secretory IgA
<b>SRD</b>	Single radial diffusion
<b>SRH</b>	Single radial haemolysis
<b>ssRNA</b>	Single stranded ribonucleic acid
<b>Tc</b>	Cytotoxic T-cell
<b>TcR</b>	T-cell receptor
<b>Th</b>	T helper cell
<b>TLR</b>	Toll like receptor
<b>TNF</b>	Tumor necrosis factor
<b>VN</b>	Virus neutralisation
<b>vRNA</b>	Viral ribonucleic acid
<b>WHO</b>	World health organisation

# 1 Introduction

## 1.1 Introduction to influenza

Influenza virus is one of the major causes of disease worldwide. During the annual influenza outbreaks, it is estimated that 5-15% of the world's population are infected resulting in one million deaths every year [236, 251]. There is extensive knowledge of influenza virus and immunity following infection after many years of scientific research, but the virus still has the ability to surprise us. At unpredictable intervals, a novel influenza A virus subtype appears in man and gives rise to a worldwide influenza outbreak, termed a pandemic. This occurred three times during the 20<sup>th</sup> century, namely in 1918, 1957 and 1968 [238]. The 1918 pandemic had the highest mortality, causing approximately 40 million deaths worldwide, with an unprecedented number of deaths of young adults [180]. If a pandemic with similar mortality occurred today the number of deaths could in a worst-case scenario exceed 350 million people [164].

The transmission of a highly pathogenic avian H5N1 influenza virus to man in Hong Kong in 1997, which had a lethality rate of 33% [203, 216], resulted in a renewed interest in influenza and research into pandemic vaccines candidates. Only a resolute culling of all chickens in Hong Kong and the lack of person-to-person spread stopped a potential fourth pandemic occurring at the end of last century. Subsequently, several avian influenza subtypes have been documented which infect man, with the largest outbreak still ongoing in South East Asia [15]. The WHO has declared that all requirements for a pandemic of the H5N1 virus now circulating in Asia, but one, have been met, underlining the possible threat and danger that the world is facing [238, 239].

## 1.2 The clinical manifestations of influenza

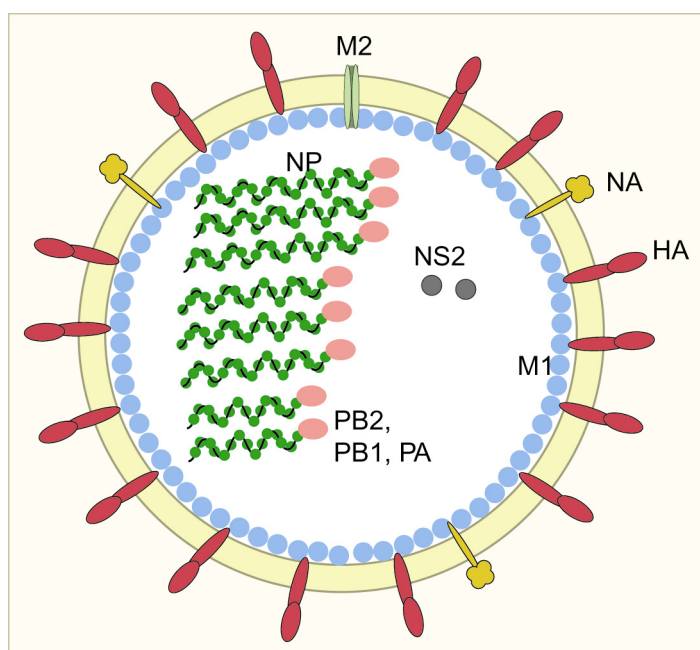
Influenza virus normally infects the epithelial cells of the upper respiratory tract. An individual can experience repeated infections throughout his or her lifetime. The reason influenza causes repeated infections is that influenza virus mutates frequently and leave an individual at best only partially protected against recurrent infection with new influenza strains. Influenza is transmitted via droplets expelled upon sneezing and coughing [155]. The incubation period is usually 2-3 days before onset of illness, but it can be as long as 7 days. The patient is generally contagious during the febrile phase, but cases of viral spread have been observed seen prior to symptoms. The illness lasts approximately one week and is normally accompanied by high fever, headache, myalgia, sore throat and rhinitis. The severity of infection is correlated with the level of viral shedding. High levels of viral shedding are often found in people with more severe illness and higher grade fever, whereas people with low levels of viral shedding have less clinical symptoms or are asymptomatic [145, 146].

Healthy people usually recover within one week of bed rest without requiring any medical intervention. In the very young, the elderly and people with underlying medical problems (e.g. diabetes, cancer, neurological diseases, kidney, cardio or respiratory diseases) influenza poses a serious risk, and infection may lead to hospitalisation and in some cases death [153]. The cause of death can be the virus itself (viral pneumonia) or secondary infection (often bacterial pneumonia) as the cells of the epithelia are damaged by virus replication. In Norway, the estimated excess mortality during an annual influenza outbreak is up to 1500 deaths per year [1]. During a pandemic, an increased number of deaths and a higher frequency of medical complications occur than in the yearly influenza outbreaks. Influenza virus replication is normally confined to the respiratory tract, but the virus that caused the H5N1 outbreaks in 1997 [223] and 2003 [170] has been shown to have an unusually broad cellular tropism with virus detected in lungs, spleen, heart, brain and colon of diseased individuals [86]. A special composition of the cleavage site of the haemagglutinin (HA), which needs to be cleaved to produce an infectious virus, may have contributed to the systemic spread of H5N1. Similar to the virus that caused the 1918 pandemic [122], H5N1 has also resulted in high systemic cytokine levels, which may have contributed to the pathogenicity observed [223].



### 1.3 The Influenza virus

Influenza belongs to the family of *Orthomyxoviridae* [65]. The first virus was isolated in 1933 and is referred to as A/Puerto Rico/33/8 or PR/8 (Figure 1) [202]. There are three influenza genera, *Influenzavirus A*, *Influenzavirus B* and *Influenzavirus C*, which are divided on the basis of antigenic differences in the internal proteins, matrix (M) and nucleoprotein (NP). The three genera differ in epidemiology, host range and pathogenicity. Influenza A and B viruses are important human pathogens, whereas influenza C infection results only in a mild respiratory infection in man. Influenza C viruses are only rarely isolated, but by early adulthood 96% of the human population have antibodies directed against influenza C, indicating that infection with influenza C is common [159]. Influenza B viruses are mainly found in man, whereas influenza A viruses are found in a range of vertebrates with waterfowl being the most important host [157]. The influenza A genus is further subdivided based on the antigenic properties of its surface glycoproteins, the HA and the neuraminidase (NA) (Table 1). Currently, there are 16 HA and 9 NA subtypes recognised by the WHO [240]. The 16<sup>th</sup> HA subtype was detected in gulls in Sweden in 2005 [70]. The guidelines for



**Figure 1.** A schematic figure of the virion of influenza A. Three proteins are detected in the viral envelope, Haemagglutinin (HA), Neuraminidase (NA) and the matrix protein M2 ion channel protein. The M1 matrix protein, the nucleoprotein (NP), the polymerase complex (PB1, PB2 and PA), as well as the Non Structural 2 protein (NS2 or NEP). NS1 and PB1-F2 proteins are not included in the virion.

influenza virus nomenclature established by the WHO [233] names the virus isolate in the following order: the influenza genus (e.g. A, B or C), then the host (omitted if human), followed by the place of isolation, strain number and year of isolation. For influenza A viruses, an abbreviation of the HA and NA subtypes are included in brackets. For example: B/England/5/66, A/Panama/2007/99 (H3N2) and A/Chicken/Italy/13474/99 (H7N1). Influenza B and C viruses are not divided into subtypes. The work of this thesis has used influenza A viruses and all subsequent text refers to influenza A, unless otherwise stated.

**Table 1. Influenza A subtypes in different species**

Species	HA subtype	NA subtype
Man	H1, H2, H3	N1, N2
Swine	H1, H3	N1, N2
Horse	H3, H7	N7, N8
Birds	H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16	N1, N2, N3, N4, N5, N6, N7, N8, N9
Whales, Seals	H4, H7	N5, N7

### 1.3.1 Viral structure and genome

Influenza has a negative sense single stranded (ss) RNA genome. The virion has a viral envelope derived from the plasma membrane of the host cell (Figure 1 and 4). The genome of influenza A viruses has 8 segments, each coding for one or two proteins, in all 11 proteins (Table 2). The largest segment is assigned segment number 1 and the remaining segments are numbered according to decreasing segment size. Each segment is encapsulated by the NP to form a ribonucleoprotein complex (RNP). Each RNP has a viral RNA polymerase attached to it, consisting of the three viral gene products that make up the RNA polymerase complex. There are three integral viral proteins located in the viral envelope of influenza A viruses; the HA, NA and an ion channel protein named M2. The peripheral matrix protein (M1) lines the viral envelope in close proximity to the viral genome and is hypothesized to interact with cytoplasmic tail of the surface glycoproteins [127]. The virion is pleomorphic in structure, with a spherical particle (80-120nm in diameter) as the dominant form, but also

filamentous and bean like structures are found. The morphology depends on the host cell type, the staining process used for electron microscopy [190] and the M segment (M1 and M2 proteins), which seem to be the main viral determinant of morphology [24, 101].

**Table 2. Influenza A gene products and their functions**

Segment number	vRNA segment length, nt <sup>a</sup>	Gene product	Polypeptide length, aa <sup>b</sup>	Function
1	2341	PB2	759	Polymerase activity, RNA cap binding
2	2341	PB1	757	Transcriptase activity
		PB1-F2	87	Modulates immune response
3	2233	PA	716	Kinase and transcriptase activity, chain elongation
4	1778	HA	566	Receptor binding, entry, fusion with endosome, trimeric structure in the virion
5	1565	NP	498	Encapsidates RNA in RNP
6	1413	NA	454	Virion release, receptor cleavage, tetrameric structure in the virion
7	1027	M1	252	Viral matrix protein
		M2	97	H <sup>+</sup> ion channel, tetrameric structure in virion
8	890	NS1	230	Controls mRNA splicing and transport
		NS2	121	RNP nuclear export

a) nt, nucleotide; b) aa, amino acid

### 1.3.1.1 Polymerase complex (PB2, PB1 and PA) and PB1-F2

The viral proteins encoded by the three longest segments (1, PB2; 2, PB1, PB1-F2; and 3, PA) are a part of the RNA polymerase complex and are also included in the virion where they are associated with the RNP. The polymerase complex is required for both viral transcription and replication, and transcribes viral mRNAs from the (-)ssRNA genome, as well as full-length segment copies (+)ssRNA that serves as the template for production of the new (-)ssRNA genomes. The three proteins differ in their isoelectric points and have been named PB1 and PB2, which are basic, and PA, which is acidic. In order to initiate the transcription of viral mRNAs, the polymerase complex needs a primer. This primer is taken from the 5' cap of cellular mRNAs in a process called "cap-snatching". Crosslinking experiments have shown that PB2 is responsible for removing the 5' cap from cellular mRNAs. After acquiring a 5' primer, the PB1 initiates the RNA elongation [127]. The PB2 also influences the pathogenicity of the virus, as a mutation in position 627 on PB2 was

found to increase the lethality of the virus in mice [86]. PA was found to be essential for (+)ssRNA dependent (-)ssRNA synthesis [149], whereas a temperature sensitive PA mutation only affected (-)ssRNA synthesis, indicating that PA activity is not essential for mRNA synthesis [124]. PA may be important in maintaining the correct conformational arrangement of the PB1 and PB2 in the transcription complex [149]. There has been found indications that PA could also have a serine protease activity, but the importance of this remains to be elucidated [82]. A novel protein has recently been isolated from influenza A, but not influenza B [35]. It is coded for by a +1 reading frame in PB1 (87 residues) and therefore termed PB1-F2. The protein has been detected primarily in the mitochondria. PB1-F2 is hypothesised to be able to down regulate the immune response, both down regulation of the intercellular antiviral responses and also extracellularly, by inactivating recruited host immune cells [35].

### *1.3.1.2 Haemagglutinin (HA)*

The haemagglutinin is translated from segment 4 and is the most abundant protein in the viral envelope. Haemagglutinin is named after its ability to agglutinate erythrocytes. HA has two major functions in the virus' life cycle; HA binds to the receptor on the host cell surface bringing the virion in close proximity to the cell membrane and HA is responsible for the fusion of the endocytosed virion with the endosomal membrane allowing release of the genome into the cytoplasm. HA is the major antigenic determinant and has a high mutation rate. The receptor-binding site, however, is highly conserved. For the HA segment, the amino acid identity is up to 39% between influenza A and B and up to 79% between influenza A subtypes [126].

