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Influenza A Virus, H10N4, Naturally Pathogenic for Mink (*Mustela vison*)

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Abstract

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Key words: aerosol, experimental infection, H10N4, H10N7, immunohistochemistry, influenza virus, interstitial pneumonia, mink, morphometry, oligonucleotide mapping, virus isolation.

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Influenza A Virus, H10N4, Naturally Pathogenic for Mink (*Mustela vison*)

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I am become a name;
For always roaming with a hungry heart
Much have I seen and known: cities of men
and manners, climates, councils, governments,
Myself not least, but honoured of them all;
And drunk delight of battle with my peers,
Far on the ringing plains of windy Troy.
I am part of all that I have met;
Yet all experience is an arch wherethro'
Gleams that untravelled world, whose margin fades
For ever and for ever when I move.

from Ulysses
Lord Tennyson (1809-1892)

To those who love me

Abstract

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Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Klingeborn, B., Englund, L., Rott, R., & Juntti, N. 1985. An avian influenza virus killing a mammalian species - the mink. *Arch. Virol. 86*: 347-351.

II. Englund, L., Klingeborn, B., & Mejerland, T. 1986. Avian influenza virus causing an outbreak of contageous interstitial pneumonia in mink. *Acta vet. scand. 27*: 497-504.

III. Berg, M., Englund, L., Abusugra, I.A., Klingeborn, B., & Linné, T. 1990. Close relationship between mink influenza (H10N4) and concomitantly circulating avian influenza viruses. *Arch.Virol. 113*: 61-71.

IV. Englund, L., & Hård af Segerstad, C. Two avian H10 influenza A virus strains with different pathogenicity for mink (*Mustela vison*). Submitted.

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Abbreviations

a.	aerosol
ABC	avidin-biotin-complex
ADP	area density of pneumonia
BHK	baby hamster kidney
c.	contact
CD	cluster of differentiation
CE	chicken embryo
chicken/49	A/chicken/Germany/N/49 (H10N7)
CO_2	carbon dioxide
CTL	cytotoxic T-lymphocytes
EID_{50}	mean egg infectious dose
ELISA	enzyme-linked-immuno-sorbent assay
fowl/85	A/fowl/Hampshire/378/85 (H10N4)
gal	galactose
HA	hemagglutinin
HA-test	hemagglutination-test
HAU	hemagglutination units
HI	hemagglutination-inhibition
i.n.	intranasal
IFN	interferon
IgA	immunoglobulin A
l.n.	lymphnode
M	matrix protein
mallard/85	A/mallard/Gloucestershire/ 374/85 (H10N4)
MDBK	Madin-Darby bovine kidney
MDCK	Madin-Darby canine kidney
MEM	minimum essential medium
MHC	major histocompatibility complex
MiLu	mink lung
mink/84	A/mink/Sweden/3900/84 (H10N4)
mRNA	messenger ribonucleic acid
NK	natural killer
NA	neuraminidase
NeuAc	N-acetyl
NeuGc	N-glycolyl
NI	neuraminidase-inhibition
NP	nucleoprotein
NS	nonstructural protein
ON	oligonucleotide
PA	polymerase protein
PB	polymerase protein
PE	post exposure
PLPG	periodate-lysine-paraformaldehyde-glutaraldehyde
RNA	ribonucleic acid
SA	sialic acid
VSV	vesicular stomatitis virus
vRNP	viral ribonucleoprotein

Introduction

The mink

The mink (*Mustela vison*) is a predator and member of the Mustellidae family within the order Carnivora, together with species like the ferret (*Mustela putorius*) which is anatomically similar to the mink (Nes *et al.*, 1988). The mink species originates from North America where the wild animals were captured in the latter half of the nineteenth century, reared in farms and selectively bred for increased reproduction, colour, size and fur quality. Farmed mink were imported to several European countries for fur production during the early 1900s (Bowess, 1996; Nes *et al.*, 1988). During the wars, when the access to mink feed was limited, groups of American mink were set free, completely replacing the corresponding European species, the marsh otter (*Putorius lutreola*).

Mink reproduction is strictly seasonal and farmed mink in northern Europe usually give birth to their kits in early May. Kits are weaned at around eight weeks of age and animals intended for fur production are killed ("pelted") in November, when the fur quality is at its best. This seasonal production means that the farms have the maximum stocking density between July and November, whereas only the breeding stock is kept during the rest of the year.

