Influenza Vaccination with Focus on the Immunobiology of the Upper Respiratory Tract

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Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

2008

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Edvard Munch: Self-portrait after Spanish Flu (Selvportrett etter spanskesyken), 1919 – 20. Oil on canvas, 59 x 73 cm, The Munch Museum, Oslo, Norway.

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Acknowledgements

The present work was carried out at the Department of Otorhinolaryngology, Head and Neck Surgery, Haukeland University Hospital in collaboration with Section for Otorhinolaryngology, Head and Neck Surgery, Department of Surgical Science, Faculty of Medicine, University of Bergen and The Broegelmann Research Laboratory, The Gade Institute, University of Bergen. I thank the Faculty of Medicine at the University of Bergen and the Broegelmann Foundation for financial support.

I started my specialisation in ENT surgery as a resident at the department in 1998, and soon came in contact with Åke Davidsson MD PhD, who together with Karl Albert Brokstad PhD at the Broegelmann Research Laboratory involved me in the research on influenza. Thanks to their including attitude and friendly atmosphere, I joined their research group and have now finished this thesis. My warmest thanks go to them for making this work possible.

I would also like to thank the persons in charge of the departments who have encouraged us all with their enthusiasm and support during our research. Prof. Jan Olofsson and Prof. Roland Jonsson deserve special thanks.

Without the help of all the staff at the Outpatient clinic and the Day care surgery, at the ORL-HNS-dept at Haukeland our work would have been a nightmare. I am truly thankful for your optimism and faith in our work. Special thanks to nurse Hildur who stood by us and helped us with the inclusion of our patients for the first study. Furthermore, I also thank all the doctors at our department who have helped me with our research project.

I am grateful to Marianne, Turid and Hilde at the Broegelmann Research Laboratory for their help with the tissue samples and analyses, and for teaching me about laboratory work, and Kate who keeps track of everything and everybody.

At the Influenza Centre at the Gade Institute, University of Bergen, Rebecca Jane Cox PhD has done a lot of work with the tissue samples and together with Prof. Lars Haaheim and Ewa Szyszko helped with their massive knowledge about the influenza virus and vaccination.

I send some warm thanks to the laboratory for clinical chemistry (Laboratorium for klinisk kjemi) at Haukeland University Hospital for their friendly attitude to researchers in general and especially for performing the extra pre-operative blood sampling smooth and gentle.

The University Library staff at the medical faculty also deserve my thanks for their smooth handling of my requests for textbooks and copies of articles, and for providing access to full-text articles of almost every medical journal.

Last but not least I thank my fiancée and life partner, Gunn, and our two children Ulrik and Nora for their support on the home arena and for all their patience with a sometimes absent minded partner and father.

List of publications

This thesis is based on the following papers:

I Brokstad KA, Cox RJ, Eriksson J-C, Olofsson J, Jonsson R, Davidsson Å.

High prevalence of influenza specific antibody secreting cells in nasal mucosa.

Scand. J. Immunol. 2001 Jul-Aug;54(1-2):243-47.

II Brokstad KA, Eriksson J-C, Cox RJ, Tynning T, Olofsson J, Jonsson R, Davidsson Å.

Parenteral vaccination against influenza does not induce a local antigen specific immune response in the nasal mucosa.

J Infect Dis. 2002 Apr 1;185(7):878-84.

III Eriksson J-C, Davidsson Å, Garberg H, Brokstad KA

Lymphocyte distribution in the tonsils prior to and after influenza vaccination.

Vaccine. 2003 Dec 8;22(1):57–63.

IV Eriksson J-C, Cox RJ, Szyszko E, Davidsson Å, Brokstad KA

Local and systemic cytokine and chemokine responses after parenteral influenza vaccination.

Influenza and Other Respiratory Viruses. 2007 Jul;1(4):139–146.

Abbreviations

APC	Antigen Presenting Cell
ASC	Antibody Secreting Cell
CD	Cluster of Differentiation
CTL	Cytotoxic T Lymphocyte
ELISA	Enzyme Linked Immuno Sorbent Assay
ELISPOT	Enzyme Linked Immuno Spot
ENT	Ear-Nose-Throat
HA	Haemagglutinin
HAI	Haemagglutination Inhibition Assay
HEF	Haemagglutinin-Esterase Fusion protein
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
LAIV	Live Attenuated Influenza Vaccine
M1	Matrix protein 1
M2	Matrix protein 2
MALT	Mucosa-Associated Lymphoid Tissue
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
MSIS	Surveillance System for Communicable Diseases (Meldingssystem for smittsomme sykdommer)
NA	Neuraminidase
NALT	Nasopharynx (or nose)-Associated Lymphoid Tissue
NEP	Nuclear Export Protein
NP	Nucleoprotein
NS1	Nonstructural Protein 1
NS2	Nonstructural Protein 2
NSAID	Non-Steroidal Anti-inflammatory Drug
ORL-HNS	Otorhinolaryngology - Head and Neck Surgery
OSA	Obstructive Sleep Apnoea
PA	Acidic Polymerase Protein
PB1	Basic Polymerase Protein 1
PB2	Basic Polymerase Protein 2

PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
RSV	Respiratory Syncytial Virus
S	Secretory
SIgA	Secretory Immunoglobulin A
Th	T helper Cells
TMC	Tonsillar Mononuclear Cells
TNF	Tumor Necrosis Factor
UK	United Kingdom
v	Viral
WHO	World Health Organization

1. Introduction

The following part is a general introduction to the field of influenza virus infection and vaccination, and the mucosal immunology of the upper aerodigestive tract. It is therefore not extensively cited.

The majority of the adult population has an opinion of what an influenza virus infection or ”the flu” is, and they will most likely have experienced one or more influenza-like illnesses during their lifetime, with typical symptoms like fever, cough, nasal congestion, headache and myalgia (61). However, many of these febrile episodes referred to as influenza are often caused by other pathogens than the influenza virus (99). During the last years there has been a tremendous public interest in influenza virus infection, mainly because of the new subtypes of avian influenza virus that have infected humans, and the potential threat of a new pandemic influenza virus.

1.1 Historical overview of influenza

Influenza has been known for centuries, and causes seasonal epidemics in the Northern Hemisphere almost every winter.

Hippocrates may provide the first report of what today is interpreted to be an influenza outbreak, in Greece in 412 BC (41). Since then there are numerous other reports that appear to describe influenza infection. The first description of what is believed to be epidemic influenza was in 1173 (10).

The name of the virus is derived from the Italian word *influentia*, which is connected to the belief in 1300 that the disease was influenced by astrological constellations. The initial search in modern time for the aetiology of influenza started in the beginning of the 19th-century, and the bacteria *Haemophilus influenza* became first associated with the disease. During and after the influenza pandemic in 1918, an

intense search started, for the cause of influenza (4). The first virus was isolated in swine by Richard Shope in 1931 (81). In 1933 Wilson Smith published his article of the influenza virus isolated from humans (84). Today, we know that the influenza virus Shope isolated from swine in 1931 descended from the 1918 (H1N1) virus.

There have been 3 (4 with the re-emergence of H1N1 in 1977) pandemics of influenza during the last 100 years, which in total have killed more than 50 million people world-wide. So far, the worst pandemic was in 1918, known as The Spanish Flu (H1N1) and this alone may have killed more than 40 million people. Later, in 1957 came the Asian Flu (H2N2) and in 1968 the Hong Kong Flu (H3N2). In 1977 the H1N1 virus re-emerged as the Russian Flu, and since then both the H1N1 and the H3N2 have been circulating in the human population.

There is a common view among health authorities that a new influenza pandemic is imminent. In latter years there have been numerous reports and predictions about this threat (23). In the media we have all learned about the highly pathogenic avian influenza outbreaks in Asia (6). According to the latest update (WHO, April 2008) there have been 348 cases of laboratory-confirmed H1N5 avian influenza in humans and 216 deaths world-wide (107). Luckily, there has so far been very few cases of human to human spread of this avian influenza virus (110).

1.2 The Influenza Virus

Influenza viruses belong to the family Orthomyxoviridae, and are enveloped RNA viruses with a segmented genome. Influenza A and B viruses have eight gene segments, while the C virus has seven gene segments. They also differ in the antigenic properties of the internal proteins (matrix and nucleoproteins).

Characteristics	**Influenza A**	**Influenza B**	**Influenza C**
No. of gene segments	8	8	7
Surface glycoproteins	HA and NA (Haemagglutinin and Neuraminidase)	HA and NA (Haemagglutinin and Neuraminidase)	HEF (Haemagglutinin-Esterase-Fusion)
Host range	Wide (humans, pigs, horses, whales, seals and birds)	Humans (but also isolated from e.g. seals)	Mainly humans, but also found in swine

Table 1: Some major differences between the influenza viruses. From (22).

Influenza virus C is genetically a relatively stable virus and is rarely isolated due to its low clinical significance. Infections with this virus give mild symptoms similar to the common cold. Most adults have antibodies against this virus, reflecting that infections with influenza virus C are common in childhood. The C virus differs from A and B viruses by having only one surface glycoprotein, namely the HEF protein.

Influenza virus B is mainly a human pathogen, although it has been isolated from some other species, e.g. seals. It is a relatively stable virus and has caused local and epidemic outbreaks, but has not caused pandemic outbreaks. The annual influenza vaccine also contains influenza virus B antigens.

It is only influenza A virus which causes pandemic influenza. Influenza A virus is found in a wide range of vertebrates, and has aquatic birds as its natural reservoir, where the influenza A infection unlike in humans is an asymptomatic intestinal infection (109). Both the type A and B viruses have 2 surface glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA), but the variety in surface molecules in the A virus is one magnitude higher than in the B virus. The A viruses are therefore divided into subtypes based on genetic and antigenic variability. So far, 16 serologically distinct HA and 9 NA have been identified in aquatic birds.

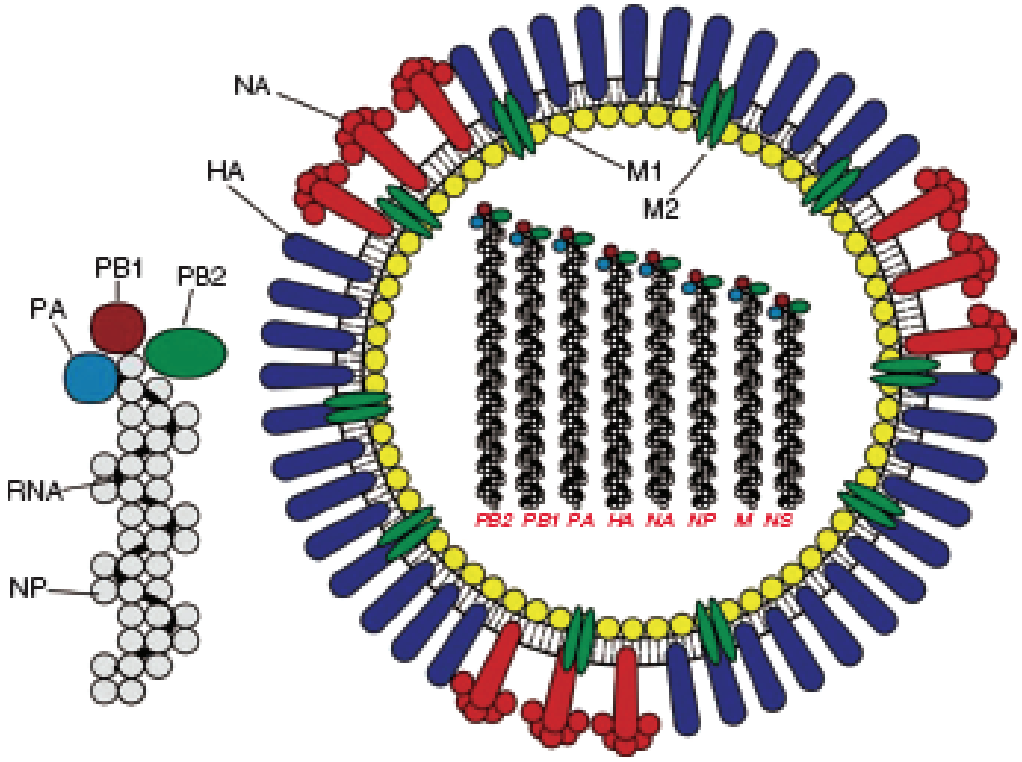

Figure 1: A diagrammatic representation of influenza A virus showing protein and genomic RNA complexes. HA, haemagglutinin; NA, neuraminidase; M1, M2, matrix proteins; NP, nucleoprotein; NS, non-structural proteins; PA, PB1, PB2, proteins involved in virus replication. From (25). Illustration reproduced with permission.

1.2.1 Classification and nomenclature of influenza viruses

The naming consensus of the influenza virus is as follows: Type of influenza virus (A, B or C) / Host (if not human) / Place of isolation / Strain number / Year, and for influenza A also the HA and NA subtype, abbreviated H and N. Example: A/duck/USSR/695/76 (H2N3) and C/Paris/1/67 (105).

1.2.2 Structure of the influenza virus

Influenza A and B viruses are enveloped negative-sense single-stranded RNA viruses with 8 genome segments, coding for up to 11 viral gene products. Each genome segment is encapsulated by nucleoproteins (NP) to a ribonucleoprotein structure (RNP). The virus envelope consists of the host cell membrane, and incorporates 3 viral encoded surface proteins. The haemagglutinin (HA) and the neuraminidase (NA) protrude from the surface with about 500 molecules per virus (54), while the M2 proteins are integrated into the membrane. These proteins are the most important antigens of the influenza A and B virus, regarding the adaptive immunity against influenza. The HA binds to sialic acid containing receptors at the cell membrane of the host cell, and the NA aids in viral exit from the cell. The M2 protein is an ionic channel that is important in modulating the pH in the virion.

Underlying the lipid bilayer are approximately 3000 copies of the M1 protein (54), and within the matrix are the 8 segmented RNP complexes, together with the viral transcriptases; polymerase B1 (PB1), polymerase B2 (PB2) and PA, and probably low amounts of NS2 (NEP) (Reviewed in (22)). The functions of the different viral proteins are summarised in table 2, and the virus architecture is illustrated in figure 1.