HA is a type I membrane protein consisting of 566 amino acids (see Figure 2). The C-terminal acts as the anchor domain and there is a short tail into the interior of the virion. After translation, the precursor termed HA0 is post-translationally modified. The HA0 is modified by addition of a number of sugar residues (depending on cell type and virus strain) and acetylation, which has been shown to be important for infectivity, at least for H7 viruses [229]. Addition of carbohydrates to HA has been shown to be important for the correct protein folding in the endoplasmic reticulum [56]. The HA0 is then cleaved by cellular proteases into two subunits, HA1 (328 residues) and HA2 (221 residues), but remain linked

by a disulphide bridge. During the process of cleavage, a number of amino acids are removed (varies from one to six amino acids). The cleavage of HA0 is a prerequisite for the conformational change in the HA which occurs upon low pH and this change is essential for release of the viral genome into the cytosol [121, 129]. The amino acid sequence at the cleavage site may be important for the virulence of the highly pathogenic avian H5N1 viruses. The cleavage site has a basic amino acid sequence, which allows a wider range of cellular proteases to cleave HA1 and HA2 [206]. As a consequence some avian strains have a wider cellular tropism contributing to the higher degree of pathogenicity observed.

The structure of HA has been determined by X-ray crystallography and by nuclear magnetic resonance (NMR) and it has been shown to be a spike like structure with a globular head and a transmembrane stalk (Figure 2) [193, 246]. The spike-like structure consists of three cleaved HA monomers, forming a non-covalently bound trimer. The globular head is composed of the larger HA1 subunit, which mainly has a  $\beta$ -sheet secondary structure. The secondary structure of the HA2 subunit has long  $\alpha$ -helices that gives HA its transmembrane stalk-like structure and extends HA 135Å from the surface of the virion.

There are three receptor-binding sites, one buried on each HA1 subunit, and they are protected and inaccessible to antibodies. Amino acid residues forming the receptor-binding



**Figure 2.** The three dimensional structure of A/Aichi/2/68 (H3N2). The HA is shown in its trimeric form. The globular head (top) is made up of HA1, whereas the stalk (bottom) consists of HA2 (shown in paler colours). [193]

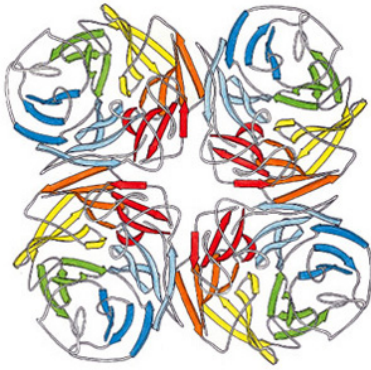
site are largely conserved between different virus isolates. However, only one amino acid substitution is needed to change the receptor specificity from a SA $\alpha$ -(2,6)Gal to a SA $\alpha$ -(2,3)Gal, which is an important factor in viral host restriction [60, 75]. Five antigenic sites have been identified using monoclonal antibodies on HA. These sites cover much of the surface of the globular head and binding of antibodies results in neutralisation of the virus [243].

### 1.3.1.3 Nucleoprotein (NP)

The nucleoprotein is translated from segment 5 and is named after its major function, which is to bind and protect RNA. NP and RNA together constitute the RNP. NP is one of the genus specific proteins that separates the *Influenzavirus A*, *B* and *C* genera. NP contains 498 residues and has a nuclear localisation signal that allows the protein to actively migrate to the nucleus. Although the NP can be phosphorylated, it is not clear if it is essential for its function [127]. Proteins that bind RNA often have conserved basic motifs, but this is not the case for NP, which has multiple sites for RNA interaction [127, 173]. Both (+)RNA and (-)RNA associate with NP, whereas mRNA does not bind to NP. The ends of all genome segments contain identical conserved sequences (13 nucleotide (nt) at 5' and 12 nt at 3') that might function as an encapsidation signal, although this has not been conclusively proven. As one of the most antigenically stable proteins in the virion, NP is also the most cross-reactive between different strains and influenza A subtypes, but not between genera.

### 1.3.1.4 Neuraminidase (NA)

The neuraminidase, encoded by segment 6, is the second most important antigenic determinant in the viral envelope. The protein consists of 453 residues and 4 identical monomers form the functional NA in the viral envelope. The monomer forms a stalk and globular head with 6 central  $\beta$ -sheets in a mushroomed shape (Figure 3) [228]. The enzymatic site is located directly over the  $\beta$ -sheets in the globular head, one on each of the four monomers, and is specific for N-acetyl-neuraminic acid. Each NA has one hydrophobic domain per subunit and NA can easily be separated from the virion by protease treatment, while retaining the enzyme activity [41]. NA is a class II membrane protein with the N-terminal end acting as a combined signal/ anchor domain spanning the lipid bilayer. NA does not undergo protolytic cleavage, but can be post-translationally modified by addition of



**Figure 3.** Topview of the tetrameric NA. Modified data originating from [228]

carbohydrates [42]. The protein has four known antigenic sites [39]. The function of NA is to enzymatically remove sialic acid from the cell surface and thereby promoting release of the virion from the cell [127]. It is also important in creating a pathway through the mucus layer in the respiratory tract and helping the virus to gain access to the surface of the epithelial cell. There is large variation in the sequence identity between genera; the globular head of NA has only 30% sequence identity between influenza A and B viruses, whereas within a subtype the identity can be as high as 97% [40]. Currently, nine subtypes of influenza A NA have been identified based on seroreactivity of post infection sera (Table 1) and NA is a target for antiviral therapy.

### 1.3.1.5 Matrix proteins (M1, M2)

Segment number 7 is bicistronic and codes for two different proteins, M1 and M2. M1 is a matrix protein and lines the viral envelope providing rigidity to the membrane. As the most common protein in the virion, it interacts with the cytoplasmic tails of HA, NA and M2, as well as the RNPs [127]. The matrix protein consists of a single polypeptide chain of 252 residues, which is translated from an unspliced mRNA and it is highly conserved between influenza A subtypes. The M1 protein is thought to be important in the morphological structure of the virion [24, 101] and to facilitate transport of the newly synthesised RNP from the nucleus to the cytosol [48]. In addition, for influenza B M1 proteins, it has been shown that a single amino acid mutation confers mouse adaptation and increased virulence [137].

The second protein encoded by segment 7, is the M2 protein. M2 is translated from a singly spliced mRNA in a +1 reading frame. The protein overlaps the M1 by 14 amino acid residues and is detected in the virion envelope as an integral membrane protein. The polypeptide undergoes modifications, such as phosphorylation and palmitoylation and is functional as a homotetramer. This tetramer, at least in influenza A viruses, has ion channel

properties and is involved in acidification of the virion during the initial infection process. M2 is the target for the antiviral influenza drugs amantadine and rimantadine [87].

#### ***1.3.1.6 Non structural proteins (NS1, NS2)***

Segment number 8, the shortest of the eight segments, is also bicistronic with two proteins translated. As the name implies, NS1 and NS2 proteins were not thought to have structural functions, i.e. excluded from the virion. The largest protein, NS1, is translated from an unspliced mRNA and the protein is normally phosphorylated. The NS1 protein is abundant in infected cells associated with polysomes and also in the nucleus and nucleolus, but it is not found in the virion. NS1 normally consist of 237 residues, but the number of residues may vary significantly between strains. This functional dimer has several functions, some unique to influenza A [125]. NS1 selectively retains cellular mRNA in the nucleus by inhibiting the polyadenylation machinery in the nucleus, which increases the quantity of 5' caps that are available for "cap-snatching" [36, 158]. This is essential for two reasons, it increases viral mRNA transcription by providing cellular 5' caps and it leads to degradation of the competing cellular mRNAs. Another important activity is the arrest of the protein kinase R (PRK) pathway (see 1.5.1) [125].

The NS2 protein is translated from a singly spliced mRNA and is translated from a +1 reading frame and has a length of 121 residues. In contrast to NS1, the NS2 protein has been detected in the virion, although in small numbers, associated with the M1 protein. The NS2 has been demonstrated to be a nuclear shuttle protein involved in transporting mRNAs out of the nucleus and to the ribosomes [162]. It has therefore been proposed to be renamed to nuclear export protein (NEP) [162].

#### ***1.3.1.7 Variation in encoded proteins between influenza genera***

The three influenza genera show some variation in the proteins expressed by different gene segments. For instance, the M2 ion channel protein is encoded on segment 7 in influenza A viruses, whereas a membrane protein from influenza B viruses is translated from the NA segment in an overlapping reading frame and is designated NB, which may have ion channel properties [127]. However, more recent investigations have shown that the protein responsible for the acidification of the virion could be the BM2 protein, encoded by segment 7, similarly to influenza A [169]. Although the function of BM2 has not been definitely



confirmed, it is an integral protein in the viral envelope, thus influenza B viruses have four membrane proteins [144]. In contrast, influenza A has only three proteins in the viral envelope [127]. Another difference in protein expression is the newly discovered PB1-F2 protein, which has only been identified in influenza A and not influenza B viruses [35].

The *Influenzavirus C* genus, has only 7 segments in its genome, in contrast to *Influenzavirus A* and *B*, and a single protein called haemagglutinin-esterase fusion (HEF) protein, is responsible for both the receptor binding and the fusion activity of HA, and the enzymatic receptor destroying activity of NA [127]. HEF is, similarly to HA, cleaved into two subunits by a cellular protease, which remained linked by a di-sulphide bridge. The receptor binding specificity of HEF is the 9-O-acetyl N-acetylneuraminic acid and as a consequence of this, the HEF enzymatic activity is a neuraminase-O-acetyl esterase. This is different from influenza A and B NAs, which are a N-acetyl-neuraminic acid neuraminidase. The proposed viral envelope ion-channel protein for influenza C is the CM2 protein, which is coded for by a spliced M segment, similarly to influenza A viruses.