In Sweden, farmed mink are kept outdoors in rows of cages covered only by a roof to provide shade and shelter. The feed, consisting of uncooked beef, chicken or fish by-products mixed with cereals, is administered on top of each cage, and is thus freely accessible for birds perching on the netting. Approximately one third of the Swedish fur production originates from the dense farming area on the south-east coast where an influenza A virus, H10N4, caused an outbreak of pneumonia in 1984. This farming area is situated close to the migratory routes used by several species of birds and close to farming areas where domestic fowl are kept.

Certain colour types (those homozygous for the aleutian gene) have a defective immune system, Chediak-Higashi syndrome, causing these colour types to be more sensitive to infections (Prieur, 1996). Aleutian disease is a persistent parvovirus infection, named after the immunologically defective colour type most severely affected, and used to be common in farmed mink of all colour types. The infection has negative effects on reproduction and survival through immune complex depositions and plasma cell infiltration in the filter organs (Hunter, 1996). Control test programmes can now detect the infection early and eradication of the disease is possible through repeated serological testing of the whole herd (Cho & Ingram, 1972; Hunter, 1996).

The influenza virus

The virus species commonly called "influenza viruses" belong to the family *Orthomyxoviridae*, a family of minus sense, single-stranded RNA viruses with segmented genomes. The infectious virus particle (virion) is pleiomorphic, spherical or filamentous in shape, and 80-120 nm in diameter. Within this family two genera are found: Influenza virus A and B (influenza A and influenza B viruses) and Influenza virus C (influenza C virus). Influenza A and B viruses are distinguished between by antigenic differences in their nucleoproteins and matrix proteins (Fenner *et al.*, 1993; Murphy & Webster, 1990). Influenza B and C viruses will not be discussed further in this thesis.

The influenza A virus genome is divided into eight segments, each coding for a single protein (segments 1-6) or two proteins (segments 7-8) (Lamb, 1989). Based on the properties of the major antigenic determinants, the surface antigens hemagglutinin (HA) and neuraminidase (NA), influenza A viruses are divided into subtypes, named after the serological variants of H and N antigens (Webster, 1994). Fourteen different subtypes of HA (H1-H14) and nine subtypes of NA (N1-N9) have been described (Yewdell *et al.*, 1994). Influenza A virus has been isolated from several mammalian species, including man, as well as from birds. All subtypes of HAs and NAs have been found in birds, whereas only a limited number of H and N antigen combinations have been isolated from mammals including humans (Rott, 1985; Webster *et al.*, 1992). Virus isolates are normally named after type, animal species (not included for human strains), place of isolation, identification of the isolate, year of isolation and the H and N subtypes. The influenz A virus isolated from Swedish farmed mink in 1984 is consequently referred to as A/mink/Sweden/3900/84 (H10N4) (I).

The fact that the virus genome is segmented allows for reassortment of segments if two or more virus strains simultaneously infect the same host cell. When such a reassortment involves the genes for hemagglutinin and/or neuraminidase, major antigenic differences are produced. This type of variation is called antigenic shift and can produce a virus subtype previously unknown to the exposed population, thus providing the basic requirement for a pandemic (Scholtissek, 1994). Less dramatic antigenic differences, antigenic drift, are caused by mutations in these genes. Reassortment and mutations can also involve any other gene or a number of genes, sometimes resulting in variations in host range, pathogenicity, growth patterns, or other traits of importance for virulence.

Structure and function of viral components

Influenza virus genes, gene products and replication have been reviewed in detail concerning structure and function by Lamb (1989) and concerning viral replication by Krug (1989). This section is a brief overview based on their papers and others, which are specifically referred to in the text. The

major surface antigen of influenza A virus, the glycoprotein hemagglutinin (HA), protrudes from the viral lipid bilayer envelope as trimers evenly distributed around the virion. The second surface antigen, neuraminidase (NA), is also a glycosylated protein but it is presented as tetrameric, mushroom-like structures, probably organized in groups, on the viral surface. The third membrane protein, M2, is an integral membrane protein present in low numbers in the viral envelope (Hay, 1992). Matrix protein (M1) is the most abundant internal protein and covers the inside of the viral membrane surrounding the nucleoprotein (NP)-covered viral RNA (the nucleocapsid). Three polymerases (PA, PB1 and PB2) are also attached to the nucleocapsid. The viral genome also codes for two nonstructural proteins (NS1 and NS2) translated in the infected cell. NS1 is not included in the progeny virion (Birch-Machin *et al.*, 1997). The internal proteins have low variability, in contrast to the highly variable surface antigens. Internal proteins do not cause any long-term protective antibody-mediated immune response, but do evoke a T-cell mediated response (Webster *et al.*, 1992).