Segment	Encoded Protein	Function
1	PB2 Basic Polymerase Protein 2	Part of the RNA polymerase complex. Transcriptase. Generates cap structures for the viral mRNAs (from the hosts mRNA)
2	PB1 Basic Polymerase Protein 1	Part of the RNA polymerase complex. Transcriptase. Elongation of RNA
	PB1-F2 Basic Polymerase Protein 1-Frame 2	Alternative reading frame of PB1. Regulation of the immune response. Mitochondrial protein.
3	PA Acidic Polymerase Protein	Part of the RNA polymerase complex. Transcriptase. Exact function poorly understood.
4	HA Haemagglutinin	Surface protein that binds to sialic acid on the host cell surface. Fusion of the viral and cellular membrane.
5	NP Nucleoprotein	Encapsidates the vRNA and the polymerases to RNP. Transport of vRNA
6	NA Neuraminidase	Surface protein. Release of new viral particles from the host cell.
7	M1 Matrix Protein 1	Form a layer between the viral membrane (host membrane) and the RNP. Essential for nuclear export of RNP.
	M2 Matrix protein 2	Membrane protein that is an ion channel, and responsible for pH regulation.
8	NS1 Non-structural Protein 1	Regulatory protein on the cellular and viral protein expression. Not essential? Inducer of proinflammatory cytokines in human macrophages. The only non-structural protein?
	NS2 (NEP) Non-structural Protein 2 (Nuclear Export Protein)	Interacts with the M1-protein in the export of RNP. Low amounts in the virus.

Table 2: Influenza A genome segments sorted after their size and an overview of the function of their products.

1.2.3 Replication

In humans, the influenza virus primarily infects the epithelial cells of the airways, where the HA binds to sialic acid residues on the epithelial cells. This binding results in endocytosis, and the formation of an endosome in the cell cytoplasm. The low pH inside the endosome triggers the fusion of the endosome membrane and the virus membrane, and results in an uncoating of the virus. The M2 proton channel in the virus facilitates an acidification of the interior of the virus, weakening the association of the M1 protein layer and resulting in release of the viral genetic material (RNP) into the cytoplasm of the infected cell. The RNP complexes are then transported into the nucleus of the infected cell, where the viral transcriptases PB1, PB2 and PA transcribes the viral RNA into messenger RNA for the synthesis of viral proteins. The viral RNA itself is also copied to form new RNPs for new viruses. The viral proteins HA, NA and M2 are transported to the cell membrane, where the M1 protein coats the inner part of the cell membrane, and then the new RNPs attach to the M1 and the

influenza particles bud through the membrane. This process is summarised in the figure 2. After the epithelial cell has produced thousands of new viruses, the cell dies, and this results in a desquamation of the epithelium. (103)

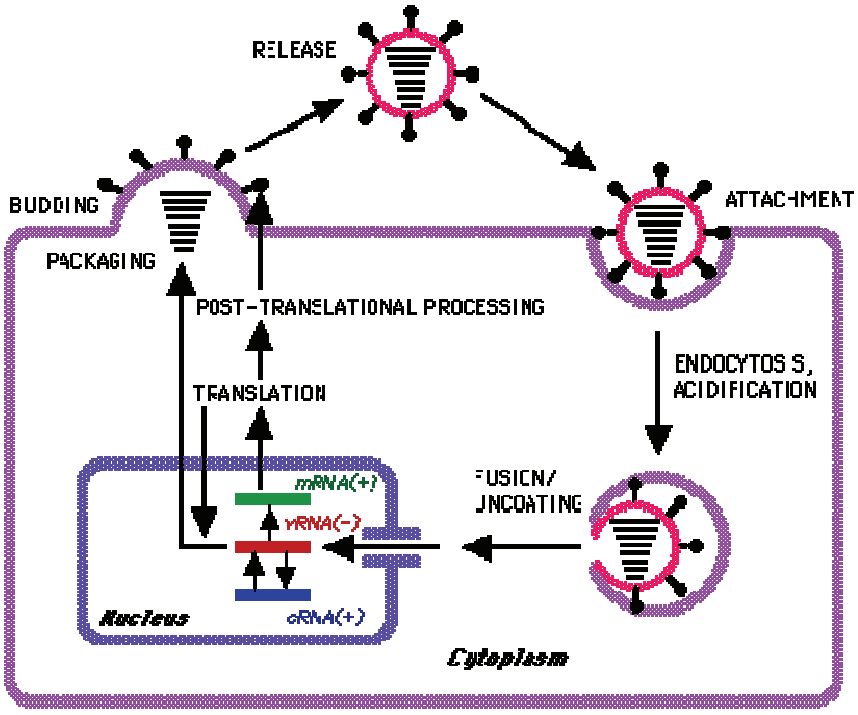

Figure 2. Illustration of the replication of the influenza virus. (Illustration courtesy of Dr A. J. Cann, University of Leicester, UK.)

1.3 Antigenic Drift and Shift

Both the A and B viruses are constantly mutating due to hosts selection pressure. These mutations can result in antigenic changes in the important surface glycoproteins HA and NA, but also other antigenic viral proteins (73) that are important for the virus to escape the hosts' earlier acquired immunity. Antibodies to HA neutralise the virus, and are therefore important in the hosts' ability to prevent infection and clearance of the virus. Accumulation of minor changes in the antigenicity causes antigenic drift. This means that the antigenic specificity of the antibodies produced are reduced, but not absent. If a new subtype of influenza virus

HA or NA emerges this is often due to genetic reassortment with an avian virus. If the HA (and/or NA) are exchanged with a human influenza virus, the acquired immunity to the surface glycoproteins is absent and this change is called an antigenic shift. Only the A virus has reportedly been able to genetically reassort (shift). If an influenza A virus reassorts either or both of the HA or NA segments, this may be the initiation of a new pandemic strain to which the population does not have any immunologic memory.

1.3.1 Antigenic Drift

The replication of the influenza A virus is an error-prone process**.** The virus has no system for proof reading of the transcription of its genes. In laboratory experiments the estimated influenza virus mutation rate is on average $1{,}5x10^{-5}$ mutations per nucleotide per infectious cycle (70;87). There are a total of approximately 14.000 nucleotides in the influenza genome, giving an average of one point mutation in every 5^{th} virus. Most mutations are silent, some may result in developing non-functional viruses (negative mutations), but a few may alter the immunological properties to more favourable regarding survival of the virus. By this mechanism the viruses adapt to the immunologic pressure of the host. This is the reason for the annual influenza outbreaks, and why the composition of the influenza vaccine has to be evaluated and updated each year.

The influenza B (and C) viruses also drift, but at a much slower rate.

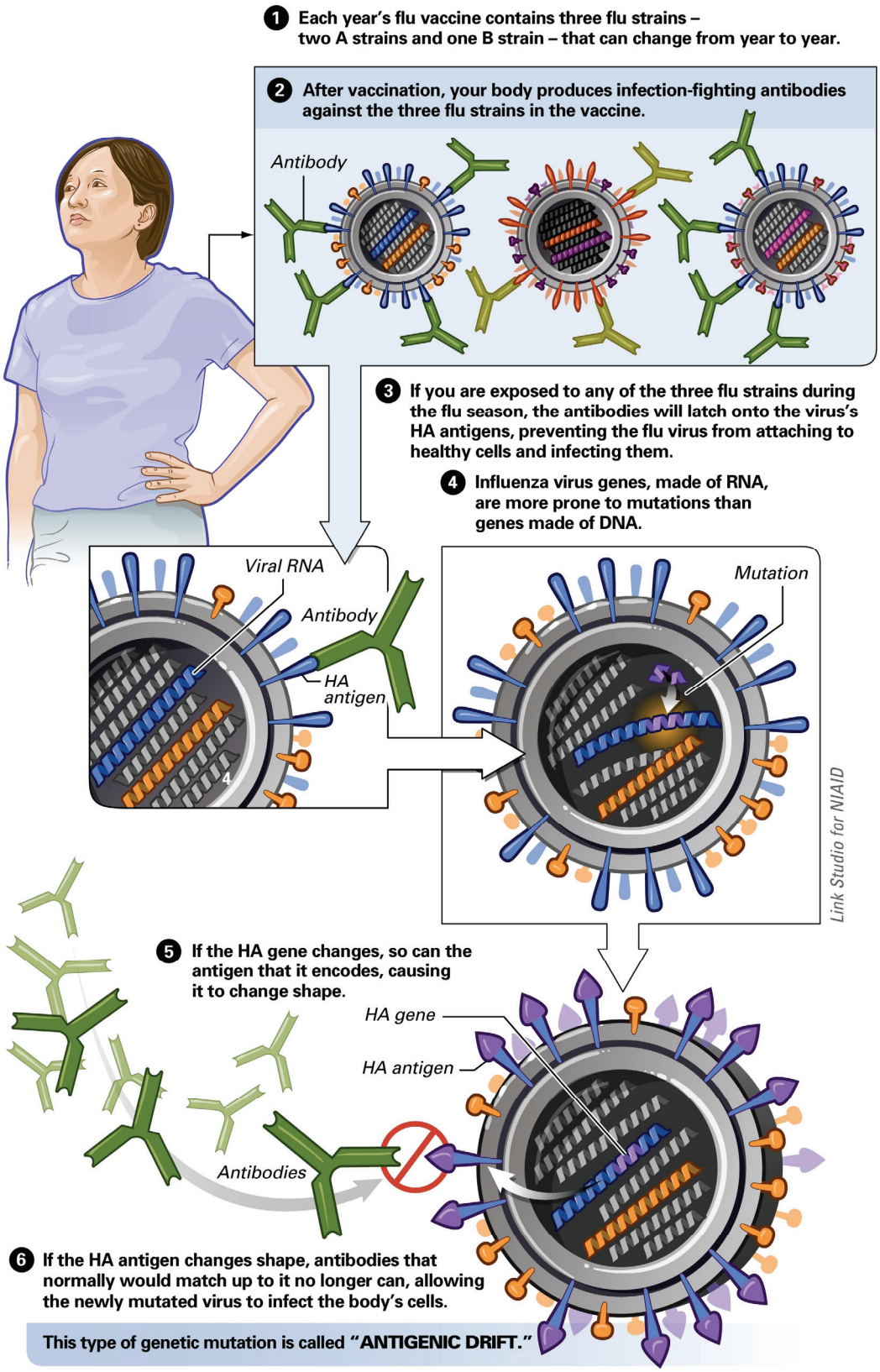

Figure 3. Antigenic drift. Illustration courtesy of "National Institute of Allergy and Infectious Diseases" (93)

1.3.2 Antigenic Shift

The natural reservoir for influenza A virus is aquatic birds (with a stronghold in China and South-East Asia), where all the 16 different HA and 9 different NA are found. The avian and the human virus HA have slightly different affinity to sialic acid residues on the epithelial cell membrane. In humans the main target is the respiratory epithelium in the upper and lower airways. The background for the low infectivity of avian influenza in humans is the requirement for a different receptor configuration on the host cells. The human influenza HA preferentially binds to sialic acid with galactose in a α2,6 configuration, while the avian influenza virus prefers a α2,3 configuration. Pigs has both the α2,3 and the α2,6 configuration of sialic acid in the upper respiratory tract mucosa, and can therefore be infected with both human and avian influenza viruses. If a pig cell is simultaneously infected with a human and an avian influenza virus, it can act as a mixing vessel for the two viruses, and this can result in a reassorted humanized influenza virus with new avian genes (Fig 4). This virus can infect humans that have no pre-existing immunity (antibodies) against the new virus and this can then result in a pandemic.

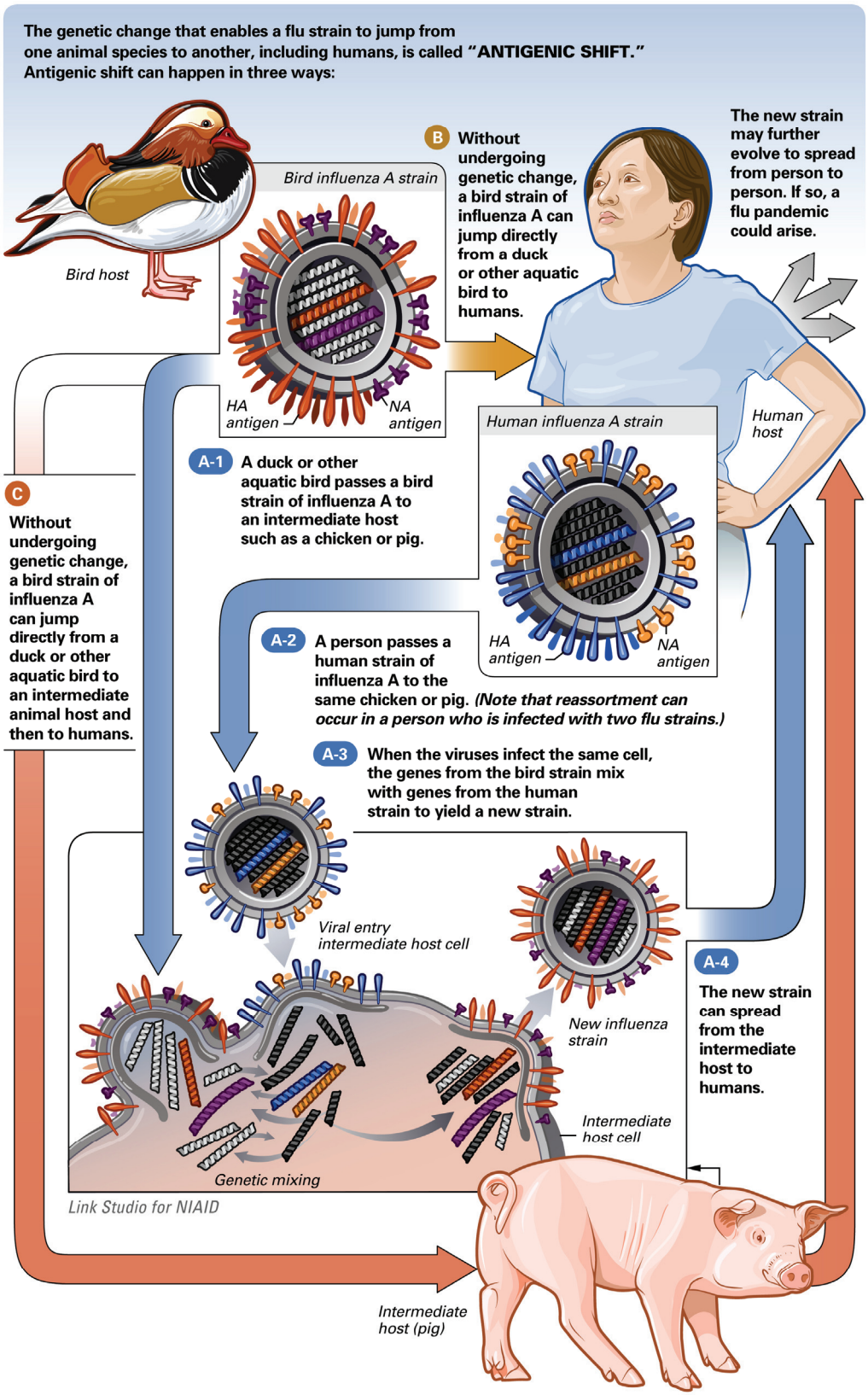

Figure 4. Antigenic shift. Illustration courtesy of "National Institute of Allergy and Infectious Diseases" (94)

1.4 Upper Airway Mucosal Immunology

The defence against pathogens can be divided into two different systems, the innate (non-specific) and the adaptive (specific) immune system. It can also be viewed as lines of defence, where the first line of defence is the "peaceful" existence of our microbes, the so-called normal flora. Our body is inhabited by microbes in a number which is a tenfold higher than the number of human cells (79). The normal flora prevents new colonisation by potentially pathogen microbes. The second line of defence is the mechanical barrier of the epithelium, together with the mucus, the olfactory system and mucociliary function. The third line of defence represents the non-specific cell response from epithelial cells, phagocytic cells (APC's) like macrophages and dendritic cells, and natural killer cells. The forth and last line of defence, is the specific response to antigens by B and T lymphocytes and their collaborating cells, cytokines and other signalling factors and pathways.