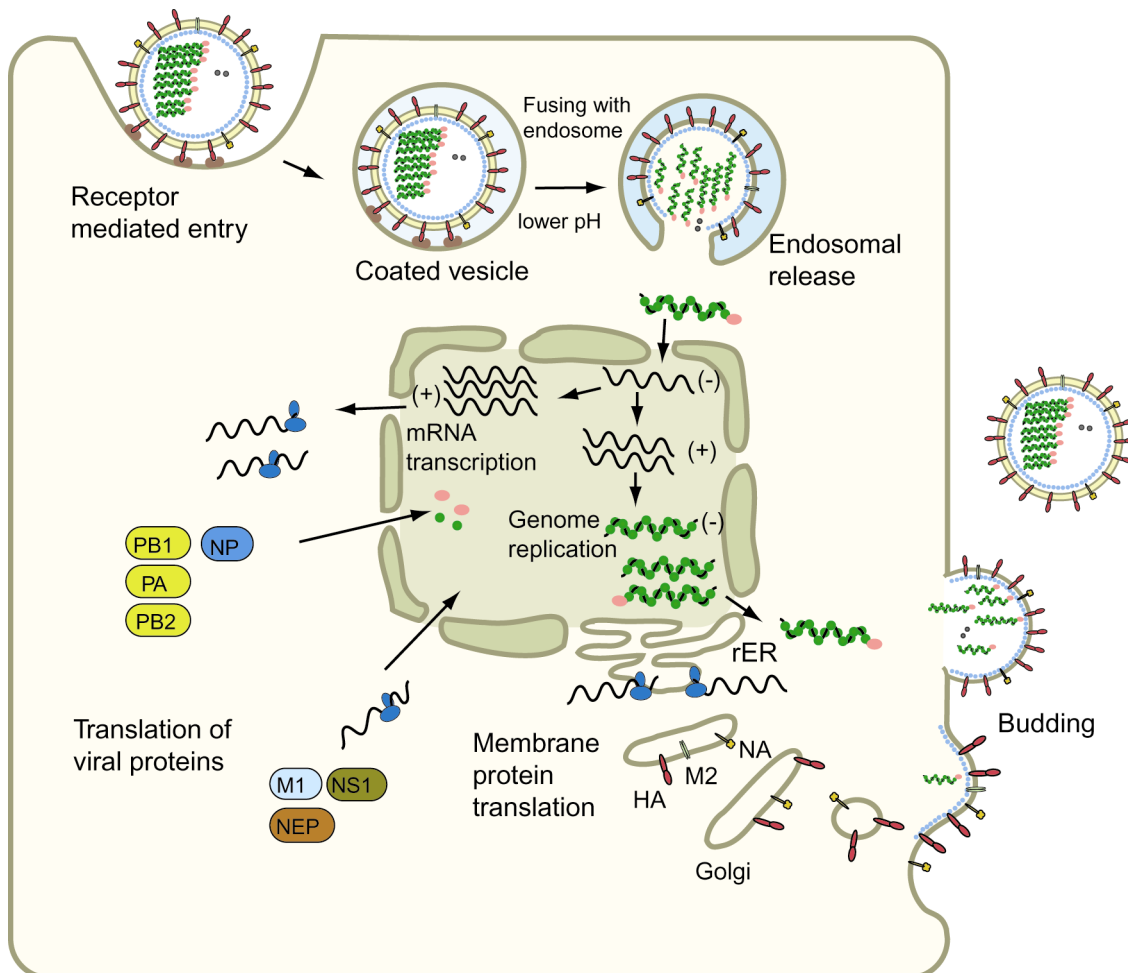
### **1.3.2 Viral lifecycle**

Influenza virus is transmitted through aerosols and infects the mucosal epithelia in the upper respiratory tract. The enzymatic activity of NA creates a pathway for the virus through the mucus layer where HA subsequently binds to the sialic acid containing viral receptor on the cell surface. This leads to receptor-mediated endocytosis where the virus is engulfed and taken into the cell in a vesicle, before it fuses with acidic endosomes (see Figure 4) [127]. The M2 ion channel protein lowers the pH inside the virion causing the RNP to become disassociated from the M1. The low pH in the endosome also sets off a conformational change in the HA molecule, which leads to the fusion of the viral envelope and the endosomal membrane. This exposes the interior of the virion to the cytosol and the dissociated RNPs can be transported to the nucleus by the NP, which has a nuclear localisation signal (NLS) [161]. The polymerase complex is associated with RNP, but each of the proteins in the polymerase complex has its own NLS and is independently transported to the nucleus [48]. The (-) sense vRNA genome serves as a template for both transcription of mRNAs and replication of new viral genomes (see Figure 4). In order to initiate transcription of viral mRNA, the viral polymerase complex needs a 5' RNA primer. This

primer is taken from the 5' ends of newly synthesised cellular mRNAs, in a “cap-snatching” process using the cap specific endonuclease activity of PB2 [88]. The cap fragment ( $m^7GpppNm_{n10-13}$ ) is then elongated [127]. At the 5' end of the template, the viral polymerase complex “stutters” at a stretch of uracil nucleotides and the poly(A) tail is transcribed. The mRNAs encoding for NP and NS1 are the first to be translated whereas the M1 mRNA transcription is delayed [127]. Early translation of NS1 is important as the NS1 protein inhibits pre-mRNA splicing and arrests cellular mRNAs, thereby stopping the cellular mRNA transport from the nucleus [125]. This may allow sufficient numbers of cellular mRNAs to be available for “cap-snatching” and also stop cellular mRNAs from being translated. In the later phase of infection NEP exports the RNP complexes to the cytosol [162], with the aid of M1 [135]. The initiation of replication of the viral genome does not require a primer and is not terminated at the poly(A) site, but template RNAs are synthesised as full-length RNAs. The template viral RNA is of sense (+) polarity (i.e. complementary to vRNA) and is copied once more to produce a new (-) single stranded viral RNA. Since shift from mRNA synthesis to replication requires protein synthesis, it is hypothesised that a viral protein, possibly NP, is necessary to avoid the need of primers and/or anti-termination at the stretch of uracil nucleotides [173]. It is also possible that the replicase is different from the transcriptase as the latter is independent of the presence of NP.

Only viral RNA (both polarities, but not viral mRNAs), are encapsidated by NP [127]. This may be linked to the conserved 5' and 3' ends on all segments, which are not transcribed in viral mRNA. The HA, NA and M2 are translated on the rough Endoplasmic Reticulum (ER), the other viral proteins are translated in the cytosol on free ribosomes. The translation of the influenza A protein PB1-F2 is not known in detail, but *in vitro* experiments have suggested that the protein is a membrane channel protein [32], but there is no indication of incorporation into the virion. From the ER the proteins are transported through the Golgi apparatus and undergo various degrees of modification before being transported to the cell membrane. M1 and RNP migrate to the cell membrane and interact with the cytoplasmic tails of HA (in its cleaved form) and NA. The M1 protein further interacts with the viral glycoproteins and shapes the cell membrane to form the viral envelope before the budding virus particle is pinched off by an unknown mechanism. If HA is not cleaved prior to budding or if the enzymatic activity of NA is inhibited [41], no free infectious virus particles are produced.

Influenza A has eight genome segments, but the mechanism for controlling that all segments are included in the virion is not known in detail. Cis-acting signals, possible on the 5' and 3' ends, have recently been found to be involved in segment packaging [131], whereas others have suggested random packaging of 9-11 segments in each virion [11]. Most virions produced are not viable; only one virion out of 20-50 virions is capable of replication in cell



**Figure 4.** Influenza A virus life cycle. See text for further details.

culture [68].

## 1.4 Influenza epidemiology and ecology

In the northern hemisphere the annual influenza outbreaks usually starts during the winter months. In the tropic and subtropics on the other hand, influenza virus is isolated all year

around. The number of suspected influenza cases in periods of known influenza spread, designated Influenza like illness (ILI) is a frequently used measure of epidemiological activity by international and national authorities [67]. The numbers of ILI cases are reported by general practitioners (GP) with patients suffering from typical influenza symptoms and the number of ILI is a good estimate of the magnitude of circulating influenza [207]. The definition of ILI that corresponds best with laboratory confirmed influenza is a sudden onset of fever, cough and fatigue [222], but other case definitions are also used [23, 234]. Additionally, GPs may take nasal/throat samples from ILI patients and in many countries centralised influenza centres isolate virus in order to identify the type, subtype if appropriate, and strain of influenza virus circulating in the community. Based on the number of ILI and/or laboratory confirmed influenza infections, the term of “No activity”, “Sporadic activity”, “Local outbreaks”, “Regional outbreaks” or “Widespread outbreaks” are used. The term pandemic is only used when an antigenic shift occurs by a novel influenza A subtype infections humans and global widespread outbreaks result in substantial morbidity and mortality (see 1.4.3).

Influenza related deaths are under-reported during an outbreak as influenza often exacerbates underlying disease [155]. The number of influenza related deaths is therefore often reported as the number of excess deaths compared to a period without (known) influenza spread. During a pandemic, but also during the annual influenza outbreaks, an excess of morbidity and mortality is reported. People that die from influenza infection are usually from the “at risk” groups; children, the elderly and people with certain chronic medical conditions. The estimate for influenza related deaths worldwide is about 1 million people each year [236, 251] and in USA about 60-70% of deaths occur in people above 65 years old [171]. However, the total number of influenza related deaths worldwide is difficult to estimate, due to a lack of knowledge about influenza epidemics in developing countries [240]. The HIV epidemic in the developing countries further obscures the situation. The total impact of influenza deaths in developed countries, including Norway, is not known in great detail or accuracy, as updated mortality rates are often not readily available.

During an influenza outbreak there is substantial morbidity. A conservative estimate is that every influenza season resulting in an economic loss of 12-17 billion dollars (sick leave, loss of production) in the USA alone [240, 245]. Influenza morbidity results in significant strain on health care systems. In the yearly influenza outbreaks in the USA, most of the mechanical

ventilators in American hospitals are in use by patients that need breathing aid and very little spare capacity exists to tackle a major influenza outbreak [164]. Morbidity after influenza infection has rarely been investigated in population studies, but an outbreak of influenza in Boston, USA, in 1976 and 1977 resulted in an estimate of 37% absenteeism in school children during a 5-week period [76, 77]. Large retrospective cohort studies investigating hospitalisation rates over 20 years in USA, found that young, otherwise healthy children (<5 years old) had a hospitalisation rate similar to adults with “high risk” of influenza complications [151, 171, 221], showing the substantial impact of influenza infection also in children.

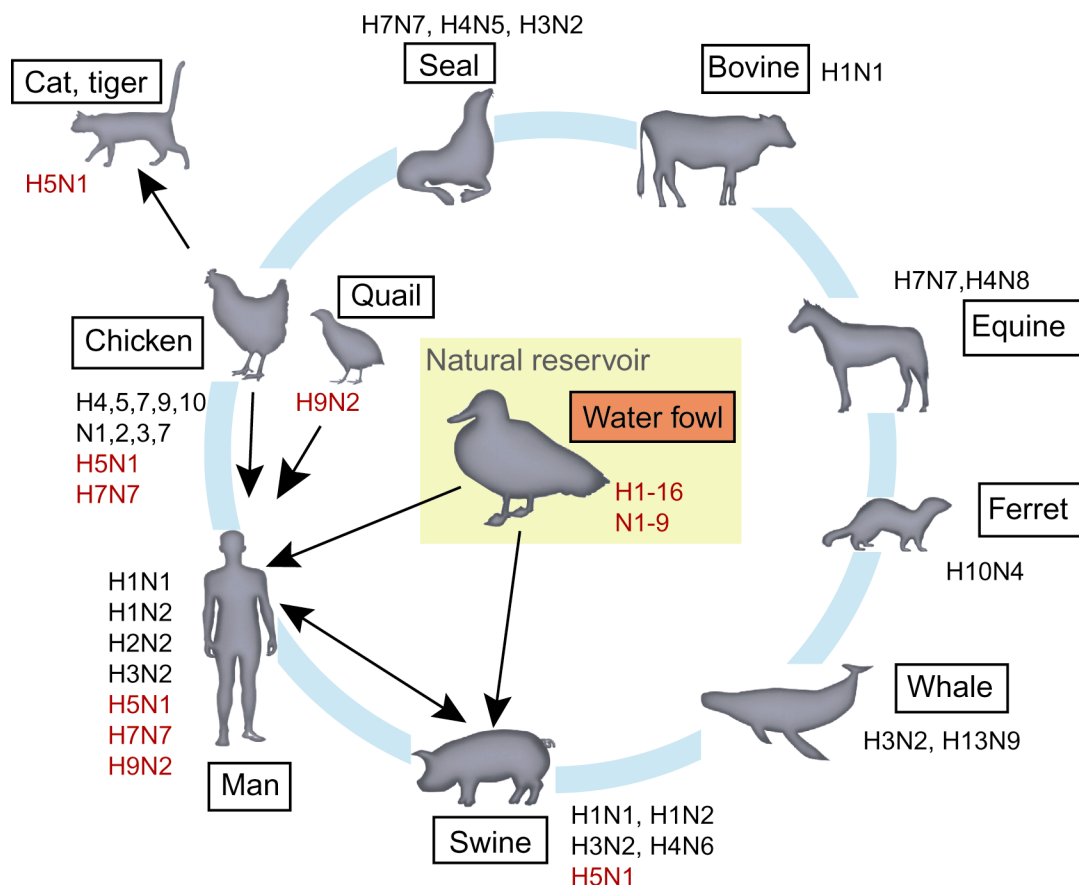
### **1.4.1 Evolution and host range**

Influenza C virus is isolated almost exclusively from man and is associated only with a mild respiratory infection and therefore only infrequently isolated. Influenza B viruses are detected in humans, usually causing milder illness and infection results in one quarter of the number of hospitalisations that influenza A virus causes [146]. Influenza B virus has also been isolated from seals [163]. In contrast, influenza A viruses have been found in a wide range of species; man, birds, seals, horses, camels, whales, ferrets, pigs [146]. The different influenza A subtypes and B viruses, have different severity and duration of illness: Influenza A H3N2 results in the most serious illness, B viruses intermediate and influenza A H1N1 gives normally the mildest illness [139]. Data on the newly isolated human reassortment influenza A H1N2 (see 1.4.3) is scarce, but it appears to cause a mild illness [61].

Influenza A has been detected in a range of species (Figure 5, Table 1) with birds as the largest reservoir. As chronic infection has not been detected [146], influenza A viruses have therefore to infect a large number of birds to maintain the viral spread. This is possible due to a vast number of juvenile birds hatching every year, which are immunological naïve to influenza virus [105]. Normally, avian influenza in birds is an asymptomatic intestinal infection and not a respiratory tract infection. The virus is secreted in high titres through the cloaca for a period up to 30 days [95] and has also been detected in unconcentrated lake water [230]. The practice in Asia of many households feeding poultry in or near the family house has been implicated as a probably route of transmission of virus to man, in China alone there are 13 billion poultry [164].