Infection is initiated by the binding, of the receptor binding site on HA, to sialic acid-containing receptors on the target cell surface followed by transfection of the virion into the cell through the endocytic pathway. Respiratory epithelial cells are the prime targets for influenza A virus in mammals. The attached virion particle is endocytosed from the cell surface enclosed in endosomal vesicles. A lowering of the pH in the endosome, brought on by fusion with an acidic lysosome (Murphy & Webster, 1990), induces a conformational change in the HA, provided that the HA has been proteolytically cleaved, leading to fusion of viral and vesicular membranes (Wharton *et al.*, 1989; Whittaker *et al.*, 1996). The low pH also activates the M2 channels in the viral membrane allowing proton entry and subsequent lowering of pH in the virus particle. This induces a dissociation of the nucleocapsid from the M1 (Hay, 1992; Whittaker *et al.*, 1996). The viral ribonucleoprotein (vRNP) then spills out into the cytoplasm and rapidly migrates into the host cell nucleus through the nuclear pores, utilizing the cellular import machinery (Whittaker *et al.*, 1996). Transcription of virion -sense RNA to viral messenger RNA (mRNA, +sense) is initiated in the cell nucleus by the polymerases, while translation of host cell mRNA is blocked. This process is initiated by so-called "cap-snatching" by the influenza virus. A capped sequence in the 5' end of cellular mRNA is detached, by an endonuclease associated with the PB2 protein, and used as a primer for transcription of viral +sense mRNA from the virion -sense RNA (Fenner *et al.*, 1993). Viral replication is subsequently achieved by transcription of full length, +sense, template RNA which is copied into new -sense virion RNA. This latter synthesis occurs without a primer.

The mRNA is transported out into the cytoplasm where ribosomal translation of the different viral proteins take place. During the first hours translation of

NP, NS1 and polymerases take place, whereas the other viral proteins are synthesized later (Whittaker *et al.*, 1996). Of the proteins so produced HA, NA and M2 are transported through the exocytic pathway to be inserted into the cellular membrane. It has been determined that at least one of the glycoproteins, HA, is biosynthesized, becomes glycosylated, and acquires its quarternary structure within the endoplasmic reticulum, before transport to the Golgi apparatus where the final alterations of the oligosaccharide side chains occur (Roth *et al.*, 1989). It is reasonable to assume that NA and M2 follow the same pathway. From the Golgi apparatus, the influenza virus glycoproteins are moved through the *trans*-reticular network, believed to be responsible for directing the glycoproteins to the appropriate domain of the polarized epithelial cell (Roth *et al.*, 1989). The other synthesized influenza virus proteins (NP, PB1, PB2, PA, NS1, NS2, and M1) are transported into the nucleus to partake in transcription or to be included in the packaging of the vRNP. The vRNP is finally exported from the nucleus and transported to the cell surface where it is surrounded by M1 protein, which prevents re-entry of the vRNP into the nucleus (Whittaker *et al.*, 1996). The new virus nucleocapsid buds off from the host cell surface. The viral envelope of the progeny virion is formed by the host cell membrane, into which viral HA, NA and some M2 proteins have been inserted.

NA cleaves the link between a terminal sialic acid and an adjacent D-galactose or D-galactoseamine. The complete functional role of the NA is not fully known, but important functions are to free the HA from sialic acid-containing structures in the respiratory mucosa, to facilitate mucus penetration, prevent self-aggregation, and to break the linkage between HA and cellular receptors to free the progeny virus particle from the host cell surface. (Colman, 1989; Morein & Bergman, 1972; Webster *et al.*, 1992; Yewdell *et al.*, 1994).

Factors influencing pathogenicity and host range

The sialic acid receptors for hemagglutinin vary between host species both in type of sialic acid, i.e. N-acetyl (NeuAc) or N-glycolyl (NeuGc) (Ito *et al.*, 1997a), and in linkage between the sialic acid (SA) and the galactose, thus differentiating between NeuAcα2,3Gal, NeuGcα2,3Gal, NeuAcα2,6Gal and NeuGcα2,6Gal (Couceiro *et al.*, 1993; Ito *et al.*, 1997a; Ito *et al.*, 1997b). Influenza isolates have been shown to have an increased affinity for receptors of the types present in the epithelium of the species from which the virus was isolated (Connor *et al.*, 1994). Equine and avian influenza strains preferentially bind to SAα2,3Gal, whereas human strains bind to SAα2,6Gal (Rogers & Paulson, 1983). Substitution of single amino acids in positions 226 and 228 in influenza HA of subtypes H2 and H3 have been shown to alter the receptor specificity between NeuAcα2,3Gal and NeuAcα2,6Gal (Connor *et al.*, 1994; Rogers *et al.*, 1983). When the sialic acid preferences for human and animal influenza strains were further investigated it was suggested that avian and equine influenza viruses were able to recognise NeuGcα2,3Gal, whereas human isolates did not (Ito *et al.*, 1997a). Studies also suggest that

human strains isolated after 1973 also recognise the NeuGcα2,6Gal receptor whereas the earlier strains preferentially bound to NeuAcα2,6Gal (Higa *et al.*, 1985; Ito *et al.*, 1997a).