Much of the immunological knowledge related to influenza is obtained from research in small animals. The mouse model is very popular, but influenza virus infections does not naturally infect mice (29). The anatomical distribution of the local lymphoid structures are also different in mice and men (88). Therefore, caution should be exercised in the translation of results from animal studies into human use (45;71).

1.4.1 The nose and nasal mucosa

The nose is the entrance to the respiratory tract, and is therefore vulnerable to airborne infectious agents. Influenza virus can spread by droplets and aerosols in the air produced by infected hosts as they sneeze and cough. The nasal mucosa is normally the site where respiratory viruses meet the immune system of the host and is a potential site of infection.

In human nose breathers about 20,000 litres of air passes through the approximately 10cm (anterior-posterior-direction) of the nasal mucosa (or 150cm^2) every day. The air is filtered for all particles larger than 2-5μm, heated up to 32 degrees Celsius and

moisturised to approximately 98% humidity. The nasal mucosa has the ability to rapidly respond to different stimuli by increasing the blood flow, mucosal thickness and secretory capability.

The respiratory epithelium is ciliated with tight junctions, and this represents a mechanical barrier to inhaled agents. There is also a high turnover of epithelial cells in the mucosa. The mucus covering the epithelium contains a range of different substances, and both specific (SIgA) and non-specific anti-microbial agents protect the epithelium from infection. If the influenza virus reaches the cell surface it binds to sialic acid and can then infect the epithelial cell and replicate (30).

The role of antibody secreting cells (ASC) in long-term immunity of the mucosa, their life span and migration is not clearly understood, although it is more than 60 years since they were first discovered (47). In mice infected intranasally with influenza virus, antigen specific ASC's are up-regulated in the nasopharynx-associated lymphoid tissue that line the nasal passage – the so called diffuse NALT (D-NALT), and has a lifelong effect (56). These murine ASC's mainly produce IgA. Mice immunised with respiratory syncytial virus (RSV) intranasally maintains a RSV-specific plasma cell population in the NALT, which induces protective immunity against subsequent RSV exposure. This nasal immunisation is even "better than nature" because infection with RSV only gives short-lived up-regulation of RSV-specific ASC in D-NALT and does not protect from later infection (36;82).

1.4.2 Mucosa associated lymphoid tissue/Tonsils/Waldeyer's ring

Mucosa associated lymphoid tissue (MALT) is a secondary lymphoid organ that consists of aggregates of lymphoid cells organised beneath the epithelium of the airway and digestive tracts. The organised lymphoid structures in the upper aerodigestive tract (Fig.5) are anatomically located in and around the pharynx. They contain the palatine (Fig.6), the nasopharyngeal, the lingual and the tubal tonsils that together with minor lymphoid aggregates found spread in the lateral and posterior pharyngeal wall and called the Waldeyer's ring (after the German anatomist Wilhelm

von Waldeyer-Hartz (1836-1921)). These secondary lymphoid structures are built up like a lymph node with B- and T-cells organised into follicles. Tonsils are important structures in the mucosal immune system as stations for immunomodulation and homing of lymphoid cells, and we have counterparts in the respiratory and gastrointestinal tracts (Example: Peyer's patches in the small intestine and the appendix) (9;71).

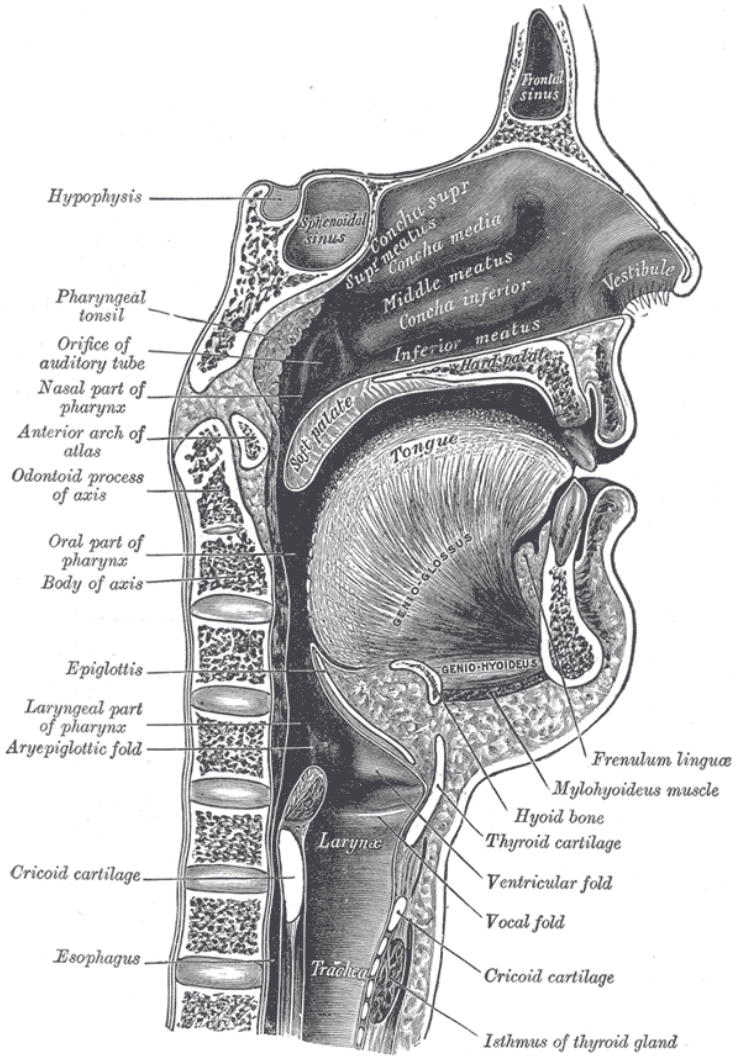

Figure 5. Lateral view of the upper respiratory tract. From Gray's anatomy.

Removing the "tonsils" is a relatively common surgical procedure. The indications for these operations are typically hypertrophy of the tonsils causing respiratory problems such as obstructive sleep apnoea (OSA) and serous otitis media ("Glue

ear") or recurrent infection e.g. chronic or recurrent tonsillitis (69;98). Surgical removal of the palatine tonsils (tonsillectomy) and the nasopharyngeal tonsil (adenoidectomy) are probably the most common operations performed on children and young adults, but is still somewhat controversial (21). There are studies showing effectiveness of tonsillectomy in children with major recurrent tonsillitis problems (69). In children with fewer upper respiratory tract infections than the indications described by Paradise et al. (69) there is no significant effect of adenotonsillectomy (57;98). In adults there are only limited data on the long term effect of tonsillectomy, and according to the latest Cochrane Library Review from 2000 (21) there is no evidence for the effectiveness. Some recent studies show a significant effect also in the adult population (3;50). Adenoidectomies and tonsillectomies are very common operations in Norway and world-wide, and the satisfaction rate of the patients and their parents is high (50;62;86;104). The standard indications for tonsillectomy in Norway are described in Guidelines for ENT-diseases: Norwegian Society of Otorhinolaryngology Head & Neck Surgery, 1998 (60).

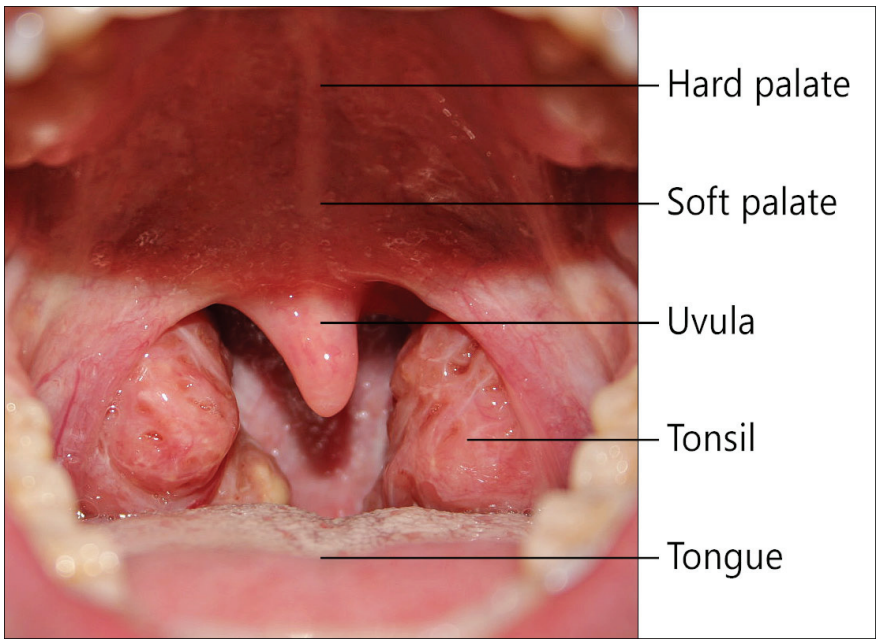

Figure 6. Photo of the fauces with the pared palatine tonsils. From Wikipedia.

The question of how adenoidectomy and tonsillectomy affects the immune system and particularly the upper airway mucosa immunology is widely debated. Since these

operations are some of the most common operations in the world, it may seem that removal of a part of the MALT in the Waldeyer's Ring does not seem to result in a major immunological disadvantage. This may be due to other parts of the local MALT replacing the function of the removed tissue. The tonsils are important sites of B-cell proliferation and differentiation, and act as both inductor and effector sites (9). Ogra observed in 1971 that the level of IgA antibodies in the nasopharyngeal fluid was reduced after adenotonsillectomy (66), and Östergaard found in 1977 low levels of IgA after tonsillectomy in both serum and saliva two years after the operation (68). Others have shown that despite changes in the immune system, there is no increase in immunological or infectious diseases (11;53;78). However in recent years a conservative attitude towards adenotonsillectomy is recommended and practised (12). Since the tonsils are the only easy accessible human lymphoid organs (65), and adenoidectomy and or tonsillectomy is a common procedure, there are numerous studies on human hypertrophied and recurrent infected tonsils and little on normal tonsils (Reviewed in references (12;80).

1.5 The immune response to influenza

1.5.1 The immune response to influenza infection

The fact that our body is constantly challenged by a vast number of microorganisms, and we seldom get infected, demonstrates the power and potency of the innate immune system. The innate immune system is fast acting, detecting and destroying influenza viruses immediately or within a short period of time (91). One component of the innate system is the mechanical barrier of the respiratory epithelium, which has a high turnover of cells and is ciliated. The cilia are the mucosal motor that moves the mucus blanket that can harbour microbes to the nasopharynx for expectoration or swallowing. The mucus itself contains a variety of potent antimicrobial factors like lysozyme, lactoferrin, peroxidases (76), secretory antibodies like SIgA (also IgG and IgM), that can neutralise the influenza virus, and other inhibitory factors that can

reduce the viruses ability to infect the epithelium. The cellular part of the innate immune system are the phagocytes, like macrophages, dendritic cells and furthermore neutrophils, eosinophils and mast cells. They can eliminate influenza virus and produce cytokines that inhibit the virus replication, cause fever and recruit natural killer (NK)-cells that limit viral spread by killing infected cells. The complement system may also play a role in the innate immune response to influenza virus infections (91).

If the virus escapes the early innate defence mechanisms, it will be recognised by the adaptive immune system. The phagocytic activities of macrophages and dendritic cells are essential in removing foreign antigen/microbes and for presenting antigen in the induction of the antigen specific response of the T- and B-lymphocytes (Fig.7). The full effect of the effector function of the adaptive immune response is revealed after some days (4-7days (30)), and starts with the inflammatory response that allows the antigen presenting cells better access to viral antigen. The APCs (mainly dendritic cells) then migrate to lymphoid tissue (e.g. MALT) where they present surface bound antigens to T-cells, and start an antigen specific clonal expansion of effector lymphocytes, which migrate back to the site of infection. This adaptive immune response can be divided into a cell mediated response conducted by the T-cells, mainly CD4+ and CD8+ T-cells, and a humoral response involving B-cells and antibody production. A highly complex signalling of different cytokines regulates and co-ordinates this immune response. In influenza virus infection there are a number of different cytokines that act pro-inflammatory (IL-1β, IL6, IL18, IFN-α/β, TNF-α) and antivirally (IFN-α/β), and are also responsible for many of the symptoms that occur during infection (83). During experimental influenza infection in humans there was an early peak in nasal lavage fluid (mucus) of IL6 and IFN-α, which correlated directly to symptoms and viral load (46).

The B-cells that migrate to the lamina propria of the mucosa at the site of infection produce mainly the dimeric IgA, which is actively transported through the epithelial cells. Since IgA does not activate the complement system and therefore is considered

to be non-inflammatory, it can bind to and neutralize virus both in the mucus, inside the epithelial cells and in the lamina propria without causing tissue damage (13). The SIgA level in the mucus correlates inversely with the virus titre (91). There is also a lower concentration of both IgM and IgG in the mucosa. IgM can also be transported through the epithelium to the mucosal surface if it contains the j-chain (bound to secretory component), but IgG can only leak (transudate) through the mucosa or via minute injuries in the epithelium. IgG is the dominant antibody in serum, and seems to have an important role in the defence against influenza viruses in the lower airways (lungs) (13).

The activation of CD8+ T-cells into cytotoxic T lymphocytes (CTLs), mediate killing of infected cells that presents foreign viral antigens on MHC class I (109).

Recovery from influenza infection is a 2-stage process. On days 5-7 T-cell dependent killing of infected cells is highly active, and there is subsequent elimination of the virus by local/mucosal antibodies (SIgA) (91).