Pigs have both the human and avian influenza virus receptor, sialyloligosaccharides SA $\alpha$ -(2,6)Gal and SA $\alpha$ -(2,3)Gal, respectively, and the pig may be a “mixing vessel” as it can sustain both avian and human influenza virus replication. When pigs and birds are housed together, often at live animal markets [231], there is ample opportunity of mixing of avian and human influenza viruses. Pig and human influenza genome segments been shown to be closely related using phylogenetic analysis for PB1, PB2, PA, NS, M and NP [230]. HA and NA segments are more varied, with some avian input as well.

Influenza virus is specific for its host and several genomic determinants for host range have been identified. The receptor binding specificity is obviously important and is determined by HA. Only one amino acid substitution in HA (226Leu→Gln) can change the receptor specificity of HA [60], shifting from SA $\alpha$ -(2,6)Gal to SA $\alpha$ -(2,3)Gal containing receptors. Humans also express a low level of SA $\alpha$ -(2,3)Gal on the non-ciliated cells of the respiratory



**Figure 5.** Subtypes marked in red have been identified as the most likely candidates to start the next pandemic. Modified from [217].

tract [198], which may be the reason some avian influenza strains can infect man directly.

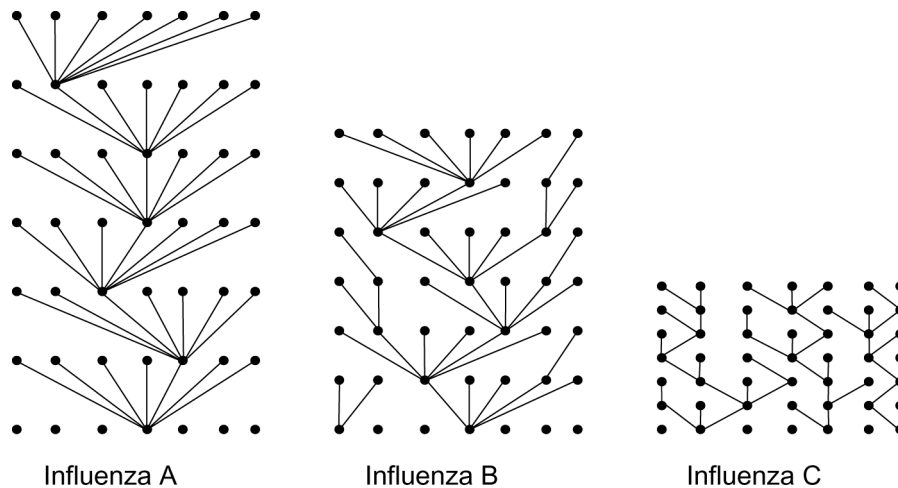
Mutations in NA affecting virulence or plaque forming ability in culture have also been observed [196]. In addition, phylogenetic analysis of NP has revealed five host specific lineages in which the NP segments are not interchangeable [72] and this may be related to host-specific phosphorylation of NP. The other internal proteins may also play a role in host discrimination; one amino acid substitution in an avian PB2 protein allowed viral replication in MDCK cells [215] and one mutation in M1 was sufficient to generate a virulent mouse influenza strain [137].

### **1.4.2 Antigenic drift**

The three influenza genera differ in the magnitude of genetic variability (Figure 6), but all genera undergo mutations leading to changes in the amino acid sequence, a process called antigenic drift. RNA viruses in general usually have very high frequencies of copy errors during replication as the RNA dependent RNA polymerase does not have the ability to proof read and influenza virus is no exception. The average number of mutations in influenza virus per genome per replication cycle is 1.0, compared to 0.0027 for yeast (*Saccharomyces cerevisiae*) [59]. More than two mutations per genome per replication often leads to extinction of a species [59]. Influenza viruses are unique among the respiratory viruses as they have a substantial antigenic variation. The mutation rate for influenza A HA and NA is 0.4% and 0.7%, respectively, per year [201]. Mutations, especially in the HA which contains the main antigenic determinants, allow the virus to escape the host's immunological memory. Viruses that are able to escape the host's antibody repertoire present in serum will have a higher fitness and hence replicate more efficiently.

The antigenic drift differs for the three influenza virus genera. New drifted influenza A variants replace previously circulating strains each year, whereas influenza B and C accumulate fewer mutations [249] and are more antigenically stable (Figure 6). Accumulation of mutations leads to annual influenza outbreaks. The slower evolution of influenza B and C viruses may be attributed to having a single host to replicate in. A hypothesis explaining this has been proposed; both influenza B and C viruses have been co-evolving with humans longer and have undergone host specific adaptations. Influenza A has

not reached its evolutionary equilibrium with man and is prevented from doing so, by frequently reassorting with viruses from other hosts [230].



**Figure 6.** Evolutionary model for antigenic drift of influenza viruses in man. Horizontal dots represent influenza variants arising in the same year. The length of the branches indicates the relative change from the preceding year. An arbitrary number of seven cycles is shown. Adapted from reference [249].

### 1.4.3 Antigenic shift

Antigenic shift occurs after reassortment of viral genome segments between two different influenza A subtypes. Two viruses co-infect the same cell and exchange segments, leading to a novel reassorted viruses with new combinations of HA and NA surface glycoproteins. When a novel virus with a novel HA (and NA), which has not recently circulated in man appears and spreads efficiently in man, the virus may cause a pandemic [230]. Evidence suggests that this has happened three times in the twentieth century, normally with the novel subtype replacing the old one. The most devastating shift occurred in 1918 and was named the “Spanish flu” [180]. The virus responsible for the 1918 pandemic was a H1N1 virus that resembled swine viruses [13, 178, 225], although a more methodical sequencing revealed that the virus most likely was transmitted to man from an avian source *in toto* and was not a reassortant [178, 179, 181, 182, 220]. The pandemics of 1957 and 1968 were the result of a reassortment between avian and human influenza viruses. For instance, the HA from the



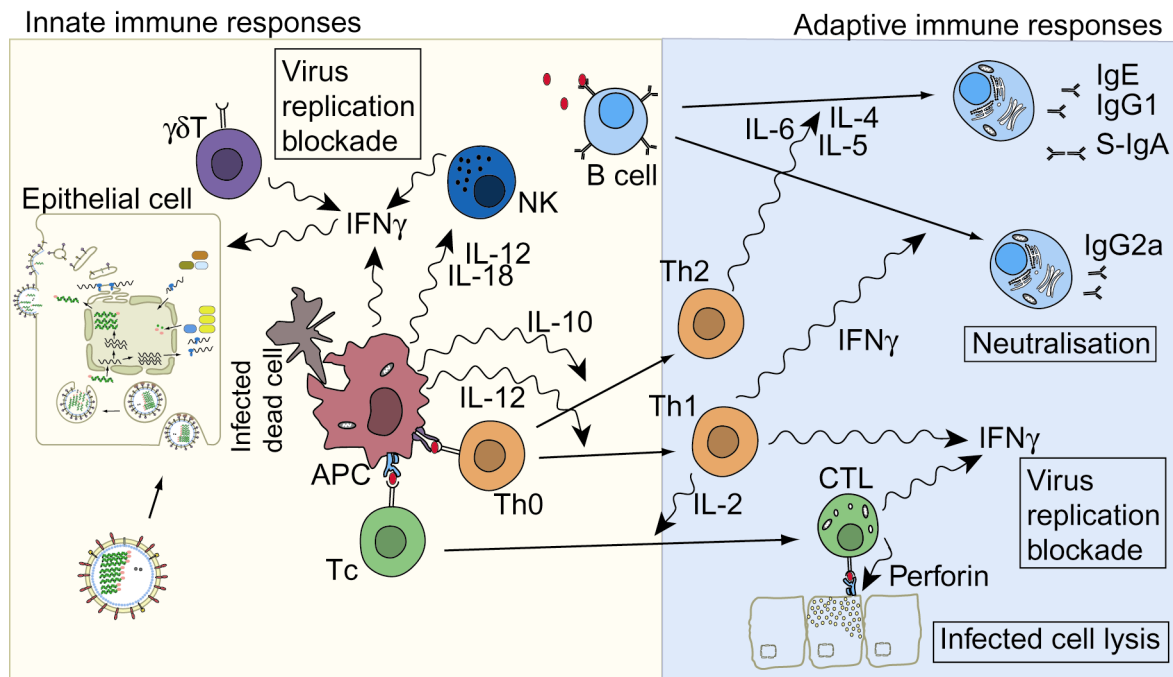
1968 pandemic virus differed by only 9 amino acids from an avian isolate [230]. The H1N1 virus reappeared in 1977, but the virus did not have superior fitness to the existing H3N2 virus, causing no excess mortality [58], and only people under the age of 20 (i.e. born after 1957) became infected with H1N1 during the first wave in 1977 [175]. Unlike the pandemics of 1918, 1957 and 1968, both H1N1 and H3N2 viruses have continued to co-circulate. The virus may have been inadvertently released after a vaccine trial using a live attenuated influenza vaccine [166] as the virus was genetically similar to a virus isolate from 1950. This may explain the mild nature of the 1977 epidemic, different from that of the other pandemics.

A double infection leading to reassortment of influenza A subtypes can also happen in man. This has been detected in isolated cases since the 1970s [114], but more recent data indicates that this happens more frequently than previously thought [97]. After the 2001-02 influenza season, a reassorted H1N2 virus has been circulating [61]. However, since both the HA and NA were of human virus origin, it did not result in an epidemiologically significant event. Epidemiological data indicates that H1N2 is drifting and evolving on its own [5], but data from autumn 2005 shows that the H1N2 virus is only rarely isolated and it may naturally disappear [241]. An antigenic shift can also occur after direct transfer of an avian virus into man. The outbreak of avian H5N1 in Hong Kong in 1997 demonstrated that avian viruses could directly infect man and did not require a “mixing vessel” [203, 216]. This has now been documented again in Asia in 2004-05 [37], but there has not been an adaptation of the novel subtype to allow sustained human-to-human spread. However, with a total of sixteen HA influenza A subtypes in birds and only three detected in man in recent memory, there is still potential for new emerging avian influenza virus to cross the species barrier and cause a new pandemic.

## 1.5 The immune system

The immune system can be divided into two interconnected parts, the innate and the adaptive system (Figure 7). The innate system responds to every antigen similarly and does not generate any immunological memory. The main function of the innate immune system is to

contain the pathogen until the adaptive immune system is activated and fully functional. The adaptive arm of the immune system, has the capacity to selectively identify a particular antigen and a memory response is induced to allow a more rapid response upon re-encounter of the same antigen. The work in this thesis has been conducted in mice and the subsequent text refers to the immune system in mice.



**Figure 7.** A schematic overview of the innate and adaptive immune system. The epithelial cell insert is from figure 4. Adapted from reference [219].

### 1.5.1 Innate immune response

The first line of defence against all respiratory pathogens is an intact mucosal membrane with the cilia and mucosal secretions that remove foreign particles. Secreted interferon (IFN) from infected neighbouring cells can turn on a range of antiviral genes in nearby cells [74]. IFN  $\alpha/\beta$ -stimulated response element (ISRE) is a promotor element that, when activated, turns on hundreds of genes with antiviral activity [150, 252]. If the virus succeeds to enter the cell and starts to replicate, a range of anti viral genes are turned on [74]. One important protein that is activated, is the protein kinase R (PKR) [106, 168]. This protein recognise dsRNA, which does not normally exist in a cell, and is therefore utilised as a hallmark of a viral infection. An activated PKR phosphorylates the translation initiation factor, eIF2 $\alpha$ ,

resulting in a complete shutdown of protein synthesis and consequently viral replication. Recently, a toll like receptor (TLR) –7 in the endosomes was found to bind influenza (ss)RNA and induce IFN- $\alpha$  production [55].

Natural killer (NK) cells are involved early in fighting infection and an increase in NK activity is detected after influenza infection [130]. NK cells have several functions; producing cytokines (e.g. IFN- $\gamma$ ) and mediating cellular cytotoxicity. The recognition strategies used by NK cells are diverse, including both up-regulated self-proteins and down-regulated self-proteins on the surface of all cell types, as well as recognition of viral proteins [177]. The Nkp46 and Nkp44 receptors found on NK cells are reported to bind HA [134]. NK cells additionally have Fc receptors (FcR), which binds to the Fc portion of antibodies and can thereby initialise a NK mediated lysis of target cells expressing antigen bound to antibody (Antibody dependent cell cytotoxicity, ADCC) [69]. Other cells are also involved in early recognition of viral infection, like macrophages which can opsonise antigen and secrete a variety of cytokines. Among them is tumor necrosis factor (TNF)- $\alpha$ , which is cytotoxic for tumor cells and stimulates innate immunity, and interleukin (IL)-1 and IL-6, which promote inflammatory responses and induce fever. There are several granulocytic cells, which are named because of their cellular morphology. Neutrophils are the most important as they directly attack microorganisms using their phagocytic capability, similarly to the role of eosinophil cells in defence against parasites. Basophils and mast cells are important in inducing an inflammatory response [10].