The NeuAcα2,6Gal is present on the surface of ciliated epithelial cells in the human trachea, the primary target for human influenza virus (Couceiro *et al.*, 1993) and the same receptor has been suggested to be present also in ferret airways (Leigh *et al.*, 1995). An α2,3Gal receptor-variant of human influenza virus was less virulent, but elicited a similar antibody-mediated immune response in ferrets, when compared to wild type α2,6Gal-binding virus (Leigh *et al.*, 1995). Differences between receptor types in MDCK cells, chicken embryo allantoic cells and chicken embryo amniotic cells have been shown to influence the receptor specificity of human influenza virus strains and to cause a selection for SAα2,3Gal specificity after multiple egg passages (Ito *et al.*, 1997b). Neither the receptor preferences of H10 influenza virus strains nor the receptor prevalence in mink airways have been defined.

A prerequisite for the fusion between endosomal and viral membranes, and thus for infectivity, is that HA0 must be cleaved into HA1 and HA2 (Wharton *et al.*, 1989) to expose the hydrophobic N-terminus on HA2 which interacts with the endosomal membrane (Palese & Garciá-Sastre, 1994). The HAs of some highly virulent avian strains of subtypes H5 and H7 are cleaved by ubiquitous proteases intracellularly in all organs in the host (Rott *et al.*, 1995; Webster *et al.*, 1992). Multiple basic amino acids, e.g. arginine, at the cleavage site have been shown to cause the increased cleavability which is correlated to increased pathogenicity in chicken (Bosch *et al.*, 1981; Perdue *et al.*, 1997; Rott *et al.*, 1995; Wood *et al.*, 1993). The cleavage may occur in the *trans*-reticular network through which the HA is transported from the Golgi to the cellular membrane (Roth *et al.*, 1989) and is probably mediated by furin, an endoprotease located in this compartment (Boycott *et al.*, 1994; Stieneke-Gröber *et al.*, 1992). These viruses can thus undergo multiple replication cycles in tissue culture and cause systemic infection in birds (Klenk & Rott, 1988).

In contrast, apathogenic avian influenza strains and mammalian influenza viruses are normally cleaved extracellularly, on budding or after being released, by proteases secreted by a restricted number of cell types in the respiratory or enteric tract, causing localized, often mild infections (Boycott *et al.*, 1994; Rott *et al.*, 1995; Webster, 1994). One such identified protease, cleaving extracellular HA, is tryptase Clara, produced by the non-ciliated secretory epithelial cells in the distal airways of rats (Kido *et al.*, 1993). The mammalian and the apathogenic avian influenza viruses have a single basic amino acid (arginine) at their HA cleavage site, indicating restricted cleavability. However, no correlation has been observed between amino acid sequence and pathogenicity in mammalian influenza strains (Webster, 1994).

Coinfecting bacteria have, however, been suggested to provide the proteases necessary for increased pathogenicity *in vivo*, by cleaving the HA of viruses that do not display signs of increased cleavability in cell cultures (Feldmann *et al.*, 1988; Rott *et al.*, 1995). Studies of one mammalian influenza virus strain (A/WSW/33, H1N1) have shown that the HA of this virus, in contrast to other mammalian influenza viruses, is cleaved intracellularly at the stage of viral entry (Boycott *et al.*, 1994). The cleavability of HA is also influenced by the location of carbohydrate side chains which can interfere sterically with protease accessibility and thus cleavage and virulence (Kawaoka *et al.*, 1984; Deshpande *et al.*, 1987; Rott *et al.*, 1995).

When the nucleotide sequences and glycosylation patterns of A/mink/Sweden/84 and A/chicken/Germany/49 were compared both viruses had nucleotide sequences typical for restricted cleavability, oligosaccharides in the same positions, and neither virus produced cleaved hemagglutinin when cultured in MDCK, MDBK and CE cell cultures (Feldmann *et al.*, 1988).

A single amino acid mutation in the HA2 gene has been shown to lower the pH optimum for virus-endosome fusion by 0.2 units and was suggested to increase the viral resistance to extracellular inactivation by acidic lung secretions (Smeenk *et al.*, 1996). The same mutation also increased the resistance to nonspecific inhibitors of hemagglutinin, present in inflammatory exudates.