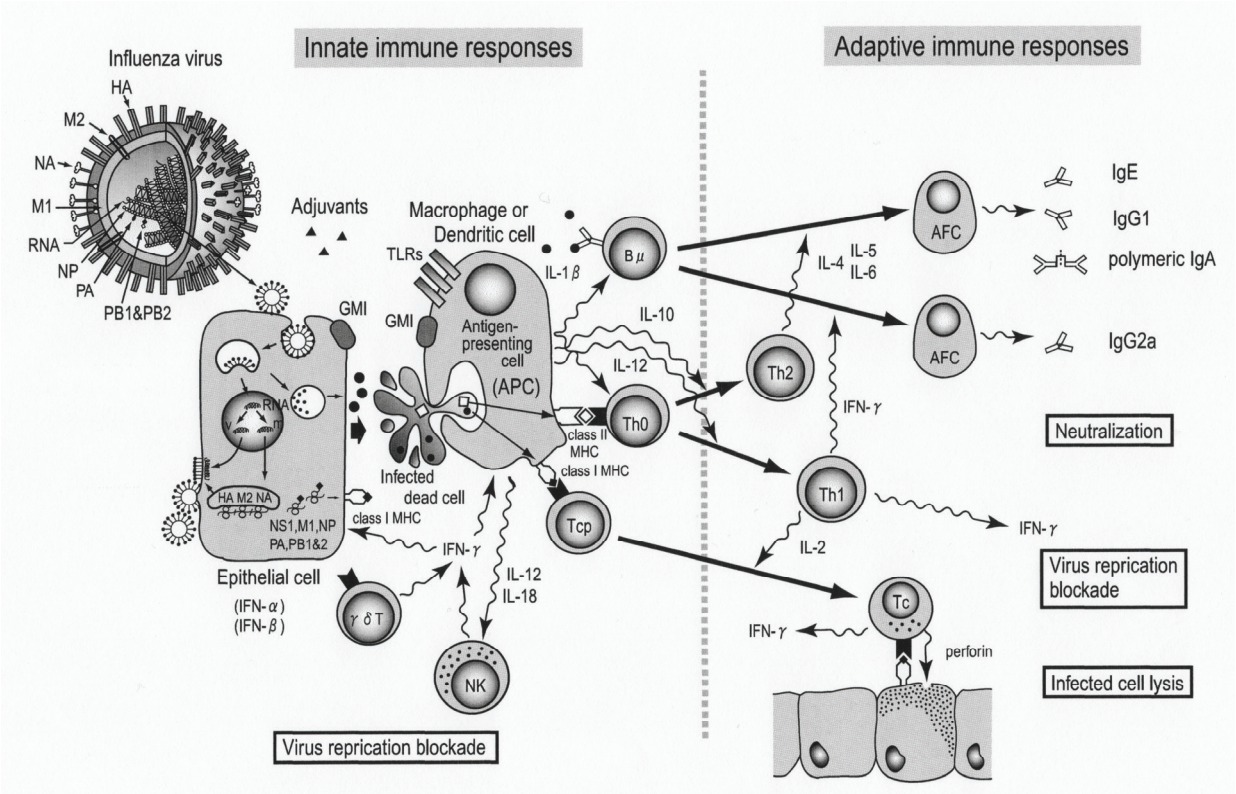

Figure 7. Overview of the defence mechanisms induced by influenza virus infection. From (91). Reproduced with permission. (Illustration courtesy to Japanese Journal of Infectious Diseases and Shin-ichi Tamura, PhD, Tokyo, JAPAN.)

1.5.2 The immune response to influenza vaccination

The efficacy of influenza vaccination varies by multiple parameters, e.g. vaccine type, vaccination route, immunologic status, age and match of vaccine to the circulating strains (40). Most adults have experienced several influenza infections during life and the immune system have memory to the subtypes of the virus circulating. Therefore one dose of the commonly used inactivated influenza vaccine will normally provide in protective levels of antibodies 60-90% of cases (Reviewed in (25). Young children, which may be immunologically naïve to influenza, may need more than one dose to get properly immunized. The different vaccines on the market have different immunogenic properties, and can also vary from year to year.

The vaccines with killed virus, given as an injection subcutaneously or intramuscularly, will in primed individuals provide a rapid systemic humoral immune response in the blood, and a weaker humoral local response in tonsils and oral fluid (Fig. 8). In serum the antibody response is dominated by influenza specific IgG-antibodies, and lower amounts of IgM and IgA antibodies towards the surface antigens HA and NA (32). Accordingly, the ASCs found in blood are also mainly IgG positive, with minor IgA and IgM positive cells (108). In saliva the main antibody response is of SIgA1 type (18). In influenza naïve children IgM dominates the systemic immune response, and there is very low concentration of SIgA induced in saliva (32).

LAIV vaccines given nasally induces a stronger local immune response, but a weaker systemic response (25). However, LAIV vaccines have the advantage that they also stimulate a cellular immune response, inducing influenza specific CD8+ T memory cells. A newly published study in mice showed that the combination of one intranasal followed by one intramuscular immunization gave the best immune response, compared to 2 intramuscular, 2 intranasal or one intramuscular followed by 1 intranasal vaccine (97).

The proposal of an universal influenza vaccine targeting more stabile epitopes like the M2 proton channel may have several advantages (28). This could allow influenza vaccination to be more like the vaccination programs for other pathogens (e.g. rubella, polio etc), with a few vaccinations in a lifetime. A recent interesting study in mice shows that pulmonary vaccination is a new potential route of influenza vaccination, and seems superior over the intranasal, intramuscular and oral route in that it elicits a strong immune response both locally and systemically (58).

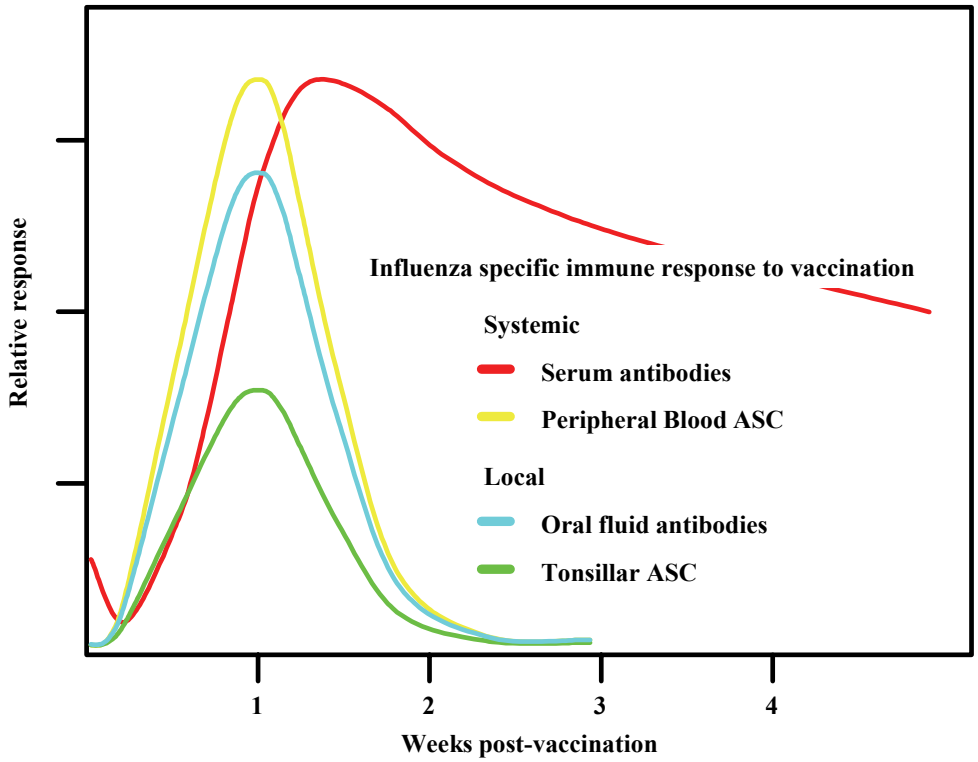

Figure 8. The figure shows an outline of some of the main immunological events (and kinetics) after an influenza vaccination, both systemically and locally (17;26). Most adult subjects have detectable serum antibodies (AB) prior to immunization. This is seen in many of our studies, e.g. [Paper I, II]. Immediately after vaccination the serum levels drops, which may be due to quick complexing of anti-influenza AB with influenza antigen. The serum AB rises quickly again and are significantly higher at 4-5days post vaccination (PV). The peak in serum AB levels occurs after 8-9 days PV, and then it falls slowly, but is still relatively high after 3-4 weeks PV. The number of influenza specific antibody secreting cells (ASC) in blood and tonsils is very low before vaccination. These cells proliferate quickly and are significantly higher in number at 4-5 days PV, with a peak after 7 days, and a rapid drop to basal levels after 2 weeks. The proportion of influenza specific ASC is much higher in blood than in tonsils, but may not represent a higher total number. The salivary AB against influenza follows the tonsillar ASC, but since we only detects AB produced and secreted in a limited time period, the salivary AB levels drops together with the local influenza specific ASC levels.

1.6 Influenza the disease

There are a large number of viruses which cause respiratory tract disease in humans. The symptoms vary from very mild clinical and sub-clinical, to the more severe disease caused by the influenza A virus.

The influenza season in Norway usually starts in November-December and ends in February-March, although there is a great annual variation in this pattern (63).

For most people, a true influenza virus infection is a self-limiting disease that has the typical pattern of fever, headache, myalgia and cough. The clinical picture can be quite different for individuals with chronic diseases or immune dysfunction.

1.6.1 Pathogenesis

For many viruses asymptomatic or sub-clinical infection is most common, but for influenza virus infection most infected individuals also become ill (96). In general the pathogenesis of influenza depends on many interacting factors of the host and of the viruses. The most important host factors in humans that determine the pathogenicity and severity of the clinical picture are age and co-morbidities, e.g. immunocompromised individuals, elderly people (especially those living in close contact in nursing homes), young children with a naïve immune system and people with serious chronic diseases (e.g. heart, lung and metabolic diseases). The different subtypes of influenza A virus are also associated with different levels of morbidity and mortality, e.g. the H3N2 subtype is associated with higher mortality than the H1N1 (101).

The transmission of influenza from person to person is primarily by aerosols and droplets, but direct and indirect contact is also a possible route of transmission. Data suggest that the smaller droplets (1-4μm in diameter), mainly produced by sneezing, enter deeper in the airways and require less virus particles to be infective than larger droplets that are deposited in the nasal cavity (5;14).

The incubation period varies from 1-4 days and infected adults normally shed virus for 3-5 days. Children and immunocompromised persons can shed virus for a much longer period (14).

In humans, influenza virus infection mainly targets the epithelial cells of the upper respiratory tract, where it starts replication and causing tissue destruction. The disruption of the epithelial cell barrier and the additional local inflammatory response leads to the common symptoms of influenza: coughing, sneezing, sore throat, runny and blocked nose. The systemic symptoms of influenza are associated with cytokines produced in the inflammatory process. Many of these cytokines, such as IL-1, IL-6 and TNF-α, are endogenous pyrogens. When they reach the hypothalamus via the bloodstream, they stimulate prostaglandin E2 production, inducing symptoms like fever, sleepiness, anorexia, myalgia and headache (19). The infection itself normally does not spread to other tissue or organs outside the respiratory mucosa in humans.

1.6.2 Clinical manifestations

Influenza infection can be either asymptomatic or symptomatic. Many people show increased antibody-titres for specific influenza viruses without knowing that they have been infected. According to the WHO, the annual influenza epidemics affect approximately 5-15% of the population, which results in a significant increase in morbidity and mortality and an associated socio-economic burden (106). In the United States, the influenza epidemics typically occur during the winter months and have been associated with an average of approximately 36,000 deaths per year during 1990—1999 (95), mainly in the elderly population. A similar trend is also observed in Norway. The typical influenza illness is, in otherwise healthy individuals, a febrile infection lasting for approximately a week with typical symptoms of fever, cough, nasal congestion, headache and myalgia (61). Uncomplicated influenza is a self-limiting disease that may need to be treated with bed rest for some days, absence from work and symptomatic treatment with analgesics and astringent nose drops. Small children which are immunological naïve to the influenza virus, patients with

chronic lung, heart or immune disease and elderly people are considered the risk groups for complicated influenza infection. In these groups the infection may be more serious and widespread and the mortality rate increase during an influenza epidemic (42).

Influenza associated pneumonia, and secondary bacterial pneumonia are among the most common complications. Pneumonia with Staphylococcus aureus is linked to serious disease and high mortality (101). Other respiratory complications like acute sub-glottic laryngitis (pseudo-croup), otitis media and bronchitis are common in children and exacerbation of known respiratory diseases like asthma, cystic fibrosis and chronic obstructive pulmonary disease are also common. Other major complications are seldom present, but myo- and pericarditis, heart arrhythmias, encephalitis, Guillain-Barré-syndrom, myositis and rhabdomyolysis and Reye's syndrome have been described (63).

1.6.3 Diagnosis of influenza

The diagnosis of influenza is normally based on patients' history and clinical presentation and findings. Normally doctors do not take an aspirate from the upper airway ("virus sample") in order to diagnose influenza virus infections, if it is known that influenza is circulating in the community. Studies have shown that during an influenza epidemic the clinical diagnosis alone was correct in about 3 out of 4 patients (111). The symptoms that were the best predictors for influenza infection were acute onset of cough and fever, with a positive predictive value of 79% (61). A recent study shows that depending on the setting the relationship between the clinical diagnosis and the laboratory verification of the virus can be very low (49). There are rapid tests for detection of influenza antigen available, but their sensitivity is generally low. However, these tests can be useful in some settings (75). The single most important factor in making the correct diagnosis is to know whether influenza virus is circulating in the community or not (111).

In Norway and other countries there are multiple general practitioners that act as watchtowers in the surveillance of influenza. At present there are 201 offices and emergency units in Norway that report all cases of influenza-like illness each week during the autumn, winter and spring season in the typical influenza period in the northern hemisphere. These data are then made commonly available through weekly published reports (MSIS). This surveillance system contributes to the knowledge of the influenza activity and severity.

In addition, 70 general practitioners submit nasopharyngeal samples from patients for influenza testing to The Norwegian Institute of Public Health (Folkehelseinstituttet). Together with reports from the microbiological laboratories in Norway this gives important information on the viruses circulating in the community. This surveillance is done in collaboration with the WHO and the European Influenza Surveillance Scheme (EISS).