A particular subset of T-cells,  $\gamma\delta$ T-cells, most devoid of either CD4 or CD8, expresses an alternative T-cell receptor (TcR)  $\gamma\delta$ TcR. This receptor can react with soluble non-protein antigen not presented by the MHC complex, but the exact role in defence against respiratory viruses is not clear.  $\gamma\delta$ T-cells may function as immune regulators and mediators, as they secrete cytokines (e.g. IFN- $\gamma$ ) and thus may provide a link between the innate and the adaptive immune system [30], but they may also have a role to play in tissue repair after infection [17]. Another system linking the innate and the adaptive immune system is the complement system. Complement is important in resolving and solubilizing antigen-antibody complexes facilitating their elimination. The complement system also recruits phagocytic cells by acting as an opsonising and inflammatory initiator. Additionally, complement together with antibody, permeabilizes membranes and contributes to the destruction of target cells.

## 1.5.2 Adaptive immune response

The adaptive immune response, which induces memory, consists of two interlinked parts, the humoral and the cell-mediated immune system (see Figure 7).

### 1.5.2.1 Cell mediated immune response

The cell-mediated immune response consists of two main cell lineages, CD4 and CD8 positive T-cells. The main function CD4<sup>+</sup> T-cells or T-helper (Th) cells, are regulation and control of the immune system by secreting cytokines. Th cells have a T-cell receptor, which can recognise antigen that is bound to the major histocompatibility complex (MHC) II on professional antigen presenting cells. After recognising an antigen, Th cells begin to divide and give rise to effector cells, whose main task is to secrete cytokines. Based on the cytokines they secrete, Th cells are divided into two subsets responsible for effector phase, Th1 and Th2 [142] and several subsets responsible for regulation [205], which will not be discussed further. The major Th2 cytokines in mice are IL-4, IL-5, IL-6 and IL-10, which stimulate B-cells to produce antibody and induce a humoral immune response (see 1.5.2.2) [142, 205]. A Th1 response induces a different cytokine profile with the most important cytokines being IL-2 and IFN- $\gamma$  [142, 205]. An important function of IFN- $\gamma$  is to increase the expression of FcR [63]. It also up-regulates the poly immunoglobulin receptor (pIgR) expression [172] and activates cytotoxic T lymphocytes (CTL) [205]. After Th cells have been activated by antigen, long-lived Th memory cells are produced and maintained [51, 102, 214], even 60 years after smallpox vaccination both antibody and memory B-cells have been detected [49]. Addition of adjuvants that shift the Th response after immunisation have been reported, but have not yet reached clinical use in man [33, 34, 64, 141].

CD8<sup>+</sup> T-cells (also known as cytotoxic T-cells, Tc) and macrophages are activated and supported by Th1 cells [16]. After an antigen is presented by MHC I and recognised by the TcR, the activated CD8<sup>+</sup> cell differentiates into a CTL. These activated CD8<sup>+</sup> lymphocytes mediate killing of self-cells presenting a foreign antigen on MHC I, which unlike MHC II, is expressed on nearly all cells in the body. The mechanism of cell lysis has been described as two-fold [191]. Firstly, the perforin/granzyme mediated pathway, which is similar but not identical to that of NK cells, CTL releases perforin that form a pore in the target membrane allowing the granzyme pass into the target cell, leading to cell death. Perforin activity has

been shown to be important in protection from influenza infection as knock-out mice for perforin (-/-) show an increased susceptibility to influenza infection and prolonged viral shedding [133]. Secondly, CTLs also use the Fas pathway, which leads to a recruitment of Fas associated death domain (FADD) protein and start of apoptosis [7]. Following activation, some of the effector Tc differentiate into memory CD8<sup>+</sup> [51, 232]. These memory cells, both CD4<sup>+</sup> and CD8<sup>+</sup> cells, can be reactivated if the same antigen is re-encountered. This reactivation will give rise to a faster immune response with a subsequent faster elimination of the antigen.

### *1.5.2.2 The humoral immune response*

The humoral immune system consists of B-cells that produce and secrete antibodies. After encountering an antigen, B-cells differentiate into plasma cells and memory cells, a process that is aided by cytokines produced by Th-cells (see Figure 7). Activated B-cells secrete antibodies, even before they become plasma cells [85]. Fully differentiated plasma cells are normally short-lived and secrete large quantities of antibody, up to several thousand antibodies per second [93], although a subset that is long-lived probably migrates to the bone marrow and contributes to the serum antibody pool [9, 160, 199]. There are five different classes of immunoglobulins (Ig) and four different subclasses of IgG in mice. Mature B-cells have membrane bound IgM and IgD that upon antigen recognition, undergo affinity maturation and class switching to IgG, IgA and IgE [38], described below for mice. IgE is involved in allergic reactions and not important in defence against viral pathogens. In mice there are four IgG subclasses, IgG1, IgG2a, IgG2b and IgG3 [62] (in man these are called IgG1 to IgG4) and only one subclass of IgA (in man there are two subclasses, IgA1 and IgA2). There is no correlation between the IgG subtypes in mice and man.

IgA is the major class of immunoglobulin in the mucosa of both mouse and man (e.g. respiratory and intestinal tracts). Antibody secreting cells (ASC) located in the mucosal tissue synthesise a peptide called J chain, which allows the IgA to dimerize and be actively transported across the mucosa, utilizing the pIgR on epithelial cells [108, 143]. IgM ASC also expresses this J chain, but IgM might not be actively secreted across mucosal surfaces in rodents [187, 227]. IgG is the major immunoglobulin class in the serum and the different IgG subclasses have different properties. The IgG2a subclass (in mice) is especially efficient at recruitment of cytotoxic T-cells, macrophages and NK cells, thus creating a link between

the innate, humoral and cell mediated immune responses. IgG2a antibody binds the FcR [94, 100] and mediates complement activation and ADCC [119]. The IgG2a subclass is important in resolving viral infection in mice and is indeed detected at high serum antibody concentrations after a range of viral infections [43], including influenza [66]. The IgG2a dominates the serum response after a Th1 response and can be used as a marker for a Th1 response [141, 213]. IgG1 is the other main IgG subclass (IgG2b and IgG3 are normally detected in lower concentrations) and is observed after a Th2 response, which mediates the class switch to IgG1, IgA and IgE. The IgG1 subclass can be used as a marker for a Th2 response.

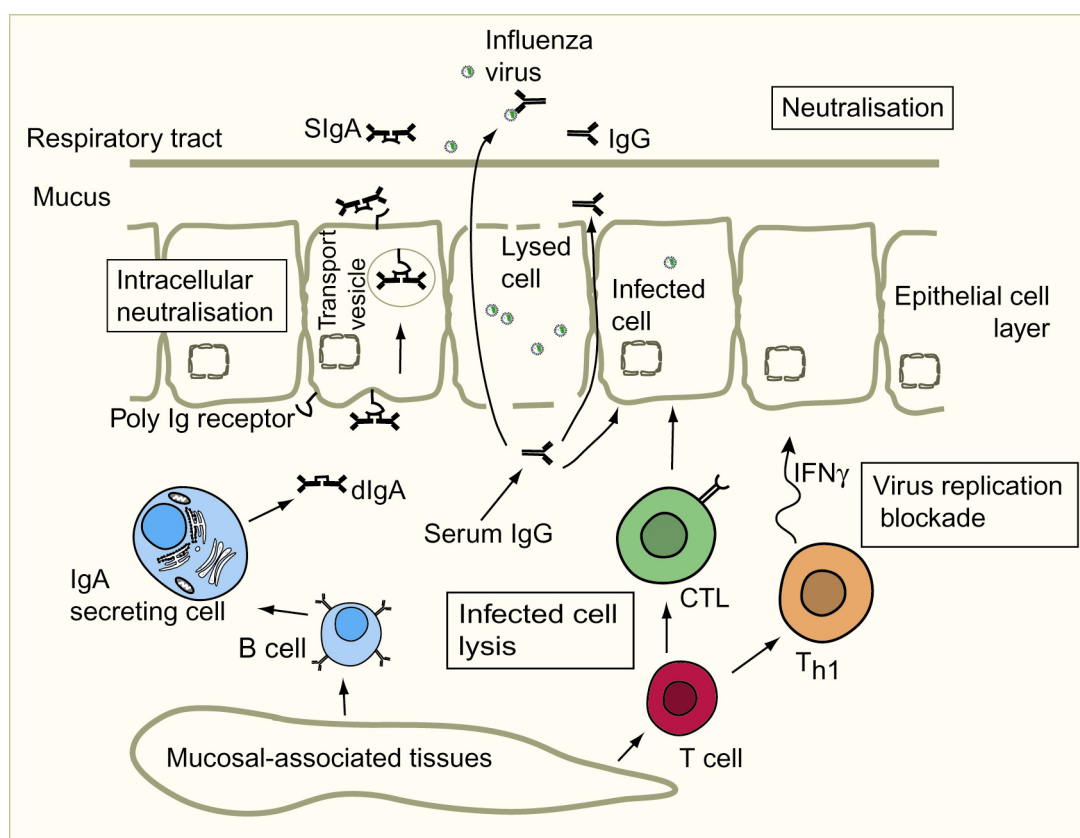
### **1.5.3 Mucosal immunity**

Mucosal immunity is not an independent part of the immune system, rather a function of the innate, humoral and cell-mediated acting in concert. The mucosa is very important in viral defence since viruses as diverse as influenza virus, rotavirus, corona virus, human immunodeficiency virus (HIV) and measles virus, enter the body via the mucosal route. Immunity to mucosal viruses is complex with a range of complementary and compensatory functions. For instance, the contribution by different immunoglobulin classes and cells of the immune system against respiratory pathogens has been difficult to decipher [16, 17, 104, 123, 136, 152, 167, 183-185, 194, 244]. It has also been hypothesised that because of the difficulty of maintaining a very high level of mucosal immunity over long periods of time, the major function of the mucosal immune system may be to reduce the severity of infection by aiding the clearance of virus [147].

In the case of influenza, the cells of the mucosa are the site of both infection and the immune response to the virus (see Figure 8) [219]. IgA is constantly secreted in its dimeric form, dIgA, across the epithelial surfaces of the mucosa. Epithelial cells have a poly immunoglobulin receptor (pIgR) that binds antibody containing a J chain and the antibody is secreted after a protease has cleaved the pIgR, leaving secretory component, SC. This dIgA with bound SC is termed S-IgA. S-IgA and the passively derived IgG from serum neutralise virus by forming antigen-antibody complexes, which block the receptor-binding site on HA. S-IgA can also bind viral proteins when it is transported through the epithelial cell (intracellularly) and thereby reduce the efficiency of the viral assembly.

Whether IgA is critical for protection against viral infection has been intensely debated for years. An IgA knock-out mouse will still have some immunity to influenza infection, possibly by a compensatory mechanism involving IgG [17]. Another complicating factor has been how the outcome is measured, as prevention of viral shedding, viral pneumonia or protection against a lethal infection all seems to correlate with different aspects of the immune system. The consensus now emerging is that IgA is important in immunity of the upper respiratory tract whereas IgG prevents viral pneumonia, clinical illness and viral shedding [22, 99, 167, 183, 184, 219]. In the lower respiratory tract, IgG more readily diffuses across the alveolar wall than across the mucosa [147] and the pIgR, vital for S-IgA secretion, is only sparsely expressed in the lungs [188].

A virus-infected cell expresses MHC I and presents viral antigens on its cell surface. These antigens can be recognised by CTL, which eliminate infected cells. Lysis of infected cells by CTL greatly reduces viral shedding and is believed to be important for viral clearance [219].



**Figure 8.** The mucosal immune system. There are four important mechanisms by which the mucosal immune system inhibits viral replication. 1) Extracellular virus neutralisation by antibody; 2) intracellular virus neutralisation by transported IgA; 3) IFN- $\gamma$  induce an antiviral state which inhibits viral replication in the cells of the mucosa; 4) lysis of infected cells by CTL. Adapted from [219]

Epithelial cells can only present viral antigens on MHC I following a productive infection and consequently CTLs do not prevent infection. Damage to the epithelial cell layer by CTL mediated lysis, will aid the passive transport of IgG derived from serum. Th1 cells will secrete cytokines in a process called delayed type hypersensitivity (DTH) reaction, among them IFN- $\gamma$ , which induces an antiviral state (see 1.5.1) in the epithelial cell layer and recruits the cells of the immune system.