The NA may have a role in the determination of host range, since changes in NA have been shown to be the major determinant of neurovirulence in mice and mouse neuroblastoma cells (Nakajima & Suigura, 1980; Sugiura & Ueda, 1980; Ward, 1995) and to influence the plaque formation in tissue culture (Schulman & Palese, 1977). Blocking of the neuraminidase activity in cell culture also caused aggregation of progeny virus on the host cell surface, which in turn prevented multicycle infection, since the progeny virus particles could not spread to other cells (Liu *et al.*, 1995). Intracellular HA-cleavage of certain H1N1 strains in MDBK cells have been shown to be NA dependent (Boycott *et al.*, 1994). Certain NA subtypes also appear to be linked to certain hosts and certain HA subtypes, but the specific mechanisms for any influence by NA on host range or virulence have not been identified (Truyen *et al.*, 1995).

The PB2 polymerase is active in the initiation of virion -sense RNA transcription to mRNA in the host cell nucleus (Webster *et al.*, 1992). A single amino acid substitution in position 627 in the PB2 gene has been shown to alter the host range of human influenza A virus *in vitro*, restricting the replication in MDCK cells, and to restrict the replication *in vivo* in non-human primates and adult humans (Subbarao *et al.*, 1993). The same group also analyzed a variety of influenza viruses and found that the amino acid in

position 627 was glutamic acid in every avian influenza virus tested (8 strains, isolated 1934-1985) and lysine in every tested human strain (12 strains, isolated 1933-1988), which indicates an influence by this amino acid on host range.

The NP gene is the major determinant of species specificity and molecular analyses of relationships between nucleotide and amino acid sequences of strains have divided the influenza viruses into two main lineages, containing all avian and all human strains, respectively (Gorman *et al.*, 1991; Scholtissek, 1996; Scholtissek *et al.*, 1993).

Mutations of two amino acids in the M1 gene have been connected to increased recruitment and infection of macrophages, spread of virus into alveoli and increased viral replication in mice and were suggested to control the viral replication in tissues beyond the bronchioles after the first day of infection (Smeenk *et al.*, 1996). Mutations in either the PB1 or the PB2 gene also influenced macrophage recruitment and spread of virus in the mouse lung (Smeenk *et al.*, 1996). The M and NS genes have also been shown to have an influence on virus growth in mouse cells at stages after the initiation of infection (Nakajima & Suigura, 1980).

Certain constellations of genes have been suggested to provide the necessary basis for high virulence (Rott, 1979), and recombination can result in a progeny virus more pathogenic than either parent virus (Scholtissek *et al.*, 1979). Virulence can be the result of an additive effect from several mutations in different viral genes (Brown, 1990). Reassortment between two human influenza strains for vaccine production is suspected to have produced a reassortant which was apathogenic for humans but caused fatal epizootics among camels (Yamnikova *et al.*, 1993). This incident shows that the recombination of gene segments can have unexpected effects, which are difficult to predict or control.

Pathogenesis

In his article written in 1919, already thirteen years before the isolation of human influenza virus, MacCallum described the histopathology of cases of pneumonia during the Spanish Flu epidemic in American military camps, concluding that the multiple cases of fatal bacterial pneumonia seemed to have an underlying, unknown causative agent similar to the also unknown cause of measles (MacCallum, 1919). From the characteristic leucopenia he also concluded that the unknown agent lowered the resistance to bacterial infections. During the next pandemic, when the influenza virus had been identified (Smith *et al.*, 1933), it was concluded that human cases of primary viral pneumonia were rare and mostly seen in patients with an underlying cardiac or pulmonary disease (Louria *et al.*, 1959), a concept which is still relevant (Webster, 1994). These patients developed an acute fatal pneumonia

characterized by haemorrhages, oedema and hyaline alveolar membranes, whereas in most other fatal cases the viral lesions were masked by a secondary purulent bronchopneumonia (Louria *et al.*, 1959).

The prime targets for the influenza virus, in the mammalian host, are the epithelial cells in the respiratory tract, sometimes as far down as the small bronchi, resulting in rhinitis and tracheobronchitis and often destruction of the bronchial mucous gland epithelium (Dunnill, 1982; Nicholson, 1992). The virus first infects scattered epithelial cells from which infectious progeny virus will later spread to adjoining cells in the airways. If terminal bronchioles are affected, and become plugged by desquamated and inflammatory cells, the air flow from the alveoli is impaired which can lead to atelectasis, oedema, haemorrhage and subsequent inflammation also in the peripheral lung tissues (Dunnill, 1982; Kobzik & Schoen, 1994). Regeneration of the respiratory epithelium in human patients starts after approximately five days and full differentiation of the epithelium is achieved after about three weeks (Dunnill, 1982).