1.6.4 Social and economic impact of influenza

The economic considerations of diseases and their treatment are becoming more important in the Norwegian Health Care system, similarly to the situation in the USA. The cost-efficacy or cost-benefit of treatment and cost of illness are important parameters for the health-care providers in their decision of where to spend the money. As in all fields of medicine there are difficult moral and ethical questions raised, and it is not unproblematic to put a price tag on life, suffering and death.

An estimate from the WHO is that there are annually 3-5 million cases of severe influenza illness, and 250,000-500,000 die from it in the industrialised world. This of course varies from year to year, but data from USA over the last decade reveals an average annual mortality rate of 120/million inhabitants and hospitalisation rates of 670/million. The majority of patients hospitalised or dying from influenza are elderly (102).

The cost of the annual influenza epidemics is difficult to calculate, and depends upon multiple parameters. A new study from Centers for Disease Control and Prevention has systematically estimated the costs of annual influenza in the USA to $87.1 billion, where medical costs alone counted for $10.4 billion (59). The majority of the costs arise from work absenteeism and lives lost.

Several studies have shown that vaccination is not only cost-effective but even cost-saving, also in the non-risk groups (1;89).

1.7 Prophylaxis and Treatment

The cornerstone of influenza prophylaxis is vaccination, especially for risk groups. The WHO has set the criteria for the risk groups which should be implemented in the influenza vaccination program.

Treatment of an influenza infection depends on the severity of infection and of the related complications. Generally influenza is a self-limiting disease that with or without the help from the doctors is symptomatically treated with bed-rest, analgesics and astringent nose-spray/drops. Other general recommendations during the influenza season are good hand hygiene, and avoid coughing and sneezing on others. Those who are ill should stay at home and rest.

Complications of influenza infection are relatively common, especially in children and elderly patients and patients with chronic diseases.

The use and development of anti-influenza drugs are still in its infancy. Although some of them are effective short term, they do not encompass the cost-effectiveness of influenza vaccination (2;7;63).

1.7.1 Vaccination

Influenza vaccination is today the primary method for preventing influenza and severe complications related to the disease. Numerous reports and studies have shown the effect of vaccination on morbidity and mortality (52;74;85)

Beneficial cost-efficacy relationships have been shown for influenza vaccination. Recently (77), the need of influenza vaccination in the at risk-group of patients in the 25 countries in the European Union (not including associated members such as Norway) was estimated. The authors estimated that 49.1% of the total population (223.4 million people) should be vaccinated annually. They postulated that if 100% of persons in the at risk-groups received influenza vaccine, this would reduce the number of influenza cases by 7.22 million, 1.92 million reduced primary care visits, 796.000 hospital admissions and 68.500 fewer influenza related deaths. Not only would the morbidity and mortality be reduced, but the costs of this vaccination-program would be saved by reduced primary care visits and hospitalisations (77).

Influenza vaccine development started shortly after the influenza virus was discovered. Inactivated influenza vaccines were introduced in the 1940s, and this is still the most common formulation of influenza vaccine. The virus strains of the vaccines are updated twice a year by the WHO's Global Influenza Surveillance Network, for the northern and southern hemisphere. The vaccines are made by propagating the influenza viruses in embryonated hen's eggs. The annual influenza vaccines contain H1N1, H3N2 and B virus antigens.

There are several types of influenza vaccines, and the main variants are the live attenuated and the killed virus vaccines. The killed virus vaccines may be whole viruses (whole-virus vaccine), disrupted viruses (split-virus vaccine) or the purified surface antigens (sub-unit vaccines). The killed virus vaccines are mainly given as an injection subcutaneously or intramuscularly. Live attenuated virus vaccines are administered as a nasal spray and this vaccine is for the time being not available in Europe. In Norway, only split and sub-unit vaccines for influenza immunization are

available. Live attenuated influenza vaccines (LAIV) have for many years been used by the Russians and in the latter years also in the USA ("Flumist®").

The WHO has set a goal of 75% vaccine coverage in the influenza at risk group within 2010. Norway has one of the lowest vaccination rates of the at risk groups, with approximately 45% of the individuals vaccinated (7). In a large study of the macroepidemiology of influenza vaccination in 56 countries, Norway was in 30th place, among countries like Slovenia and Uruguay, and far behind the leading countries Canada, Korea and USA (92). The reason for this low vaccine coverage in the at risk groups in Norway may be multifactorial, but the main reason is probably that these people do not see the need for vaccination and that influenza is not seen as a serious illness (7). Lack of information and recommendation from doctors may also be a reason for this. In this context the vaccination rate among health-care workers is very low, despite the recommendations from WHO and studies showing significant reduction in mortality among patients when the staff are vaccinated (Reviewed in reference (20). The attitude among health-care workers towards influenza-vaccination can also be a reason for the general low influenza vaccine coverage in Norway (7).

Side reactions after influenza vaccination are commonly minor, e.g. tenderness, oedema and erythema at the injection site. Some people experience a minor influenza like reaction, but serious side effects of vaccination are extremely rare. Among 3.5 million influenza immunizations in Norway, 4 people had to be hospitalised. One person died, without a clear relationship to the components of the vaccine (7).

Children are more prone to influenza infection, while serious illness and deaths are higher among persons aged $\geq$65 years, children aged <2 years, and individuals of any age who have medical conditions that place them at increased risk for complications from influenza.

In Norway the following groups are considered at high risk (about 900,000 persons) and are recommended for annual influenza vaccination (The Norwegian Institute of Public Health):

1. Persons that are 65 years or older
2. Adults and children with serious airway conditions, especially persons with decreased lung capacity
3. Adults and children with chronic heart and blood vessel diseases, especially persons with serious heart failure, low minute volume or pulmonary hypertension
4. Adults and children with decreased resistance against infections
5. Adults and children with diabetes mellitus (both type 1 and type 2)
6. Adults and children with chronic renal failure
7. Persons living in nursing homes for the elderly

The latest recommendations of the Advisory Committee on Immunization Practices (ACIP), CDC, USA from 2007 now promotes a more active vaccination program including more conditions in the at risk group (38). The most important differences are that the CDC recommendations include young children down to 6 months of age, persons over 50, pregnant women and health care workers.

1.7.2 Antiviral drugs

Antiviral drugs against influenza have been on the market for some time, but their use is limited, and they are not a substitute for influenza vaccination. There are two main indications for the use of antiviral drugs against influenza; in prophylaxis and treatment of influenza. When treating influenza with antiviral drugs, the medication should be started within 48 hours of the onset of symptoms, and only if influenza virus is verified (by rapid tests) or undoubtedly the reason for the symptoms. The duration of the influenza can be shortened by 1-4 days of treatment. Prophylactic use could be indicated to prevent influenza in a community or closed facility like a

nursing home, or if an at risk person is allergic to eggs, and therefore can not receive the vaccine.

There are two principle types of anti-influenza drugs available. The M2-channel inhibitors, amantadine and rimantadine, are only effective against influenza A. The use of the M2-channel-blockers is not approved in Norway, and their use is associated with substantial side-effects. The other group of antiviral drugs against influenza is the neuraminidase inhibitors, zanamivir (Relenza®) and oseltamivir (Tamiflu®). They are effective against both influenza A and B viruses, and are both registered for use in Norway (2).

According to the guidelines from the UK National Institute for Clinical Excellence in 2003 (64), the only people that should be treated with antiviral drugs, are patients in the at risk groups, and they do not recommend the use of amantadine (and rimantadine, which is not available in the UK).

2. The Study

2.1 Aims of the study

The main objective of this study was to examine the local and the systemic immune response after parenterally administered inactivated influenza vaccine in adults. Our focus was on the immune response in the palatine tonsils, nasal mucosa, saliva and peripheral blood/serum. This work is a follow up of studies performed by the influenza research group in Bergen (15;31).

The specific aims of the four publications were:

1: To determine the basal level of influenza-specific antibody secreting cells (ASC) in the blood (systemic compartment), tonsils (local lymphoid organ) and nasal tissue (local mucosa).

2: To evaluate the effect of parenteral influenza vaccination on the number of influenza virus specific ASCs locally in the tonsils and nasal mucosa.

3: To investigate the effect of influenza vaccination on the distribution of lymphoid cells in the palatine tonsils. Since parenteral vaccination induces humoral immune responses in the tonsils, we wanted to examine if vaccination has an impact on other immune competent cells in the tonsils.

4: To study the levels of cytokines and chemokines produced locally and systemically after influenza vaccination of patients undergoing tonsillectomy.

2.2 Materials and Methods

2.2.1 Patients

This study is based on 4 clinical trials, summarised in table 3. All our patients were recruited from the dept. of ORL-HNS at Haukeland University Hospital. The patients were referred to our department by an ENT-doctor who had judged them to have indications for a tonsillectomy because of recurrent tonsillitis and/or hypertrophic tonsils. From this cohort we included suitable patients who conferred to our inclusion criteria. Our patients were with exception of their tonsillar problems healthy, not on regular medication (except hormonal contraception) and did not have allergic disease. We have obtained all appropriate approvals for our studies and the regional ethical committee approved our studies. The patients were sent written information and asked to take part in our study, and they were examined 1-2 weeks before the operation. Those who signed the informed consent form were then included in the study. Saliva and blood samples were collected from all patients at the time of inclusion. The patients in trials 2-4 were then sorted into a control and a vaccinated group. The patients included in the vaccination group(s) were subcutaneously vaccinated with a standard dose of the seasonal influenza split virus vaccine according to the manufacturers' instruction. No side reactions to the vaccination were reported or observed, apart from a few cases of minor local inflammation at the injection point.

In the first and second trials we collected a unilateral biopsy from the mid part of the inferior turbinate in the nose. This was done under general anaesthesia before tonsillectomy, with local anaesthesia (Tetracaine with Adrenaline) at the site of the biopsy.

Trial	1	2	3	4
Time of trial	Spring 2000	Autumn 2000	Autumn 2002	Autumn 2004
No. of subjects	19	23	33	25
Sex M/F	9/10	11/12	9/24	12/13
Age-range (years)	17-40	16-38	16-56	18-59
Mean Age (years)	28	26	25	27
No. of controls			9	5
Vaccine strains	None			
H1N1		A/New Caledonia/20/99	A/New Caledonia/20/99	A/New Caledonia/20/99
H3N2		A/Panama/2007/99	A/Panama/2007/99	A/Wyoming/3/2003
B		B/Yamanashi/166/98	B/Shangong/7/79	B/Jiangsu/10/2003
Vaccine Manufacturer		Fluarix® GlaxoSmithKline®	Fluarix® GlaxoSmithKline®	Fluarix® GlaxoSmithKline®
Samples	Blood/Serum Oral fluid Tonsils Nasal mucosa	Blood/Serum Oral fluid Tonsils Nasal mucosa	Blood/Serum Oral fluid Tonsils	Blood/Serum Oral fluid Tonsils
Papers	I, II, III	II, III	IV	IV

Table 3. Summary of patient groups studied.

2.2.2 Laboratory tests

We collected blood, saliva, tonsillar and nasal mucosal tissue from patients. In these studies we have used the following immunological methods to examine the immune response after vaccination:

Enzyme-Linked ImmunoSorbent Assay (ELISA):

We used the ELISA technique to analyse the level of influenza specific antibodies in the serum and saliva, before and after influenza vaccination.

Enzyme-Linked ImmunoSPOT Assay (ELISPOT)

The ELISPOT technique was used to enumerate the influenza specific antibody secreting cells (ASC) in lymphocytes from blood, nasal mucosal tissue and from tonsillar tissue. The results from peripheral blood lymphocytes were compared before and after vaccination. Since pre-vaccination samples from tonsils and nasal mucosa were not available, the levels of influenza specific ASC from post-vaccination samples were compared with data from non-vaccinated volunteers (control group).

Haemagglutination Inhibition Assay (HAI)

This is the "gold standard" method for measuring influenza specific antibodies in serum and is commonly used as a surrogate of protection. A serum HAI titre of 40 has been deemed to be protective against influenza virus infection (48).

Immunohistological staining of cell surface markers

Tonsils were cryopreserved and sectioned before immunohistological staining against 14 different cell surface markers: CD4, CD8, CD19, CD20, CD45RA, CD45RO, Mac387, CD68, CD11b, CD11c, HLA-DR, E-cadherin (CD144), IL-3rα (CDw123), CD1a.

Fluorescent labelling of influenza specific antibody secreting cells

Cryosectioned tonsillar tissue was stained with fluorescent tagged influenza antigen and counter stained. The sections were analysed using a fluorescence microscope and the influenza specific ASC counted (ASC/mm²). To our knowledge this is the first report of identifying single influenza specific ASC's in tissue.

Multiplex Bead Immuno Assay

We have analysed the concentration of 25 cytokines and chemokines directly in the serum and saliva of both vaccinated and non-vaccinated patients using the multiplex bead immuno assay. A 10-plex cytokine assay was used to analyse cytokines in supernatants from lymphocyte stimulated in *in vitro* cultures. We used a Luminex 100 instrument (Luminex Corporation) with STarStation software (Applied Cytometry Systems) to read and analyse the data.

Quantitative PCR (QPCR)

Cytokine gene expressions (10 of the most common cytokines) were measured on a 7500 Real Time PCR system (Applied Biosystems) in lymphocytes from whole blood samples that were collected in PAXgene tubes (PreAnalytix GmbH) which freezes and preserves the mRNA expression levels.

2.3 Summary of Results

2.3.1 Paper I

High prevalence of influenza specific antibody secreting cells in nasal mucosa.

This study was conducted to examine the basal level of influenza-specific antibody-secreting cells (ASCs) in the local mucosa of the upper respiratory tract. Nineteen patients scheduled for tonsillectomy were enrolled in this study, and none of whom reported an influenza-like illness the previous winter. Tonsils, blood, saliva and a nasal biopsy were sampled from all patients. The Haemagglutination Inhibition Titre (HAI) showed that nine patients had HAI-titre above the level of detection (>10) for the H3N2 virus, and two had protective levels (≥40). For the two other viruses H1N1 and B, none of the subjects had detectable HAI-titres. We also measured the concentration of total influenza specific antibodies in the serum and saliva by ELISA; which gave results comparable with the pre-vaccination concentrations observed in previous work. The level of influenza specific antibodies in oral fluid was low but detectable, and represents the production in a short time frame, whereas the serum levels represents an accumulated production over several days.

Lymphocytes were isolated from blood, nasal mucosa and tonsillar tissue, and analysed by the ELISPOT method to enumerate the number of influenza specific antibody secreting cells (ASC) per million lymphocytes. In the biopsy from the nasal mucosa taken from the mid portion of the inferior turbinate (concha inferior) we found 10-100 times higher frequency of influenza specific ASC than in tonsils and blood. This reflects the basal influenza specific ASC frequency in the nasal mucosa, which represent an important first line of defence against influenza infection.