## 1.6 Prevention and prophylaxis of influenza

The burden of annual influenza infection is substantial, both in terms of illness, lives lost and economic impact on society [236, 251]. Additionally, we are eventually facing a new pandemic that could cause unprecedented levels of morbidity and mortality, both in the developed countries, but even more so in the developing countries. Particularly in a pandemic scenario, but also for the yearly influenza outbreaks, improving immunity to current influenza vaccines and developing new antiviral drugs are of vital importance. Continued and focused research efforts are needed in order to understand the immunology, epidemiology, ecology and the aetiology of influenza viruses. Despite many years of studies, we still lack some basic knowledge about influenza and the infection it causes, and the subsequent immune response.

What we do know, is that the vaccine that is produced today provides a satisfactory protection with a protective effectiveness of 70-90 percent against laboratory confirmed influenza in healthy adults [21, 26, 47, 84, 110, 238, 242] and vaccination is cost effective [154]. However, there is still room for improvement, especially in the elderly the efficacy is not optimal. This is also true for the antiviral drugs as they too must be refined and new drugs need to be developed to fight resistant viruses.

### 1.6.1 Vaccines

The two main types of influenza vaccine are inactivated virus and live virus vaccines. Inactivated vaccines are normally administered parenterally, and are chemically treated to



ensure that the virus is not capable of replicating. Live virus vaccine is administered intranasally, and the virus is attenuated to only produce an upper respiratory tract infection and not cause any overt clinical illness.

The vaccine strain used in the current trivalent vaccine needs to be an epidemiological important virus and present in the area the vaccine is going to be used in. The candidate vaccine strain is then sequenced and a strain is chosen that is capable of growing to high titre in the system used for vaccine production (embryonated hens' eggs or cell culture). Alternatively, a high growth reassortment virus can be produced by co-infecting the proposed vaccine strain with a high growth strain in order to produce a high growth reassorted virus expressing the desired HA and NA [118]. The high growth vaccine strain is then inoculated into embryonated hens' eggs as the currently used vaccine substrate (or cell-culture) and the virus is later harvested, purified and concentrated. The virus is then processed in order to produce an inactivated or a live virus vaccine. In the case of the H5N1 circulating in Asia, it was necessary to genetically modify the virus in order to allow growth in embryonated hens' eggs, as the wild type virus was lethal to the embryo. The WHO has issued guidelines concerning vaccine production using avian influenza viruses [236, 238, 239].

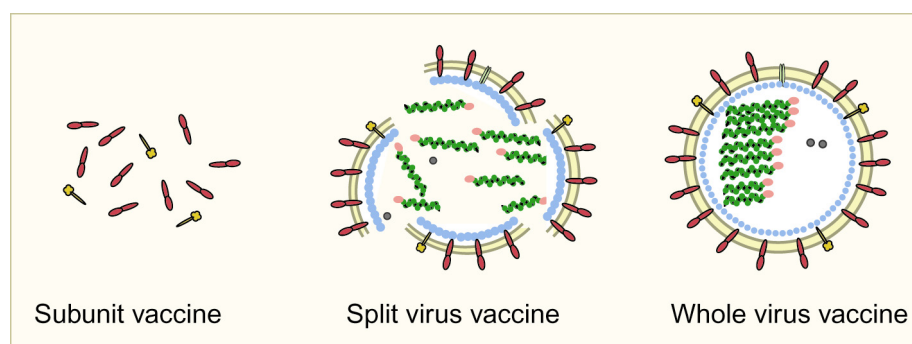
#### *1.6.1.1 Inactivated vaccines*

There are three main formulations of inactivated vaccines (see Figure 9). Whole virus vaccine is inactivated by chemical agents (e.g. formaldehyde or  $\beta$ -propiolactone) in a procedure that does not destroy the viral envelope [78]. This vaccine was widely used up to the 1970s, but its use was discontinued due to a high level of side reactions [12, 25, 79]. A split virus vaccine is produced by chemical agents (e.g. ether or tributyl phosphate) that disrupt the viral envelope [12, 54]. Immunisation with split virus vaccine produces fewer side reactions (i.e. has a lower reactogenicity), but has also a somewhat reduced immunogenicity compared to whole virus vaccine [12, 79]. An even less reactogenic vaccine is the subunit influenza vaccine, consisting of the highly purified surface antigens, HA and NA [174]. All inactivated vaccines are administered parenterally, either intramuscularly or subcutaneously (Extensively reviewed in [71]).

Standardisation of the antigenic concentration of the vaccine is carried out with reagents prepared by independent reference laboratories, using the single radial diffusion (SRD)

technique [247]. This method quantifies the amount of HA only and variation between different manufacturers can occur as not only antibodies directed against HA contribute to the vaccines immunogenicity. The normal adult human dose has a concentration of 15µg HA per strain, which means that a trivalent vaccine (two influenza A subtypes, H3N2 and H1N1, and one influenza B) contains in total 45 µg of HA.

There are also two additional experimental influenza vaccines that are not currently licensed. The use of an inactivated virus vaccine delivered intranasally has been tested with a mucosal adjuvant (cholera toxin and heat labile enterotoxin). In large-scale clinical trials, an unacceptable high frequency of side effects such as Bell's Palsy (facial paresis) occurred and further human use was discontinued [128, 148], reviewed in [98]. The other experimental vaccine that has yet to become a reality is a DNA based vaccine where the DNA coding for influenza antigens is directly injected intramuscularly [46] or intradermally [81]. This could lead to both MHC I and MHC II presentation and possibly a very good immune response. Despite good results in animal models, DNA vaccines have been poorly immunogenic in man [226].



**Figure 9.** Schematic representation of the different influenza vaccine formulations

### 1.6.1.2 Live vaccines

Live attenuated influenza virus vaccine have been extensively used in Russia and have recently been licensed in the USA [47]. The vaccine virus is a reassortment virus between the virus with the desired HA and NA and a cold adapted (ca) parent virus that has reduced ability to replicate in the epithelia cells of the respiratory tract (similarly to the reassortment described in 1.6.1). The ca vaccine is delivered intranasally by spray and the ca virus

replicates to a low viral titre in the respiratory tract. The immune response elicited by a live virus vaccine should be very similar to that induced by natural infection. Currently, the live vaccine used in the USA is not recommended for children and the elderly [84], two of the “at risk groups” that have the highest rate of complications after influenza infection. Another hypothetical concern is the possibility of new reassortments between the live vaccine and a simultaneous wild type influenza infection. This could lead to a new pandemic virus if the reassorted virus had a influenza subtype novel to man, or that the ca virus could revert to wild type virulence, much like the case with live polio vaccine [117]. The potential danger of live influenza vaccine, was demonstrated in Asia in the 1970’s leading to new subtype of influenza A (H1N1) virus appearing in 1977 [166], although little is known in detail about the incident.

The clinical experience with live influenza vaccines has demonstrated that live vaccines provide satisfactory protection. A large meta-analysis of several challenge experiments after vaccination with live or inactivated influenza vaccines, concluded that they had a similar vaccine efficacy [21]. Similar levels of systemic side reactions were found after live influenza vaccines (6.2%) and inactivated influenza vaccines (6.3%) [21]. However, there might be a difference in the immune mechanisms of protection as the live virus vaccine induced more local IgA antibodies and the inactivated vaccine elicited more serum IgG antibody. Live influenza vaccines are not currently licensed in Norway or Europe.

### *1.6.1.3 Target groups for vaccination*

The WHO has issued a position paper on influenza and background information for the use of inactivated vaccines together with a priority list of the “at risk groups” which have increased risk of influenza complications. These “at risk groups” should be annually vaccinated with influenza vaccine [235, 237]. The WHO has not issued any recommendations on the use of live influenza vaccines [240]. The “at risk groups” are:

- Residents of institutions for the elderly or the disabled;
- Elderly non-institutionalised individuals with 1 or more of the following chronic conditions, chronic cardiovascular, pulmonary, metabolic or renal disease, or who are immunocompromised;
- Other individuals (adults and children aged > 6 months) in the community who have chronic cardiovascular, pulmonary, metabolic or renal disease, or are immunocompromised;

- Individuals who are above a nationally defined age limit irrespective of their medical risk status (most countries define the limit of age > 65 years);
- Other groups defined on the basis of national data; and
- Those with regular, frequent contact with high-risk persons such as health care workers in contact with high-risk persons and household contacts of high-risk persons.

The WHO recommends vaccination for pregnant women [237, 240] and in the USA vaccination is also recommended for children 6-23 months old [83]. The Norwegian health authorities follow the WHO recommendations to a large extent, although they do not explicitly recommend vaccination of healthy children and pregnant women [4].

The vaccine, normally an inactivated vaccine, is administered intramuscularly in October and November in the northern hemisphere. One injection (preschool children are given two injections at one month intervals) will for most people induce satisfactory immunity against influenza. Since influenza strains are prone to antigenically drift from one influenza season to the next, annual vaccination is recommended. According to the Norwegian Institute of Public Health [4] only 8-10% of the general population and about 30% people in the “at risk groups” are vaccinated each year. A goal of annual immunisation of at least 75% of people over 65 years old by 2010, has been set by the WHO and ratified by the Norwegian health authorities.

#### *1.6.1.4 Licencing criteria for influenza vaccine*

There are a set of regulatory criteria influenza vaccines need to pass in order to obtain a marketing license in the European Economic Area. These evaluation criteria are defined by the Committee for Medicinal Products for Human Use (CHMP) [3]. The vaccine efficacy is tested by the haemagglutination inhibition (HI) [96, 115] or single radial haemolysis (SRH) assays [195]. An HI assay measures the ability of serum antibodies to inhibit haemagglutination (virus binding to erythrocytes) and the SRH assay detects the capacity of serum antibodies to activate complement-mediated lysis of virus-covered erythrocytes. An HI titre  $\geq 40$  (or an equivalent SRH  $> 25\text{mm}^2$ ) indicates 50 percent protective levels of serum antibody against influenza infection in man [96, 115]. The evaluation criteria for people aged 18-60 are:

- The number of seroconversions or significant increases in anti-haemagglutinin antibody titre should be > 40%;
- Mean geometric increase >2.5;
- The proportion of subjects achieving an HI titre  $\geq 40$  or SRH > 25mm<sup>2</sup> should be >70%

The criteria are less stringent for people over 60 as they do not respond as well as young people to influenza vaccine (seroconversions >30%; geometric mean increase > 2 and the proportion with an HI titre  $\geq 40$  or SRH > 25mm<sup>2</sup> should be >60%). Only one of the criteria must be met by each strain contained in the vaccine, in order to obtain vaccine approval. There are no requirements for inactivated vaccines to evoke a cellular immune response. Future influenza vaccines may induce a higher level of protection and/or more long-lived immunity by also targeting the cellular arm of the immune system. There are no specific criteria for pandemic influenza vaccines, but the WHO has prepared recommendations for increased preparedness and research [236], in addition to an action plan if a pandemic starts [238].

### 1.6.2 Antiviral agents against influenza

Two classes of antiviral agents have been licensed for therapeutic and prophylactic use against influenza. Drug therapy reduces the illness by 1-2 days, ameliorates disease and decreases the possibility of transmission [87, 90, 165]. One class of antivirals, the M2 inhibitors, blocks the ion channel protein M2 and prevents viral uncoating and replication. Both Amantadine and Rimantadine are M2 inhibitors, but they are effective only against influenza A viruses and have an efficacy of 70-90% in healthy adults when used prophylactically [47]. These drugs are available as tablets. The other class of anti-influenza drugs are the NA inhibitors, which prevent the release of influenza virus from the cell. NA inhibitors are effective against both influenza A and B viruses. They are available as a capsule (Oseltamivir) or as an inhalable powder (Zanamivir) with a prophylactic efficacy of approximately 80%. Antivirals provide protection for people that have either missed their annual vaccination, do not tolerate the vaccine or have increased risk of infection (household contacts, hospital staff).

Both types of agents have side effects (mild nausea and gastrointestinal complications [47]), but it is generally thought that NA inhibitors have fewer and less serious side effects, but

they are also far more expensive than M2 inhibitors [132]. Mutations in the influenza virus, rendering the virus resistant to the drugs have been observed after antiviral treatment. Resistant viruses develop more often after treatment with M2 inhibitors than after treatment with NA inhibitors and pose a real threat for the usefulness of anti-influenza agents [120, 138, 140]. However, NA inhibitor resistant viruses have compromised infectivity and replicative ability in mice compared to wild type virus [31]. Combinations of two different classes of drugs has been proposed to limit the development of resistance [132], much like the antiviral cocktail used to treat HIV infection. Although the drugs do not interfere with the normal immune response elicited by influenza vaccination [45], they do not on the other hand elicit any immunological memory and are therefore not a substitute for vaccination.