Primary viral pneumonia is seen in pigs but is relatively rare in humans and horses, where the infection is usually restricted to the upper respiratory tract (Bachmann, 1989; Hannant & Mumford, 1996; Louria *et al.*, 1959). The viral pneumonia usually shows signs of viral injury to bronchial, bronchiolar as well as alveolar epithelium and is perhaps best characterized as a bronchointerstitial pneumonia (López, 1995). During the first 24 hours after experimental infection of pigs, viral replication in alveolar epithelial type I and type II cells was followed by sloughing of these cells and accumulation of polymorphonuclear granulocytes (neutrophils) in lung capillaries (Winkler & Cheville, 1986). At 48 hours, the alveoli in the experimentally infected pigs contained macrophages, neutrophils, cell debris and oedema, but regeneration of type I epithelial cells was also seen (Winkler & Cheville, 1986). Studies in rats have indicated that a pulmonary surfactant, mainly produced by type II pneumocytes, inhibits the proteolytic cleavage of HA induced by tryptase Clara from the non-ciliated secretory cells in rat airways (Kido *et al.*, 1993). It has also been suggested that the surfactant neutralizes influenza virus through binding of sialic acid-containing molecules in the surfactant to the receptor binding sites on HA (Benne *et al.*, 1995). The destruction of type II pneumocytes thus interferes with surfactant production which in turn negatively affects the protective mechanisms in the pulmonary tissues.

The influenza pneumonia in pigs is very similar to the pneumonia caused by influenza A virus A/mink/Sweden/84 we describe in the experimental study in paper IV. The early lesions in this viral pneumonia are caused by the innate nonspecific immune response. Macrophages are normally present in the alveoli to phagocytose foreign particles and injured tissue cells and are the first cells of the natural, non-specific immune system to visibly react to an

influenza infection in the alveoli. In ferrets, macrophages have been suggested to be the most important factor in the prevention of alveolar influenza infection (Bird *et al.*, 1983). Besides, macrophages take part in antigen presentation, together with dendritic cells and B-lymphocytes. The potent capacity of the macrophage to produce pro-inflammatory cytokines is important for the antigen-presenting cell (APC)-function, in recruiting and activating a T-cell response, as well as for activation of the innate immune response (Abbas *et al.*, 1994). The activated T-cells, in turn, produce cytokines, among others interferon-γ which is a macrophage-activating factor (Abbas *et al.*, 1994).

Neutrophils are among the first leukocytes to accumulate at the site of influenza virus infection in response to chemotactic stimuli and macrophage cytokine production (Abbas *et al.*, 1994; Kobzik & Schoen, 1994; Murphy & Webster, 1990). Specific adherence of neutrophils to influenza-infected MDCK cells in vitro has been demonstrated as early as 4.5 hours after infection, which coincides with the insertion of progeny virus antigens through the host cell membranes (Ratcliffe *et al.*, 1988). It has subsequently been shown that the neutrophils adhere to the sialic acid-binding site on the HA molecule on the influenza virus (human H1N1) or HA protruding from the membrane on the infected cell (Ratcliffe *et al.*, 1993). The neutrophils, however, failed to adhere to virus bound to human antibodies, which indicated that opsonization in this case, surprisingly, reduced the effect of the primary defence mechanism (Ratcliffe *et al.*, 1993). Natural killer cells (NK cells) also contribute to the viral clearance, as part of the innate defence mechanisms, by lysing infected cells (Webster, 1994). The lytic activity of NK cells can be enhanced by type I interferon produced by most virally infected cells (Bender & Small, 1992; Ronni *et al.*, 1997).

The activity of class I MHC (MHC I)-restricted $CD8^+$ cytotoxic T lymphocytes (CTL) is evoked by recognition of peptides from most viral proteins, generally synthesized in the infected cell, which are processed in the cell and subsequently presented in association with MHC I-molecules on the cell surface (Yewdell & Hackett, 1989). A $CD8^+$CTL response is thus normally initiated by intracellular viral reproduction following natural infection or vaccination with live vaccines. It has, however, also been stimulated by certain formulations of subunit vaccines (Takahashi *et al.*, 1990) and high doses of killed influenza vaccine combined with certain adjuvants (Mbawuike & Wyde, 1993). The MHC I-molecules are present on most cells in the body. Cytotoxic T cells are required for normal recovery from influenza infection but do not prevent infection (Bender *et al.*, 1982; Renegar, 1992). They are cross-reactive between influenza A viruses of different subtypes, mainly due to the recognition of conserved T-cell-epitopes on the internal proteins, predominantly NP (Yewdell & Hackett, 1989). The CTLs peak early (after 6-8 days) in the infected lungs and are important for