2.3.2 Paper II

Parenteral vaccination against influenza does not induce a local antigen-specific immune response in the nasal mucosa.

This study was a natural follow up of the first paper, were we wanted to examine the immune response in the nasal mucosa and tonsils after parenteral influenza vaccination in 23 patients scheduled for tonsillectomy. All patients were healthy except for their hypertrophic tonsils or recurrent tonsillitis. In line with earlier results, we found that the influenza immunization induced a significant increase in influenza virus–specific serum and oral fluid antibodies at the time of the operation 7 days after vaccination. Lymphocytes were isolated from blood, tonsillar and nasal tissue. The numbers of influenza virus–specific antibody-secreting cells (ASCs) were measured by ELISPOT. In peripheral blood the number of influenza specific ASC increased significantly 1 week after vaccination. The numbers of ASCs in the tonsils and nasal mucosa were compared to data from paper I with the non-vaccinated volunteers, and for medical and ethical reasons pre-operative biopsies were not taken from the patients' tonsils or nasal mucosa. We found that there was a significant increase in the number of influenza virus–specific ASCs in the tonsils in the vaccinated group. Surprisingly, in the nasal mucosa there was no difference in the number of ASCs between the vaccinated and the non-vaccinated patients.

These findings indicate that the parenteral influenza vaccination elicits a systemic response. Since the nasal mucosa is an important tissue for the protection against influenza (and other) virus infections our findings may indicate that parenteral vaccination does not give the optimal immune stimulation and protection in the nasal mucosa. The increase in anti-influenza antibodies found in the saliva probably originates from other sources than the nasal mucosa.

2.3.3 Paper III

Lymphocyte distribution in the tonsils prior to and after influenza vaccination.

In the human pharynx the respiratory and gastrointestinal tract are joined. In this area we have the lymphoepithelial structures called the Waldeyer's ring. There are 4 tonsillar structures, namely the nasopharyngeal (epipharyngeal tonsil or "adenoids"), the tubal, the palatine and the lingual tonsils, together with smaller collections of lymphoid tissue, they form a complete ring structure. The tonsils are rich in lymphocytes and probably play an important role as a reservoir of memory and immune competent cells for the respiratory tract. The tonsils may also function as an inductor and effector site for immune responses against respiratory pathogens and foreign antigens. In this study, we examined if parenteral influenza vaccination had an impact on immunological cells in the palatine tonsils. We used histological tissue sections from the cryopreserved palatine tonsils from vaccinated and non-vaccinated patients (Trials 1 and 2) and stained these sections immunohistologically for 14 cell surface markers. The positively stained cells were counted by microscopy. We observed a significant decrease in CD4+ cells in the tonsils of vaccinated subjects. There was also a significant decrease in both naïve ($CD45RA^{+}$) and memory ($CD45RO^{+}$) T-cells after parenteral vaccination. The reason for this decrease is not known, but $CD4^{+}$ T-cells, that are the major contributor to both CD45RA and CD45RO positive cells, may be recruited to the systemic compartment where they take part in the humoral immune response. The number of macrophages with the CD68 surface marker increased in numbers in vaccinated subjects, whereas the macrophages positive for mac387 did not change.

We also stained histological tissue sections with influenza antigens labelled with a fluorescent tag to identify influenza specific antibody secreting cells (ASC) in tissue. To our knowledge this is the first report identifying single influenza specific ASC's in human tissue. We found the influenza specific ASC spread throughout the tonsillar tissue, but mainly in the follicles, and the numbers of ASC significantly increased in the vaccinated patients. This finding is in line with our findings from paper II using a

different method, and their distribution in the tonsils probably shows that the influenza specific ASC's have migrated/homed to the tonsils rather than been activated there.

Our findings show that there are dynamic changes in the tonsils after parenteral influenza vaccination, and this indicates that the tonsils play an important role in immunity to respiratory pathogens.

2.3.4 Paper IV

The local and systemic cytokine and chemokine response after parenteral influenza vaccination.

Cytokines are important mediators of immune responses, but they may also play a role in the symptoms and pathology of diseases. Since there is limited data on the cytokine response in man after influenza vaccination, we wanted to investigate the levels of cytokines produced locally and systemically after parenteral influenza vaccination. Our patients were as in our earlier trials scheduled for tonsillectomy, but otherwise healthy. Blood and saliva were sampled from all patients 1-2 weeks prior to, and at the time of operation. We also had a control group of non vaccinated patients. As a reference to earlier studies and to demonstrate that the vaccine stimulates the immune system, we measured the antibody response in serum and saliva by ELISA and the serum by HAI assay. The cytokine and chemokine concentrations were determined in both unstimulated samples (whole blood, serum and saliva) and *in vitro* influenza stimulated peripheral blood mononuclear cells (PBMC) and tonsillar lymphocyte (TMC) cultures. Influenza vaccination induced an immune response with protective levels of serum haemagglutination inhibition antibodies and a significant local antibody response in the saliva, as had been previously observed. There were no significant differences observed in the cytokine or chemokine levels between the vaccinated subjects and the non-vaccinated controls in either the serum or saliva. With Quantitative PCR we measured the gene expression levels of 10 common cytokines in PBMC of the vaccinated and non-

vaccinated subjects. IL-5 and IL-10 were below the detection limit, and for the other cytokines there were no significant differences between the vaccinated and the non-vaccinated patients. In supernatants from *in vitro* stimulated lymphocytes from peripheral blood and tonsils we found a significant increase in the concentrations of inflammatory cytokines in the vaccinated subjects. In PBMC after vaccination there were significant increases in the concentration of 8 of the 10 cytokines measured and in tonsillar lymphocytes we found significant increases in 6 of the 10 cytokines. The cytokine response in the vaccinated subjects revealed a mixture of type 1 and type 2, pro- and anti- inflammatory cytokines.

Our data shows that it is difficult to detect changes in the cytokine profile one week after vaccination. In supernatants from *in vitro* stimulated lymphocytes in blood and tonsils there is an increase in different cytokines in the vaccinated subjects that reveals a heterogeneous cytokine profile. This data will provide useful baseline information for further research in the understanding of the immune response after influenza vaccination and also in trials of novel influenza vaccines.

2.4 General Discussion

The studies presented in this thesis were performed by collecting samples from patients undergoing tonsillectomy at the ENT department. The patients were both individuals vaccinated with the recommended trivalent influenza vaccine and non-vaccinated controls. In this setting we were able to collect local lymphoid tissue (tonsils) as well as nasal mucosal biopsies, blood and saliva samples to examine the immune response induced after the influenza vaccination.

The palatine tonsils are relatively large immunological tissues in the human body, extremely rich in immunocompetent cells. The main indications for performing tonsillectomy are recurrent or chronic inflammation of the tonsils and tonsillar hypertrophy. It can be discussed whether the tonsils which are removed are healthy or not, and therefore reflects the immunological process occurring in normal tonsils.

There are numerous immunological studies of tonsils, but almost all trials are conducted in patients with diseased tonsils and an indication for tonsillectomy. Studies have shown that there are microanatomical differences in normal and diseased tonsils (43) and also cellular differences in recurrent infected palatine tonsils versus idiopathically hypertrophied tonsils (67). Probably for ethical reasons, there are few human studies that have investigated differences in normal versus diseased tonsils regarding the specific immunological response to influenza disease or vaccination. In our studies we have compared the tissue samples from vaccinated and non vaccinated patients, and consequently changes observed are most likely due to the effect of vaccination.

The nasal mucosa samples were also collected from the same patient group, but none of these patients were suffering from nasal diseases, so we consider these nasal mucosa biopsies to be from healthy mucosa.

2.4.1 Systemic and local antibody response to influenza vaccine

In earlier studies the antibody response to inactivated influenza immunization has been examined in detail (15;31). It is generally accepted that the antibodies produced locally, especially SIgA, are of major importance in the resistance to influenza disease. The HAI test provides a surrogate correlate of protection and titres of ≥40 are protective against influenza (48). In the present studies we have used the locally and systemically produced antibody concentrations and the HAI-test to show vaccine efficacy. In all our studies included in this thesis the antibody levels were comparable to our earlier studies (17;32;34). The present studies have mainly measured the total influenza specific immunoglobulin (antibody) concentrations (IgX), without sub-typing the different Ig classes or subclasses. We have however not examined the efficacy of the vaccine to protect from influenza disease in our studies. Those kind of studies require a completely different experimental set-up, e.g. larger cohort, follow up and registration of other parameters like side effects, burden of influenza in the communities etc.

2.4.2 Antibody secreting cell (ASC) response

Antibodies are produced and secreted by many differential stages of B-lymphocytes (ASC), but the antibodies are particularly secreted by terminally differentiated plasma cells. In previous studies it has been demonstrated that there is an early influenza specific ASC response in the blood and in the tonsils after parenteral influenza vaccination (17;26). We have in our studies found data supporting these earlier observations. Animal studies of mice and pigs immunized or infected with influenza virus, have shown an increase of ASCs in the nasal mucosa as well as in NALT (55;90). Since local antibodies play a major role in resistance against influenza infection, we wanted to determine the effect of parenteral influenza vaccine on the number of ASC in the nasal mucosa. We collected blood, tonsils and nasal biopsies under general anaesthesia for tonsillectomy. Due to the design of our studies and for medical and ethical limitations, we were not able to compare pre- and post-vaccination responses in tonsils and nasal mucosa of an individual patient. In the first study (Paper I) we surprisingly found a 10-100 times higher number of influenza-specific ASC in the nasal mucosa biopsies than in blood and tonsils, without recent influenza exposure. These ASCs probably have an important role in protection against influenza infection. In the animal studies the pre-infection and pre-immunization numbers of the influenza-specific ASCs in the nasal mucosa were low and seemed to return to the basal level a short time after challenge (55;90). The human situation is probably reflecting previous exposure to influenza. In our next study (Paper II) we examined the level of ASCs in blood, tonsils and nasal biopsies 1 week after parenteral influenza vaccination. In line with earlier results, we found a strong increase in the ASC level in the blood and a statistically significant increase to 2 of the 3 vaccine strains in tonsils. In the nasal mucosa there was no change in the ASC level after parenterally immunization. This indicates that the parenteral vaccination mainly stimulates the systemic immune system with an increase in ASCs and antibodies in the blood, and only partly the local immune system with an increase in tonsillar ASCs and oral antibody concentrations, but not the nasal mucosa. The high numbers of influenza specific ASCs in the nasal mucosa probably remain stable

for a long period of time, but may decline over years without local stimulation, e.g. a new influenza infection.

2.4.3 Lymphoid cells in tonsils

We wanted to examine in more detail the lymphoid cells in the tonsils. We knew from our earlier studies that the number of influenza-specific ASC is increasing in tonsillar tissue one week after vaccination. To investigate if there are other changes in the cellular pattern (Paper III), we used tonsils from both vaccinated and non-vaccinated patients, and examined cryosectioned tonsillar tissue stained with monoclonal antibodies for 14 human cell surface antigens representing the major types of cells of the immune system. We did not expect there would be any significant difference between the vaccinated and the non-vaccinated. This method of enumerating positively stained cells in complex tissue samples should not be compared with a more sophisticated method like flow cytometry. We have been extra careful in interpreting the tissue staining results from this study. For most cell markers there were no significant changes between the two groups. One of the pivotal cells in the immune system is the CD4+ T-helper cell, which controls and regulates the immune response. We found a significant decrease in this CD4+ cell population in the tonsils of the vaccinated patients. CD4+ cells are also the main contributors to the CD45 RA (naïve T-cells) and CD45 RO (memory T-cells) positive cells, which were also found at a significantly lower frequency in the vaccinated patients. A British study which examined tonsillar tissue from vaccinated and non-vaccinated patients, found that there is a shift in the CD4+ cell population from mixed CD45RA+ and CD45RO+ population to an almost exclusively CD45RO+ population (44). We can only speculate on the reason for this drop in T-helper cells after vaccination. The Th-cells may be recruited to local draining lymph nodes in the systemic compartment of the immune system, contributing to the immune response to the vaccine. In the immunohistochemical staining of the tonsillar tissue we also observed a significant increase in CD68 positive cells, as a marker for macrophages.

Another macrophage marker, MAC387 did not change. The reason for this is difficult to explain, and our results must be interpreted with caution. It might be that these two markers represent different subsets of macrophages. However, both of these markers are not exclusively specific for macrophages (37;39;72).

In paper III we also used an alternative method to detect the influenza specific ASC in tonsillar tissue. We stained fixed tonsillar tissue sections with fluorescent labelled H3N2 influenza antigen. As shown by the ELISPOT method on tonsillar lymphocytes in culture, we were able to show a significant increase in influenza specific ASC of the vaccinated patients. The ASCs were found scattered around as individual cells in the tonsillar tissue, especially around the germinal centres and mantle zones, but also outside the follicles. We believe this is as an indication on that the ASCs are homing to the tonsils from the bloodstream, rather than being locally activated. This novel method of *in situ* (tissue) detection with immunofluorescent labelled ASC has to our knowledge, not been described earlier.

2.4.4 Cyto- and chemokines

Cytokines are small protein molecules which are produced and secreted by mainly immune competent cells as a way of communication. They are mediators that interact with receptors on other cells, and regulate the immune responses in different ways. Some cytokines attract cells to the site of the immune response, so called chemo attractant cytokines or chemokines. Cytokines do not only mediate the communication between different cells, but may themselves contribute to the symptoms and the pathology of the immune response.

We have in our studies of lymphocytes from blood and tonsils shown that parenteral influenza vaccination has an impact on the number of immune potent cells. In our two trials included in paper IV, we wanted to investigate if the complex immune reaction to influenza vaccine could be determined in more detail, by examining the cytokines and chemokines involved in this process, both locally and systemically. Our patients were randomised into 3 groups: one control group of non-vaccinated

patients and two groups who were immunized 1 week or 2 weeks before the operation. We analysed 25 cytokines directly in unstimulated serum and saliva samples from all patients, 1 or 2 weeks before tonsillectomy and at the time of operation. This was done with a multiplex bead immuno assay that analysed 25 different cytokines simultaneously using xMAP technology in a Luminex 100 instrument. Generally the levels of cytokines in these samples were low, and mostly below detection limit. Surprisingly, the cytokine concentrations were higher in saliva than in serum. There was a large individual variation in the cytokine levels. Except for the cytokine IL-12p40 there was no significant differences in the cytokine levels between the groups. IL-12p40 decreased in all 3 groups at the time of the operation, compared with the level 1 or 2 weeks before, so we believe that this change may be related to stress from the operation due to neuroendocrine mediators (Reviewed in (35).