## 1.7 Immunity to influenza

Influenza virus undergoes substantial antigenic drift allowing the virus to escape the host's immune response. The host's immunological defence against viral pathogens are multifaceted, complex and involve a range of antiviral mechanisms. The primary target for influenza virus is the respiratory tract and that is also the site of the initiation of immune response, with both secretory IgA and CTL responses, as well non-specific innate immunity.

### 1.7.1 Immunity after influenza infection

After an influenza virus infection innate immunity will help to contain the viral replication until the adaptive response begins. Initially, influenza virus will start replicating in the epithelial cells of respiratory mucosa. The immune response will include local mucosal S-IgA and CTL, as well as systemic IgG and IgM [219]. The resulting immunological memory will consequently be memory IgA B-cells, but also memory IgG B-cells, directed against the main antigenic epitopes on HA and NA of the virus. The cytotoxic response is mainly directed against the internal viral proteins, NP and M1 protein [192, 218], and the cytotoxic T-cell response is important in viral clearance and recovery from infection. The internal influenza proteins are more conserved, thus the memory Tc cells may be more cross-reactive against drifted viruses and possibly across influenza A subtypes. There is, however,

conflicting data as to what provides immunity against lethal challenge, at least in mice [152]. In man, viral infection usually results in long-lived immunity [51]. This was seen after the re-appearance of H1N1 in 1977. H1N1 had not been circulating for 20 years [58]. However, people 30-35 years old had antibodies that reacted with the 1977 H1N1 influenza virus [103], possibly resulting from an early childhood infection with an antigenically similar virus as the 1977 virus was antigenically very similar to an 1950 isolate [166]. People under 20 were almost exclusively infected [175] and young adults also had a marked lower immune response to inactivated vaccines containing H1N1, suggesting that they did not have any immunological memory against the virus [2]. Immunological memory resulting from infection allows the immune system to respond more strongly and quickly the next time that an individual encounters influenza.

### **1.7.2 Immunity after vaccination**

Immunity after vaccination varies depending on the type of vaccine used. Immunisation with inactivated vaccines of individuals that are immunological naïve, i.e. the immune system has not experienced that influenza subtype before, will mainly induce low titres of serum antibody [2] with little or no T-cell response. The main antibody class will be IgG, with some IgM, but little IgA. Inactivated vaccines are efficacious in man and this is probably because we all have experienced several influenza virus infections and thus has immunological memory. If an inactivated vaccine contains antigenically similar strains to those that generated the immunological memory during a previous infection, vaccination will activate memory and a boost of ASC with a concomitant increase of antibody [27, 28, 80, 109]. The systemic response will be quicker after vaccination of primed individuals with the ASC response peaking after a week, while the serum antibody continues to increase up to 14-21 days after vaccination [44]. The main antigenic determinates are located on the HA and most neutralising serum antibodies will be directed against HA [57, 167].

Vaccination with inactivated influenza vaccine prevents laboratory confirmed influenza in up to 70-90% of vaccinees, although in the very young and old (>65) the vaccine provide lower rates of protection [21, 26, 47, 84, 110, 238, 242]. Also in years with a sub-optimal match between the vaccine strains, the vaccine is effective in preventing illness, both in children [186] and young adults [176]. If there is no pre-existing immunity, as is the case

with a new pandemic novel influenza subtype, one standard dose of inactivated vaccine will probably not be adequate. Repeated vaccination with adjuvanted vaccines is often necessary to elicit a satisfactory immune response to a novel subtype [2, 91, 92, 156, 208, 209, 212]. Rubens *et al.* demonstrated in 1973 that whole virus vaccine elicited rates of protection similar to split virus vaccine in protection against influenza illness with a 69% reduced attack rate compared to unvaccinated [189], but numerous studies have shown that whole virus is more immunogenic than split virus vaccine, especially in unprimed populations [12, 18-20, 25, 79, 107]. Annual immunisation has been shown not to compromise the immune response, i.e. there is no reason not to vaccinate annually, especially if drift viruses are prevalent in the population [112].



## 2 Aims of the study

The use of whole virus vaccines was largely discontinued in the late 1970s due to a higher level of side-effects. However, whole virus vaccine is still the most immunogenic of the available influenza vaccine formulations. Evaluation and licensing of influenza vaccines is based on their immunogenicity as demonstrated by the HI and SRH assays, which only detect serum antibodies with specificity against the vaccine virus. The methodology for investigating and understanding the immune response after vaccination and infection has evolved with techniques like Enzyme linked immuno sorbent assay (ELISA), virus neutralisation (VN) assay, Enzyme-linked immuno spot (ELISPOT) assay and a wide range of assays to detect cytokine profiles and immunological memory induced by the vaccines.

With an ever increasing pandemic threat from novel influenza subtypes crossing the species barrier into man, we need to expand our knowledge about which influenza vaccine formulation will provide the best protection in man. The novel avian candidate vaccines that have been tested in man have hitherto not been shown to be very immunogenic [91, 92, 156, 208, 212, 248]. There is thus a need for a vaccine that is more immunogenic while maintaining an acceptable level of reactogenicity (i.e. side effects). We therefore aimed to investigate how the vaccine formulation, number of doses and vaccine strength influenced the immune response and viral replication after challenge in a pre-clinical animal model.

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### 3 Overview of papers I-III

#### Paper I

This study was carried out to investigate the kinetics of the immune response to split and whole virus vaccine formulations. BALB/c mice were immunised intramuscularly with 1 or 2 doses (3 weeks apart) of 7.5, 15 or 30µg HA of monovalent A/Panama/2007/99 (H3N2) vaccine. The immune response elicited after vaccination was examined at multiple time-points after each dose of vaccine.

The two different vaccine formulations induced the peak number of ASC on similar days. The highest number of IgG ASC was detected 5-14 days after one dose of vaccine and already at 5 days after the second dose of vaccine. The number of IgM ASC after vaccination peaked more rapidly than the influenza-specific IgG ASC, but the number of IgM ASC was lower than IgG ASC. Interestingly, we also detected an earlier and higher antibody response (VN, HI, IgG and IgM) in the serum of mice after one dose of whole virus vaccine. This could be relevant to the human situation, as an immunologically naïve vaccinee may have protective levels of serum antibody earlier after immunisation with the whole virus vaccine formulation. We also observed higher numbers influenza-specific ASC in bone marrow following one dose of whole virus vaccine, which indicated a greater capacity of this vaccine formulation to induce a more long-lived antibody response.

After two immunisations, split virus vaccine induced higher numbers of IgG ASC in the spleen than whole virus vaccine, although no direct correlation to the concentration of serum antibodies was observed. The earlier ASC response after the second immunisation demonstrated that immunological memory was generated after the first dose of vaccine. Another intriguing finding was the fact that whole virus vaccine induced a predominance of IgG2a subclass with consistently higher IgG2a/IgG1 ratios in serum and ASC, which is suggestive of a Th1 response and priming for a cellular mediated immune response. In contrast, split virus vaccine produced a ratio close to 1, indicative of more of a type 2 response. The immune response after immunisation with whole virus vaccine may therefore be more similar to the immune response detected after influenza infection.

**Paper II**

The protective efficacy elicited of split and whole virus vaccines were tested in a non-lethal challenge study, which also included unvaccinated controls. Furthermore, we identified humoral immunological parameters that were associated with very low viral replication in the nasopharynx. The experiment was designed with a viral challenge nine weeks after the last dose of vaccine, to more closely mimic a human situation.

In mice immunised with one dose of vaccine, viral challenge resulted in the peak nasal wash titres that were up to 9-fold lower than in unvaccinated controls. Mice immunised with whole virus vaccine generally had lower peak nasal wash titres and sharper reduction of viral shedding on the subsequent days. After two doses of whole virus vaccine, similar levels of viral shedding were observed as after one dose of whole virus vaccine. In contrast, after two doses of split virus vaccine (especially the 15 $\mu$ g and 30 $\mu$ g vaccine strengths) the nasal titres were lower than in the whole virus vaccine group. In mice immunised with the two highest vaccine strengths of split virus vaccine elicited very high serum IgG concentrations, which might have significantly reduced viral shedding probably by passive diffusion of IgG through the damaged respiratory epithelia.

The peak numbers of IgG and IgA ASC were detected earlier after challenge in mice vaccinated with whole virus vaccine than split virus vaccine. Whole virus vaccine also primed for higher numbers of IgA ASC after challenge. Mice with no or low peak viral shedding, regardless of vaccine formulation, had higher pre challenge serum IgG and HI antibodies than mice with higher levels of viral shedding. Mice with high viral shedding also had high numbers of IgG and IgA ASC in spleen and lungs, but lower number of IgG ASC in bone marrow 21 days after challenge. Bone marrow is the site of long lived ASC and this may indicate that mice with low viral shedding had better memory recall after challenge. The ratios of distribution of IgG2a and IgG1 subclasses after challenge differed between the two vaccine formulations. Whole virus vaccine induced higher IgG2a/IgG1 ratios post challenge than split virus vaccination, confirming findings described in paper I. The Th1 response induced by whole virus vaccine, however, was not as important as pre-existing antibody in resolving a non-lethal upper respiratory tract infection.

### **Paper III**

The formulation of influenza vaccines is known to influence both the reactogenicity and immunogenicity. We extended the work described in Papers I and II, and investigated the cytokine response induced after vaccination (split and whole virus vaccine formulation) and compared it to the response after natural influenza infection. Spleen cells were stimulated *in vitro* with influenza to determine the associated Th1, Th2 and inflammatory cytokine profile.

Mice immunised with whole virus vaccine had an earlier increase in HI titres, significantly higher IgG2a/IgG1 ratios and higher concentrations of IL-12, IL-2 and IFN- $\gamma$  than split virus vaccine. This supports the finding that whole virus vaccine elicits a Th1 biased response, as we observed in Papers I and II. In contrast, one dose of split virus vaccine elicited more IgG1 antibodies and higher concentrations of IL-4 and IL-6, suggestive of a Th2 response. Immunisation with two doses of split virus vaccine increased the IgG1 concentration further and lowered the IgG2a/IgG1 ratio compared to one dose of split virus vaccine, similarly to that observed in paper I. This coincided with high concentrations of IL-5 and the prototypic IL-4, indicating a stronger Th2 biased response after two doses of split virus vaccine.

Particularly after two doses of whole virus vaccine, higher concentrations of IL-10 and IL-1 $\beta$  were induced. These cytokines have been implicated in fever and influenza-like symptoms, possibly explaining the increased reactogenicity of whole virus vaccine. Two doses of whole virus vaccine also induced higher concentrations of IL-6. This cytokine, together with IL-10, are important in inducing an IgA response, which suggests that whole virus vaccine may be better at eliciting an IgA response in mice. This offers an explanation of the higher number of IgA ASC detected after challenge in mice immunised with whole virus vaccine (Paper II). Natural infection of mice and immunisation with whole virus vaccine resulted in similar cytokine profiles with high concentrations of IL-10 and IFN- $\gamma$ .

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## 4 Discussion

The work presented in this thesis aimed to show how the formulation of an influenza vaccine affected the immune response and the protective efficacy after viral challenge. Studies of different formulations of influenza vaccine have demonstrated that vaccination with whole virus vaccine results in a higher level of side reactions in man than split or subunit vaccines (e.g. fever, malaise and myalgia) [18-20, 25, 79]. Consequently, whole virus vaccine is no longer in common use. However, as whole virus vaccine is more immunogenic than the other influenza vaccine formulations, there has been a call to investigate all possible vaccine formulations as potential pandemic vaccines [156, 211, 248].

The immune response after immunisation is dependent upon the dosage of immunogen in the vaccine and it is therefore important to investigate the effect of different vaccine strengths. In all the murine experiments described in this thesis, the vaccine strengths of either half, full or twice the human dose have been used. Although these vaccine strengths are large for a small experimental animal, there is not any direct relationship between the vaccine strength and the animal's body weight. We used vaccine strengths relevant to man so that our data can be used as a reference for the research community at large when testing new influenza vaccine candidates in a pre-clinical model, where mice are frequently used.

We initially investigated the kinetics of humoral response after vaccination, using two vaccine formulations, three vaccine strengths and one or two doses of vaccine. A detailed experimental plan was necessary to thoroughly characterise the immune response elicited by these two vaccine formulations, using a range of methods to detect the differences in the resulting humoral immunity. In designing animal experiments, it is important to remember that in any biological model system a random variation exists which could influence the results. To achieve greater statistical robustness in the kinetics study (paper I), which had 72 subgroups, all sampling points were combined to test dose and vaccine strength responses. In the challenge experiment (paper II) consecutive daily nasal washings from the same mouse were used, which largely reduced both the number of animals needed and allowed individual follow-up.