clearing the influenza infection through the destruction of infected cells and the release of cytokines (Mackenzie *et al.*, 1989; Webster, 1994; Yewdell & Hackett, 1989). The CTLs also need stimulation from cytokines produced by $CD4^+$ helper T-cells, and to some extent also by $CD8^+$ T-cells, to differentiate (Abbas *et al.*, 1994; Yewdell & Hackett, 1989). There are also $CD4^+$ T-cells with a cytolytic effect, but $CD4^+$ T-cells mostly function by stimulating other cells through cytokine production (Abbas *et al.*, 1994).

Usually within a week from infection serum antibodies to HA, and to some extent NA, can be detected (Murphy & Webster, 1990). Antibody production is generally T-cell-dependent, i.e. initiated by the interaction between APCs and T-cells, which in turn stimulates the effector mechanisms of antigen specific B-cells and eventual antibody production (Abbas *et al.*, 1994; Male, 1991). This reaction does not require viral reproduction in infected cells and will thus also be evoked by a killed vaccine. Circulating antibodies are responsible for neutralizing the infectivity of progeny virus in an infected host (Murphy & Webster, 1990). Together with the local secretory antibody response, mainly IgA in the bronchial and nasal secretion, they form an important defence barrier against subsequent infections with the same virus or serologically cross-reactive viruses (Murphy & Webster, 1990).

Avian influenza

All known subtypes of HA and NA have been isolated from aquatic birds, particularly migrating waterfowl (Webster, 1994; Webster *et al.*, 1992). The most severe manifestation of the infection is fowl plague in chicken, first described during the 19th century and shown to be caused by an influenza virus in 1955 (Schäfer, 1955; Webster, 1994). Avian influenza can cause substantial losses to the poultry industry through mild symptoms like loss of egg production or through the need for massive eradication of highly pathogenic infections (Easterday & Hinshaw, 1991). The most pathogenic forms of influenza in bird species, particularly chicken and turkeys, have all belonged to subtypes H5 or H7, but not all isolates with those HA subtypes are pathogenic and strains with high pathogenicity for one bird species can be apathogenic for other species (Easterday & Hinshaw, 1991).

Free-living birds, such as migratory ducks, can be subclinically infected with influenza virus. The infection is normally restricted to the intestinal mucosa and virus can be shed in high quantities in the faeces, contaminating the environment, thus providing means for the virus to be transferred to new avian or mammalian hosts (Easterday & Hinshaw, 1991; Webster *et al.*, 1992). Reassortment between influenza genes from birds and mammals are believed to generate the new human influenza strains which occasionally spread from China to the rest of the world (Webster *et al.*, 1992; Yasuda *et al.*, 1991).

Influenza in mammals

Influenza virus is endemic in three mammalian species; humans (H1N1, H3N2, and before 1968, H2N2), swine (H1N1 and H3N2) and horses (H3N8 and H7N7) (Fenner *et al.*, 1993; Webster, 1994). In all three species the infection typically causes nasal discharge, fever, unproductive cough and lethargy after a short incubation period. In uncomplicated cases the illness period is fairly short and recovery usually occurs within a few weeks (Bachmann, 1989; Easterday & Hinshaw, 1992; Hannant & Mumford, 1996; Nicholson, 1992). Lymphopenia is usually observed during the acute stage of the disease, followed by a neutropenia in later stages, both probably reflecting the respective migrations of these cells into the infected tissues in response to the infection (Lewis *et al.*, 1986). The viral infection is usually restricted to the upper respiratory tract in humans and horses whereas virus presence is often demonstrated in bronchiolar and alveolar epithelium in swine (Bachmann, 1989; Easterday & Hinshaw, 1992). Secondary infection with bacteria is not uncommon (Bachmann, 1989; Easterday & Hinshaw, 1991; Louria *et al.*, 1959; Martin *et al.*, 1959), particularly in horses where it can lead to severe diseases like strangles or purulent bronchopneumonia (Hannant & Mumford, 1996). Influenza infection has also been suspected to predispose for a rare form of acute encephalopathy in children, Reye's syndrome (Nicholson, 1992) and has been suggested to play a role in sudden infant death syndrome by increasing the effect of bacterial toxins (Jakeman *et al.*, 1991). Another interesting function of influenza HA has recently been suggested by Rott and Cash who showed that the influenza hemagglutinin directly stimulated polyclonal activation of mature B lymphocytes. Different HA subtypes appeared to have different ability to activate B-cells (H10 was classified as a low activator) but all 12 HAs included in the study activated B-cells (Rott & Cash, 1994). The authors speculated if this reactivation of possibly self-reactive B-cells can be important for the understanding of certain autoimmune diseases.