The supernatants from stimulated lymphocytes from peripheral blood (PBMC) and tonsils (TMC) were analysed with a 10-plex cytokine assay in the Luminex 100 instrument. We found a significant increase in INF-γ, IL-10 and TNF-α in both TMC and PBMC in patients vaccinated both one and two weeks earlier. Two weeks after vaccination there were significant increases in the levels of GM-CSF, IL-2, IL-5, IL-6 and IL-8 in either PBMC, TMC or both. Generally there were higher cytokine concentrations in the TMC than in the PBMC. In an elderly and also a young population increases were found in the INF-γ, IL-10 and IL-6 production in stimulated PBMCs after vaccination (8), and there was generally a higher production in the young population. IL-4 was found in very low concentrations in all groups, which is line with a previous report form Guthrie et al (44). It has been shown earlier that there is limited correlation between serum cytokine levels and PBMC cytokine production (51), as shown in our study.

We also examined the gene expression by QPCR of 10 cytokines in PBMC, by collecting whole blood in PAXgene tubes, which preserves the mRNA expression levels. This showed only a slight increase in most cytokines after vaccination, except

for IL5 and IL10, which were below the detection limit. For some cytokines (IL-2, IL-4, INF-γ and TGF-β) the gene expression levels were higher 1 week after vaccination than 2 weeks after. This may indicate that there is still some enhanced gene expression, but that the cytokine production is no longer activated. This supports the cytokine results from serum and saliva, that there is no extensive cytokine production at 1 or 2 weeks after influenza vaccination that can be measured without *in vitro* stimulation.

2.5 Conclusions and Future perspectives

This study is a continuation of a long term project by the co-authors (16-18;24;26;32-34), but with focus on the local immune system in the upper airway mucosa and lymphoid tissue of humans.

There has been a tremendous interest in influenza research in recent years due to the appearance of highly pathogenic avian influenza stains. This has also increased the focus on the next pandemic influenza and the prophecies for this are somewhat ominous, regarding morbidity, mortality, socio-economic implications and also our possibilities for prevention.

The most effective way to protect from influenza is by vaccination. Resistance against influenza virus infection and illness is mediated through a complicated network of immune reactions, including both the innate and the adaptive immune systems (25;27).

Influenza infects the respiratory mucosa in humans. The humoral immunity mediated mainly by SIgA in the upper respiratory tract and by IgG in the lower airways, can protect against influenza infection and is therefore of major importance in resistance. Cellular immunity mediated mainly by CTL's (T-cells) is important in clearance of influenza infection and in the avoidance of complications, but it does not prevent infection. It is likely that lymphoid organs in the upper airways contribute

significantly to the local protection against influenza as inductive, effector and memory sites near the target of influenza virus infection.

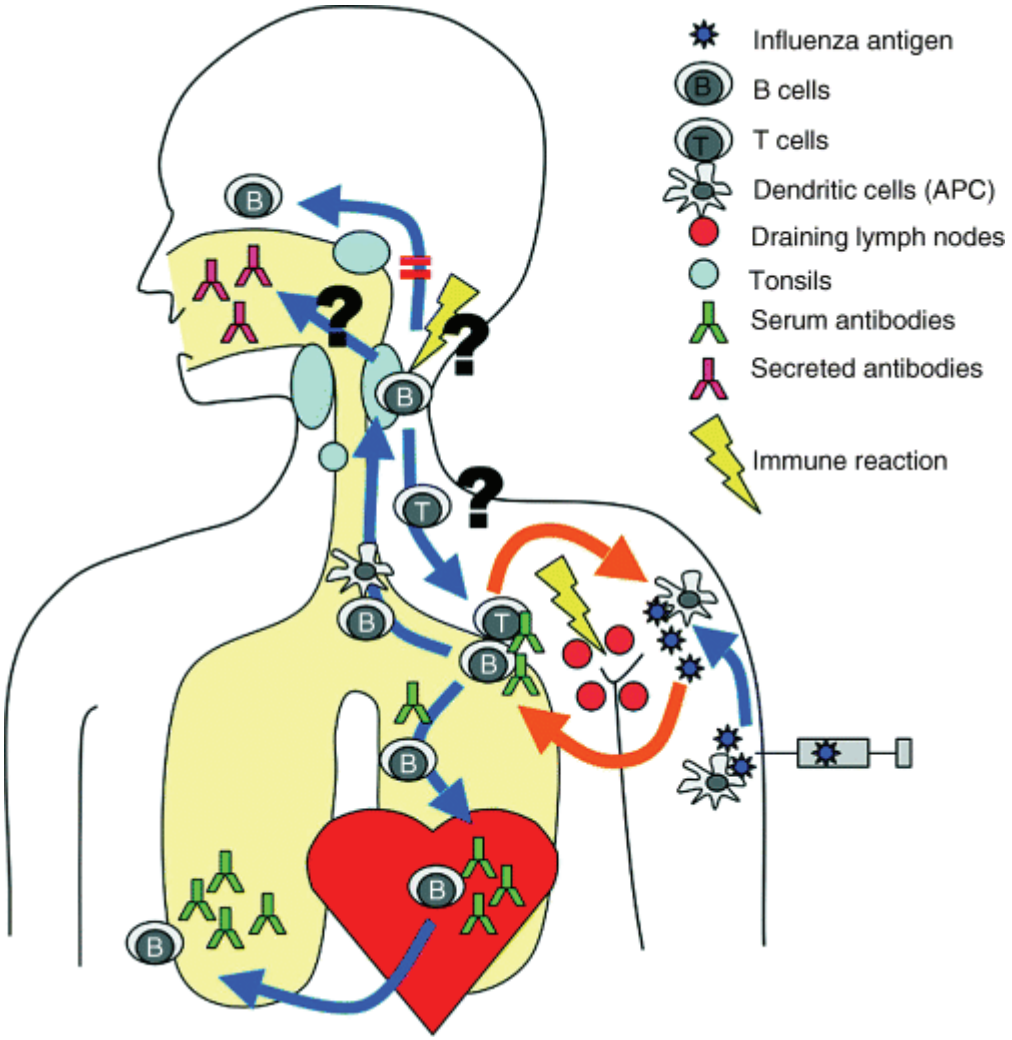

Figure 9. A simplified overview of the immunological processes occurring in connection with parenteral influenza vaccination. From (25). The injected influenza antigens are transported either as free antigen or by antigen presenting cells (dendritic cells) to the local draining lymph nodes in the armpit. The initial immune reaction is measurable in blood as elevated influenza specific antibodies and antibody secreting cells (ASC) [Paper I, II, III, IV]. Influenza specific ASC migrate to local lymphoid organs like the tonsils in the Waldeyer's ring, but not to the nasal mucosa [Paper I, II]. The origin of the antibodies in saliva may originate from local lymphoid tissue in the oral cavity. T-helper cells may migrate back from tonsils to the draining lymph nodes in the armpit, participating in the present immune response [Paper III].

Our studies have shown that vaccination with a trivalent sub-unit influenza vaccine induces a rapid and strong local and systemic antibody response (Fig.9). These antibodies are produced by ASC's. We have found a significant up-regulation of influenza specific ASC's after vaccination in tonsils and serum. In the nasal mucosa there are high numbers of influenza specific ASC's, but this does not increase after parenteral vaccination. The importance of tonsillar tissue in the resistance to influenza has been questioned (100), but we have found that vaccination induces an upregulation of ASC's and a reduction of CD4+ T-cells in tonsils. Although ENT-surgeons world-wide largely spend their working days removing this immune competent tissue, there is little doubt that the tonsils are contributing to the immune response and resistance towards influenza.

For future work there is still a tremendous amount of "unexplored land". The influenza virus is unpredictable, and there is a need for more knowledge in understanding the interactions between the immune system and the virus. With better knowledge, it will be possible to develop more efficient strategies for preventing and treating influenza.

The immunological effects of a live attenuated influenza vaccine administered as a nasal spray would be very interesting to study. Probably this route of vaccination will give a stronger immune response in the nasal mucosa. It would also be interesting to study further cellular changes in the human nasal mucosa, with reference to influenza vaccination locally and/or systemically. Further studies with different time-points of the cytokine response in the human upper airway are also needed to clarify in which way they contribute in the immune response. The rapid development of sophisticated tools for immunological studies will probably open new possibilities in examining the immune response of influenza vaccines.

We hope our results will contribute to the understanding of immunity in the upper airway and the immune response to influenza vaccination, and also help in the development of better vaccine strategies for the future.

References

1. Economic and Social Impact of Epidemic and Pandemic Influenza. Vaccine 2006;24(44-46):6776-8.

2. Andre antivirale midler. Norsk legemiddelhåndbok. 2007.

3. Alho OP, Koivunen P, Penna T, Teppo H, Koskela M, Luotonen J. Tonsillectomy versus watchful waiting in recurrent streptococcal pharyngitis in adults: randomised controlled trial. BMJ 2007;334(7600):939.

4. Barry J. The great influenza : the epic story of the deadliest plague in history / John M. Barry. Viking, New York; 2004.

5. Behrens G, Stoll M. Pathogenesis and Immunology. In: Kamps B, Hoffmann C, Preiser W, editors. Influenza Report. Paris, Cagliari, Wuppertal, Sevilla: Flying Publisher; 2006. p. 92-109.

6. Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, de Jong MD et al. Avian influenza A (H5N1) infection in humans. N.Engl.J Med. 2005;353(13):1374-85.

7. Bergsaker M, Hungnes O, Iversen B. [Vaccination against influenza--why, for whom and with which vaccine?]. Tidsskr.Nor Laegeforen. 2006;126(21):2814-7.

8. Bernstein ED, Gardner EM, Abrutyn E, Gross P, Murasko DM. Cytokine production after influenza vaccination in a healthy elderly population. Vaccine 1998;16(18):1722-31.

9. Bernstein JM, Baekkevold ES, Brandtzaeg P. Immunobiology of the Tonsils and Adenoids. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, Mayer L, editors. Mucosal immunology. 3rd. ed. Elsevier Academic Press; 2005. p. 1547-72.

10. Beveridge WI. The chronicle of influenza epidemics. Hist Philos.Life Sci. 1991;13(2):223-34.

11. Bock A, Popp W, Herkner KR. Tonsillectomy and the immune system: a long-term follow up comparison between tonsillectomized and non-tonsillectomized children. Eur.Arch.Otorhinolaryngol. 1994;251(7):423-7.

12. Brandtzaeg P. Immunology of tonsils and adenoids: everything the ENT surgeon needs to know. International Journal of Pediatric Otorhinolaryngology 2003;67(Supplement 1):S69-S76.

13. Brandtzaeg P. Role of mucosal immunity in influenza. Dev.Biol.(Basel) 2003;115:39-48.

14. Bridges CB, Kuehnert MJ, Hall CB. Transmission of influenza: implications for control in health care settings. Clin.Infect.Dis. 2003;37(8):1094-101.

15. Brokstad KA. Qualitative, quantitative and kinetic aspects of the humoral immune response induced systemically and locally after influenza vaccination / Karl Albert Brokstad. 1995.

16. Brokstad KA, Cox RJ, Major D, Wood JM, Haaheim LR. Cross-reaction but no avidity change of the serum antibody response after influenza vaccination. Vaccine 1995;13(16):1522-8.

17. Brokstad KA, Cox RJ, Olofsson J, Jonsson R, Haaheim LR. Parenteral influenza vaccination induces a rapid systemic and local immune response. J Infect.Dis. 1995;171(1):198-203.

18. Brokstad KA, Cox RJ, Oxford JS, Haaheim LR. IgA, IgA subclasses, and secretory component levels in oral fluid collected from subjects after parental influenza vaccination. J Infect.Dis. 1995;171(4):1072-4.

19. Brydon EW, Morris SJ, Sweet C. Role of apoptosis and cytokines in influenza virus morbidity. FEMS Microbiol.Rev 2005;29(4):837-50.

20. Burls A, Jordan R, Barton P, Olowokure B, Wake B, Albon E et al. Vaccinating healthcare workers against influenza to protect the vulnerable--Is it a good use of healthcare resources?: A systematic review of the evidence and an economic evaluation. Vaccine 2006;24(19):4212-21.

21. Burton MJ, Towler B, Glasziou P. Tonsillectomy versus non-surgical treatment for chronic / recurrent acute tonsillitis. Cochrane.Database.Syst.Rev 2000(2):CD001802.

22. Cheung T, Poon L. Biology of Influenza A Virus. Annals of the New York Academy of Sciences 2007;1102(1):1-25.

23. Cinti S. Pandemic influenza: are we ready? Disaster.Manag.Response 2005;3(3):61-7.

24. Cox RJ, Brokstad KA. The postvaccination antibody response to influenza virus proteins. APMIS 1999;107(3):289-96.

25. Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand.J.Immunol. 2004;59(1):1-15.

26. Cox RJ, Brokstad KA, Zuckerman MA, Wood JM, Haaheim LR, Oxford JS. An early humoral immune response in peripheral blood following parenteral inactivated influenza vaccination. Vaccine 1994;12(11):993-9.

27. Cox RJ, Haaheim LR, Ericsson JC, Madhun AS, Brokstad KA. The humoral and cellular responses induced locally and systemically after parenteral influenza vaccination in man. Vaccine 2006;24(44-46):6577-80.

28. De Filette M, Fiers W, Martens W, Birkett A, Ramne A, Lowenadler B et al. Improved design and intranasal delivery of an M2e-based human influenza A vaccine. Vaccine 2006;24(44-46):6597-601.

29. Doherty PC, Turner SJ, Webby RG, Thomas PG. Influenza and the challenge for immunology. Nat Immunol 2006;7(5):449-55.

30. Doyle WJ, Gentile DA, Skoner DP. Viral and bacterial rhinitis. Clin.Allergy Immunol 2007;19:177-95.

31. El Madhun AS. Characteristics of the humoral immune response after parenteral influenza vaccination / Abdullah Sami El-Madhun. 1998.

32. El Madhun AS, Cox RJ, Haaheim LR. The effect of age and natural priming on the IgG and IgA subclass responses after parenteral influenza vaccination. J Infect.Dis. 1999;180(4):1356-60.

33. El Madhun AS, Cox RJ, Seime A, Sovik O, Haaheim LR. Systemic and local immune responses after parenteral influenza vaccination in juvenile diabetic patients and healthy controls: results from a pilot study. Vaccine 1998;16(2-3):156-60.

34. El Madhun AS, Cox RJ, Soreide A, Olofsson J, Haaheim LR. Systemic and mucosal immune responses in young children and adults after parenteral influenza vaccination. J Infect.Dis. 1998;178(4):933-9.