## 4.1 How the vaccine formulation affects the ASC response

Antibodies are produced by B-cells, and enumeration of the number of influenza-specific antibody secreting cells is an important measure of the immunogenicity of the vaccine. We used the ELISPOT assay to detect the activated B-cells, ASC, in both the vaccination (paper I) and challenge studies (paper II) to characterise the kinetics of the immune response. The precise nature of differentiation from a B-cell to an ASC is unclear. ASC in general are capable of cell division, an element implicated in class switching [85]. ASC are sometimes mistaken for plasma cells, which are a subpopulation of ASC that are highly specialised, short lived, non-dividing, terminally differentiated cells [8, 85]. However, some plasma cells that are detected in the bone marrow, are long lived and contribute to the serum antibody pool for extended periods of time [53, 200]. Memory B-cells induced by vaccination are not directly detected in our ELISPOT assay, as 6 days of polyclonal stimulation are needed in order to stimulate differentiation of memory cells to antibody secreting cells [50, 52].

After vaccination with one dose of vaccine, no clear differences were found in the peak number of ASC detected between two vaccine formulations (paper I). After challenge of mice immunised with one dose of whole virus vaccine, higher numbers of ASC were detected at day 7, despite lower levels of viral shedding than in the split virus vaccine group (paper II). Therefore, the memory recall after challenge was probably stronger in mice that had been vaccinated with whole virus vaccine. Interestingly, whole virus vaccine induced higher numbers of IgA ASC 7 days after challenge, indicating that one dose of whole virus vaccine was better than one dose of split virus vaccine in priming for an IgA response.

An earlier and stronger ASC response occurred after two doses of vaccine as a result of the immunological memory elicited after the first dose of vaccine. Two immunisations of split virus vaccine elicited higher numbers of IgG ASC than whole virus vaccine and thus appeared to further boost the immune response than the second dose of whole virus vaccine (paper I). In man, increases in influenza-specific antibodies after vaccination are dependent on pre-vaccination antibody levels, with a lower proportion of subjects with significant increase in vaccinees with the highest pre-vaccination serum antibody concentration [111]. Since one dose of split virus vaccine induced lower serum antibody concentrations than one dose of whole virus vaccine, this may explain why two doses of split virus vaccine seemed



to boost the IgG ASC response more than two doses of whole virus vaccine and result in higher antibody concentration 9 weeks after the last immunisation.

## 4.2 The importance of serum antibody

The relative role of IgM, IgG and IgA antibodies in protection against viral replication are still not clearly demonstrated and no strong consensus has emerged. In part, this can be attributed to the different methods used to test the immunological mechanisms of protection against infection, e.g. different challenge doses and end-point measured, priming, route of delivery, and antigenic similarity between priming and challenge viruses. In the 1970s it was demonstrated that a serum HI titre above 40 is associated with protection against influenza illness in man [96, 115]. Since IgG constitutes a large proportion of serum antibody, IgG must have an important role to play in protection against illness. As illness is difficult to measure objectively in mice, we examined the level of viral shedding after challenge.

Influenza virus replicates in the respiratory tract, therefore the focus has been on locally produced IgA, which is actively secreted across the mucosa. It has been hypothesized that IgA mediates protection against viral replication in the upper respiratory tract [183-185]. However, inactivated vaccines induce mostly serum IgG, but are nevertheless effective in preventing illness in man [47] and mice [22]. Prevention of illness may be a more complex immunological process involving a complex interaction of the infection and the immune response than direct prevention of viral shedding. In our study, mice had very low serum IgA concentrations and no nasal wash IgA was detected immediately after challenge. We found that the vaccine induced IgG antibody not only protected the mice against signs of illness, but also significantly reduced the level of viral replication in the upper respiratory tract compared to unvaccinated controls. Furthermore, the highest concentrations of pre-existing serum IgG were associated with very low or no viral shedding upon challenge. Serum derived IgG was probably the effector mechanism in reducing the viral shedding, which by passive diffusion over the mucosal surface neutralised the virus and thereby prevented viral replication.

We found that whole virus vaccine induced more serum IgM antibody than split virus vaccine in mice (paper I). However, IgM might not be actively transported across the mucosal surfaces in mice [187, 227] and probably did not influence viral shedding in the challenge experiment (Paper II). Whole virus vaccine is known to induce more IgM antibody in man than split virus vaccine [19, 25] and furthermore, IgM is actively secreted across the mucosal epithelial layer in man [143]. Therefore, pre-existing IgM may well contribute to reducing viral entry. As a result, whole virus vaccine may be more effective in neutralising drifted influenza strains as the normally pentameric IgM is the most cross-reactive of the antibody classes [14, 89].

We observed that naïve mice produced faster and higher VN and HI antibody titres to whole virus vaccine than to split virus vaccine and this may also be relevant to the human situation. If a novel influenza A subtype crosses the species barrier into man, the human population will be immunological naïve to this new virus. If whole virus vaccine formulation intrinsically induces an earlier and faster serum response in a naïve immune system, it may also induce protective serum antibody concentrations in man earlier after vaccination. There are no set requirements of immunogenicity for use of pandemic vaccines in man [237, 238], and more experience with avian influenza vaccines in man is required. However, it is reasonable to assume that the higher serum antibody concentration the vaccine induces, the better the protection against infection and its associated medical complications.

Another aspect of vaccination with avian influenza vaccines is that the intrinsic immunogenicity may be poorer with avian influenza virus derived vaccines than the current H3N2 vaccines. In a pilot study we investigated the immunogenicity of an avian H7N1 whole virus vaccine in a mouse model and found that the level of HI serum antibody and ASC induced after immunisation to be much poorer than that detected after either split or whole virus H3N2 vaccine, despite the mice being immunological naïve to both the H3N2 and H7N1 subtypes (see data in the Appendix). There are difficulties using an HI assay to detect antibody against avian viruses [210], which make the virus neutralisation test a more suitable method. However, there is nevertheless a much lower HI titre and number of ASC compared to the HI titres and ASC numbers detected with the H3N2 vaccine (paper I and III) and there is possibility that avian influenza vaccine may be inherently less immunogenic, similarly to that observed in man [91, 92, 156, 208, 209, 212]. This is also a strong

indication that efficient priming and the use of adjuvant in conjunction with the avian vaccines, may be necessary to achieve protective levels of immunity in man.

### 4.3 Vaccination effectively limits viral shedding

The protective efficacy of candidate influenza vaccines can be tested by different challenge methods, e.g. homologous and heterologous viral challenge, full respiratory tract infection, limited upper respiratory tract infection or lethal infection. These methods may test different aspects of the immune system and therefore the contributions by antibodies and cellular immune effectors like CTL and NK cells in resolving the infection may vary. An upper respiratory tract challenge model is thus be a more suitable model to mimic human influenza, as the virus normally replicates in the upper respiratory tract. In the challenge study we have chosen to investigate the effect of vaccination upon the level of replicating virus in the nasal cavity of the mouse. Mice were challenged using an influenza strain antigenically indistinguishable from the vaccine virus, as the vaccine strains contained in the human vaccine usually provide a good match to the circulating influenza strains in the community [176]. Compared to a lethal challenge study, the results were more difficult and complex to analyse, but also more relevant for the human situation, where a lethal outcome is rare. Even in the most severe pandemic documented, the ‘Spanish flu’, the lethality rate was only about 3% in the most serious wave of influenza in the autumn of 1918 [73].

An initial upper respiratory tract infection after challenge was achieved by using a low challenge volume in non-anaesthetised mice, as natural reflexes and low volume allow the virus to be deposited in the nasopharynx [184, 250]. After challenge, vaccinated mice had lower levels of viral shedding (up to 9-fold) than unvaccinated control mice. This is in itself important, because in man the level of viral shedding is closely linked to the severity of illness. A vaccine that reduces the level and duration of viral replication in the respiratory tract will moderate the clinical symptoms and reduce viral dissemination [145, 146, 155]. In mice vaccinated with one dose of vaccine, both formulations reduced the level of viral shedding compared to unvaccinated control mice. Mice immunised with one dose of whole virus vaccine generally had a lower and shorter duration of viral shedding than mice immunised with split virus vaccine.

After two doses of vaccine there was a marked difference between the two formulations; the two highest split virus vaccine strengths resulted in no or very low viral shedding. In contrast, similar levels of viral shedding were observed after one and two doses of whole virus vaccine. The two highest strengths of split virus vaccine induced the highest pre-challenge serum antibody levels and this pre-existing antibody significantly altered the time course of infection. This was also demonstrated by analysis of the humoral immunological correlates in mice with low viral shedding irrespective of vaccine formulation, demonstrating the importance of pre-existing antibody in reduction of viral shedding early after infection.

Whole virus vaccine elicited a predominantly Th 1 response (papers I-III). However, this did not more effectively limit viral shedding after two doses of vaccine than after one dose. A cellular response may not be induced after a relatively mild infection if pre-existing antibodies are present and capable of significantly limiting viral replication. Viral shedding decreased from 2 days post challenge in all the vaccinated mice, which is too early for any significant number of CTL, or indeed, new ASCs to be generated from memory cells. The Th1 biased response elicited by whole virus vaccine may be more important in protection against a more severe infection. One may speculate that if we had used a lethal mouse adapted influenza strain or an antigenically drifted virus would increase the effect of the more cross-reactive IgA and CTL may have been more clearly observed. It is therefore possible that a cytotoxic response would only be a major effector mechanism against a severe respiratory tract infection, thus avoiding the unnecessary tissue damage caused by a vigorous cytotoxic response.

#### 4.4 Is whole virus vaccine the best formulation for a naïve population?

The results demonstrated that whole and split virus vaccines to some extent stimulated different arms of the immune system. Whole virus vaccine elicited a predominance of the IgG2a subclass (paper I) and a cytokine profile (paper III) that is compatible with a Th1 response. Split virus vaccine induces a more IgG1 antibody and Th2 type of cytokines. It has frequently been held that non-replicating antigens cannot be presented on MHC I and

stimulate a type 1 response. However, exogenous antigen can be presented on MHC I [116], a process known as “cross-presentation” [224]. A number of reports have in fact demonstrated that non-replicative influenza antigen can induce CTL [6, 29, 192, 197, 204, 218]. The mechanism behind this has not been elucidated, but an interesting observation is that all the influenza vaccines that induce CTL have an intact lipid membrane. An intriguing explanation may therefore be that the intact viral envelope of whole virus vaccine leads to a higher level of antigen being presented on MHC I than split virus vaccine.

Interestingly, mice previously immunised with whole virus vaccine had higher numbers of IgA ASC in the spleen, the lungs and the bone marrow after challenge, regardless of the level of viral shedding. Stimulation of IgA production is normally associated with a Th2 response. However, as described in paper III, whole virus vaccine also induced higher concentrations of cytokines that stimulate an IgA response and thus may explain why mice vaccinated with whole virus vaccine had higher numbers of influenza-specific IgA ASC after challenge. Only very low concentrations of IgA were detected in the serum after vaccination alone. However, as the major effector site of IgA antibodies are the mucosa it would be interesting to further examine the local IgA response induced in mucosal secretions or tissues, after vaccination with whole virus vaccine. Brokstad *et al.* have previously found that split virus vaccine delivered intramuscularly induced IgA ASC in the local lymph nodes in primed adults [27]. However, as humans have normally experienced a number of influenza infections and therefore will have some IgA memory cells, it is not possible to determine if the split virus vaccine itself elicited IgA ASC in man or activated pre-existing memory IgA. Nonetheless, this raises the possibility that whole virus vaccine would be better than split virus vaccine in eliciting an IgA response also in man.

The increased immunogenicity of whole virus vaccine in a naïve individual suggests that whole virus vaccine formulation may be a preferable vaccine formulation as it elicits both mucosal and systemic antibodies, as well as prime for a CTL response [113, 136, 185]. Provided this holds true also in man, whole virus vaccine would be the vaccine formulation of choice for use a pandemic scenario. Two doses of vaccine may be needed to elicit an appropriate immune response, as human trials with avian influenza vaccines have concluded [91, 92, 156, 208, 209, 212]. One possibility could therefore be to combine one dose of the broader immune response of whole virus vaccine with the one dose of the more serum IgG inducing split virus vaccine. Only further vaccine trials in man can resolve which

combination of vaccine formulations, strengths and number of doses will induce the most appropriate immune response. These trials are urgently needed, as there will be too little time to clinically test vaccine formulations and dose responses after an influenza pandemic is declared.

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