35. Elenkov IJ, Chrousos GP, Wilder RL. Neuroendocrine regulation of IL-12 and TNF-alpha/IL-10 balance. Clinical implications. Ann.N.Y.Acad.Sci. 2000;917:94-105.

36. Etchart N, Baaten B, Andersen SR, Hyland L, Wong SY, Hou S. Intranasal immunisation with inactivated RSV and bacterial adjuvants induces mucosal protection and abrogates eosinophilia upon challenge. Eur.J Immunol 2006;36(5):1136-44.

37. Falini B. PG-M1: a new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. The American journal of pathology 1993;142(5):1359-72.

38. Fiore AE, Shay DK, Haber P, Iskander JK, Uyeki TM, Mootrey G et al. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP), 2007. MMWR Recomm.Rep. 2007;56(RR-6):1-54.

39. Flavell DJ, Jones DB, Wright DH. Identification of tissue histiocytes on paraffin sections by a new monoclonal antibody. J.Histochem.Cytochem. 1987;35(11):1217-26.

40. Ghendon Y. The immune response to influenza vaccines. Acta Virol 1990;34(3):295-304.

41. Ghendon Y. Introduction to pandemic influenza through history. Eur.J Epidemiol. 1994;10(4):451-3.

42. Glezen WP. Serious morbidity and mortality associated with influenza epidemics. Epidemiologic Reviews 1982;4:25-44.

43. Gorfien JL. Comparison of the microanatomical distributions of macrophages and dendritic cells in normal and diseased tonsils. The Annals of otology, rhinology & laryngology 2001;110(2):173-82.

44. Guthrie T. Parenteral influenza vaccination influences mucosal and systemic T cell-mediated immunity in healthy adults. J Infect Dis 2004;190(11):1927-35.

45. Haley PJ. Species differences in the structure and function of the immune system. Toxicology 2003;188(1):49-71.

46. Hayden FG, Fritz R, Lobo MC, Alvord W, Strober W, Straus SE. Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. J Clin.Invest 1998;101(3):643-9.

47. Hiepe F, Radbruch A. Is long-term humoral immunity in the mucosa provided by long-lived plasma cells? A question still open. European journal of immunology 2006;36(5):1068-9.

48. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J.Hyg.(Lond) 1972;70(4):767-77.

49. Hoeven A, Scholing M, Wever P, Fijnheer R, Hermans M, Schneeberger P. Lack of Discriminating Signs and Symptoms in Clinical Diagnosis of Influenza of Patients Admitted to the Hospital. Infection 2007;35(2):65-8.

50. Hsu APP, Tan KL, Tan YB, Han HJ, Lu PKS. Benefits and efficacy of tonsillectomy for recurrent tonsillitis in adults. Acta Oto-Laryngologica 2007;127(1):62-4.

51. Jason J, Archibald LK, Nwanyanwu OC, Byrd MG, Kazembe PN, Dobbie H et al. Comparison of serum and cell-specific cytokines in humans. Clin.Diagn.Lab Immunol. 2001;8(6):1097-103.

52. Jefferson TO, Rivetti D, Di Pietrantonj C, Rivetti A, Demicheli V. Vaccines for preventing influenza in healthy adults. Cochrane.Database.Syst.Rev 2007(2):CD001269.

53. Jung KY, Lim HH, Choi G, Choi JO. Age-related changes of IgA immunocytes and serum and salivary IgA after tonsillectomy. Acta Otolaryngol.Suppl 1996;523:115-9.

54. Lamb RA, Krug RM. Orthomyxoviridae: The Viruses and Their Replication. In: Knipe DM, Howley PM, editors. Fields' virology. 4 ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 1487-531.

55. Larsen DL, Karasin A, Zuckermann F, Olsen CW. Systemic and mucosal immune responses to H1N1 influenza virus infection in pigs. Veterinary Microbiology 2000;74(1-2):117-31.

56. Liang B, Hyland L, Hou S. Nasal-Associated Lymphoid Tissue Is a Site of Long-Term Virus-Specific Antibody Production following Respiratory Virus Infection of Mice. J.Virol. 2001;75(11):5416-20.

57. Marshall T. A review of tonsillectomy for recurrent throat infection. Br.J Gen Pract. 1998;48(431):1331-5.

58. Minne A, Louahed J, Mehauden S, Baras B, Renauld JC, Vanbever R. The delivery site of a monovalent influenza vaccine within the respiratory tract impacts on the immune response. Immunology 2007;122(3):316-25.

59. Molinari NA, Ortega-Sanchez IR, Messonnier ML, Thompson WW, Wortley PM, Weintraub E et al. The annual impact of seasonal influenza in the US: Measuring disease burden and costs. Vaccine 2007;25(27):5086-96.

60. Møller P. Veileder for fagområdet Øre-nese-hals-sykdommer / Norsk oto-laryngologisk forening ; hovedredaktør: Per Møller. 1998.

61. Monto AS, Gravenstein S, Elliott M, Colopy M, Schweinle J. Clinical signs and symptoms predicting influenza infection. Arch.Intern.Med. 2000;160(21):3243-7.

62. Mui SM, Rasgon BMM, Hilsinger RLJ. Efficacy of Tonsillectomy for Recurrent Throat Infection in Adults. [Article]. Laryngoscope 1998;108(9):1325-8.

63. Nasjonalt Folkehelsehelseinstitutt. Fakta om influenza. 29-5-2007. www.fhi.no

64. National Institute for Clinical Excellence. Guidance on the use of zanamivir, oseltamivir and amantadine for the treatment of influenza. 2003. www.nice.org.uk

65. Nave H, Gebert A, Pabst R. Morphology and immunology of the human palatine tonsil. Anat.Embryol.(Berl) 2001;204(5):367-73.

66. Ogra PL. Effect of tonsillectomy and adenoidectomy on nasopharyngeal antibody response to poliovirus. The New England journal of medicine 1971;284(2):59-64.

67. Olofsson K. The surface epithelium of recurrent infected palatine tonsils is rich in gammadelta T cells. Clinical and experimental immunology 1998;111(1):36-47.

68. Ostergaard PA. IgA levels and carrier rate of pathogenic bacteria in 27 children previously tonsillectomized. Acta Pathol.Microbiol.Scand.[C.] 1977;85(3):178-86.

69. Paradise JL, Bluestone CD, Bachman RZ, Colborn DK, Bernard BS, Taylor FH et al. Efficacy of tonsillectomy for recurrent throat infection in severely affected children. Results of parallel randomized and nonrandomized clinical trials. N.Engl.J Med. 1984;310(11):674-83.

70. Parvin JD, Moscona A, Pan WT, Leider JM, Palese P. Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. J Virol. 1986;59(2):377-83.

71. Perry M, Whyte A. Immunology of the tonsils. Immunol Today 1998;19(9):414-21.

72. Pulford KA, Rigney EM, Micklem KJ, Jones M, Stross WP, Gatter KC et al. KP1: a new monoclonal antibody that detects a monocyte/macrophage associated antigen in routinely processed tissue sections. J Clin Pathol 1989;42(4):414-21.

73. Rimmelzwaan GF, Boon AC, Voeten JT, Berkhoff EG, Fouchier RA, Osterhaus AD. Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. Virus Res. 2004;103(1-2):97-100.

74. Rivetti D, Jefferson T, Thomas R, Rudin M, Rivetti A, Di Pietrantonj C et al. Vaccines for preventing influenza in the elderly. Cochrane.Database.Syst.Rev 2006;3:CD004876.

75. Ruef C. Diagnosing influenza--clinical assessment and/or rapid antigen testing? Infection 2007;35(2):49-50.

76. Russell MW, Bobek LA, Brock JH, Hajishengallis G, Tenovuo J. Innate Humoral Defence Factors. Mucosal immunology / edited by Jiri Mestecky ... [et al.].-3rd ed. 2005. p. 73-93.

77. Ryan J, Zoellner Y, Gradl B, Palache B, Medema J. Establishing the health and economic impact of influenza vaccination within the European Union 25 countries. Vaccine 2006;24(47-48):6812-22.

78. Sainz M, Gutierrez F, Moreno PM, Munoz C, Ciges M. Changes in immunologic response in tonsillectomized children. I. Immunosuppression in recurrent tonsillitis. Clin.Otolaryngol.Allied Sci. 1992;17(5):376-9.

79. Savage DC. Microbial ecology of the gastrointestinal tract. Annu.Rev Microbiol. 1977;31:107-33.

80. Scadding GK. Immunology of the tonsil: a review. J R.Soc.Med. 1990;83(2):104-7.

81. Shope RE. Swine Influenza: I. Experimental Transmission and Pathology. J.Exp.Med. 1931;54(3):349-59.

82. Singleton R, Etchart N, Hou S, Hyland L. Inability To Evoke a Long-Lasting Protective Immune Response to Respiratory Syncytial Virus Infection in Mice Correlates with Ineffective Nasal Antibody Responses. J.Virol. 2003;77(21):11303-11.

83. Sladkova T, Kostolansky F. The role of cytokines in the immune response to influenza A virus infection. Acta Virol 2006;50(3):151-62.

84. Smith W. A virus obtained from influenza patients. Lancet 1933;222:66-8.

85. Smith S, Demicheli V, Di Pietrantonj C, Harnden AR, Jefferson T, Matheson NJ et al. Vaccines for preventing influenza in healthy children. Cochrane.Database.Syst.Rev 2006(1):CD004879.

86. Soreide AK, Olofsson J. [Tonsillectomy--day surgery or hospitalization?]. Tidsskr.Nor Laegeforen. 1999;119(10):1423-7.

87. Stech J, Xiong X, Scholtissek C, Webster RG. Independence of Evolutionary and Mutational Rates after Transmission of Avian Influenza Viruses to Swine. J.Virol. 1999;73(3):1878-84.

88. Suzumoto M, Hotomi M, Fujihara K, Tamura S, Kuki K, Tohya K et al. Functions of tonsils in the mucosal immune system of the upper respiratory tract using a novel animal model, Suncus murinus. Acta Otolaryngol. 2006;126(11):1164-70.

89. Szucs TD. Medical economics in the field of influenza--past, present and future. Virus Research 2004;103(1-2):25-30.

90. Tamura S, Iwasaki T, Thompson AH, Asanuma H, Chen Z, Suzuki Y et al. Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection. J Gen Virol 1998;79(2):291-9.

91. Tamura S, Kurata T. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. Jpn.J Infect.Dis. 2004;57(6):236-47.

92. The Macroepidemiology of Influenza Vaccination (MIV) Study Group. The macroepidemiology of influenza vaccination in 56 countries, 1997-2003. Vaccine 2005;23(44):5133-43.

93. The National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health NIH. Antigenic Drift illustration. 28-5-2005. www3.niaid.nih.gov/news/focuson/flu/illustrations/antigenic/antigenicdrift.htm

94. The National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health NIH. Antigenic Shift illustration. 28-5-2005. www3.niaid.nih.gov/news/focuson/flu/illustrations/antigenic/antigenicshift.htm

95. Thompson WW. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA 2003;289(2):179-86.

96. Tyler KL, Nathanson N. Pathogenesis of Viral Infections. In: Knipe DM, Howley PM, editors. Fields' virology. 4 ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 199-243.

97. Vajdy M, Baudner B, Del Giudice G, O'Hagan D. A vaccination strategy to enhance mucosal and systemic antibody and T cell responses against influenza. Clinical Immunology 2007;123(2):166-75.

98. van Staaij BK, van den Akker EH, Rovers MM, Hordijk GJ, Hoes AW, Schilder AGM. Effectiveness of adenotonsillectomy in children with mild symptoms of throat infections or adenotonsillar hypertrophy: open, randomised controlled trial. BMJ 2004;329(7467):651.

99. Wallace LA, Collins TC, Douglas JDM, McIntyre S, Millar J, Carman WF. Virological surveillance of influenza-like illness in the community using PCR and serology. Journal of Clinical Virology 2004;31(1):40-5.

100. Wiley JA, Tighe MP, Harmsen AG. Upper Respiratory Tract Resistance to Influenza Infection Is Not Prevented by the Absence of Either Nasal-Associated Lymphoid Tissue or Cervical Lymph Nodes. J Immunol 2005;175(5):3186-96.

101. Wilschut J, McElhaney JE, Palache AM. Pathogenesis, Clinical Features and Diagnosis. In: McElhaney JE, Palache AM, editors. Rapid Reference to Influenza. 2nd ed. Edinburgh: Mosby Elsevier; 2006. p. 96-116.

102. Wilschut J, McElhaney JE, Palache AM. Social and Economical Impact of Influenza. In: McElhaney JE, Palache AM, editors. Rapid Reference to Influenza. 2nd ed. Edinburgh: Mosby Elsevier; 2006. p. 117-32.

103. Wilschut J, McElhaney JE, Palache AM. The Influenza Virus: Structure and Replication Jan C. Wilschut,, Abraham M. Palache. In: McElhaney JE, Palache AM, editors. Influenza. Edinburgh: Mosby Elsevier; 2006. p. 27-48.

104. Wolfensberger M, Haury JA, Linder T. Parent satisfaction 1 year after adenotonsillectomy of their children. International Journal of Pediatric Otorhinolaryngology 2000;56(3):199-205.

105. World Health Organisation. A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull.World Health Organ 1980;58(4):585-91.

106. World Health Organisation. Influenza Fact Sheet No. 211. 2003. www.who.int/mediacentre/factsheets/fs211/en/index.html

107. World Health Organisation. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO. 30-4-2008. www.who.int/csr/disease/avian_influenza/country/cases_table_2008_04_30/en/index.html

108. Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature 2008.

109. Wright PF, Webster RG. Orthomyxoviruses. In: Knipe DM, Howley PM, editors. Fields' virology. 4 ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 1533-79.

110. Yang Y, Halloran ME, Sugimoto J, Longini JrIM. Detecting human-to-human transmission of avian influenza A (H5N1). Emerg Infect Dis [serial on the Internet].2007 Sep. Available from www.cdc.gov/EID/content/13/9/1348.htm

111. Zambon M, Hays J, Webster A, Newman R, Keene O. Diagnosis of Influenza in the Community: Relationship of Clinical Diagnosis to Confirmed Virological, Serologic, or Molecular Detection of Influenza. Arch Intern Med 2001;161(17):2116-22.

Errata

Paper II; Page 879, first column, 4.th line from the top, should be: In addition, a nasal mucosal biopsy sample from the middle portion of the caudal medial part of the inferior turbinate was collected from 8 patients.

Paper IV; Page 145, reference number 9, Dinarello et al, was published in J Clin Invest in 1984.

Papers I-IV