Two subtypes of influenza virus, H7N7 (equine 1) and H3N8 (equine 2), have co-circulated in the equine population and have been responsible for widespread epizootics (Hannant & Mumford, 1996). Equine 2 has, however, caused all outbreaks since 1977 and is currently endemic in Europe and the Americas (Hannant & Mumford, 1996; Webster *et al.*, 1992). It has been suggested that horses may be particularly sensitive to influenza viruses of the H3 subtype, but interspecies transmission of viruses to, or from, horses has been limited (Webster & Guo, 1991). One equine influenza outbreak in China has, however, been shown to be caused by an H3N8 virus variant of avian origin, suggesting, for the first time, a direct transmission of virus from birds to horses (Gou *et al.*, 1992). Different vaccination strategies have been applied to control the spread and negative effects of influenza outbreaks, especially in race and show horses, but the effects have been limited, partly due to the

antigenic drift within the circulating strains (Abusugra *et al.*, 1985; Berg *et al.*, 1990; Hannant & Mumford, 1996; Klingeborn *et al.*, 1980).

Classical swine influenza virus, first isolated in 1931, belongs to the subtype H1N1 and is still circulating in North America while it has been replaced by an H1N1 virus of avian origin, circulating together with H3N2 virus, in European swine (Bachmann, 1989; Easterday & Hinshaw, 1992; Webster *et al.*, 1992). The two H1N1 viruses did, at least temporarily, co-circulate in the Danish swine population (Abusugra *et al.*, 1989). Swine influenza virus has been transmitted from swine to humans (Dacso *et al.*, 1984; O'Brien *et al.*, 1977), occasionally causing disease outbreaks when the virus has continued to spread between humans (Top & Russell, 1977). A reassortant between concomitant human and swine influenza has been isolated from an influenza outbreak in pigs (Sugimura *et al.*, 1980).

A review on ruminant influenza from 1984 (Lopez & Woods, 1984) mentions seven bovine H3N2 isolates and one reindeer isolate of unknown subtype from the former USSR, two H1N1 virus isolates from cattle and sheep, respectively, together with another sheep isolate of neuraminidase subtype N2 from Hungary. Very few other reports of influenza in ruminants have been published, apart from an influenza outbreak in Mongolian camels caused by a human vaccine strain (Yamnikova *et al.*, 1993). Influenza is not mentioned in a reference book on virus infections in ruminants (Dinter & Morein, 1990). Thus, influenza virus infection does not currently appear to be an important disease in ruminants.

Avian influenza virus sometimes crosses the species barrier to infect and cause disease in a mammalian species. Should the avian strain adapt to, and become established in, the new mammalian host species it poses a risk to the human population. Examples of such avian derived influenza strains which have naturally spread to mammals are H1N1 in European swine (Scholtissek *et al.*, 1983), H3N8 in horses in China (Gou *et al.*, 1992; Webster & Guo, 1991) H7N7, H4N5, H4N6, and H3N3 in harbour seals in the US (Callan *et al.*, 1995; Hinshaw *et al.*, 1984; Webster *et al.*, 1981), H13N2 and H13N9 in a pilot whale in the US (Hinshaw *et al.*, 1986) and the object of this thesis, H10N4 in mink. This virus, A/mink/Sweden/84 (H10N4), caused an outbreak of respiratory disease with very high morbidity in farmed mink in southern Sweden in 1984 but has not been isolated from mink since (I). The avian derived H3N8 strain caused at least two outbreaks of influenza with high morbidity in horses in 1989-90 but has not been reported since then, or outside China. The H7N7 and H4N5 strains were involved in two outbreaks of pneumonia in seals but at least H7N7 experimentally appeared to require a coinfection with other pathogens to cause the severe lesions seen during the outbreak (Webster *et al.*, 1981). In contrast, the new H1N1 in swine

successfully replaced the classic swine influenza and has been endemic in the European pig population since the 1980s.

Ferrets were used when influenza virus was first isolated from humans (Smith *et al.*, 1933) and are still often used in influenza research, because they respond to infection with human influenza virus with lesions similar to those seen in adult humans (Renegar, 1992). This is partly beacuse ferrets and humans probably have the same type of sialic acid-containing receptors in the airways (Leigh *et al.*, 1995). Ferrets are also used to produce immune-sera used to discriminate between influenza isolates. It has been shown that influenza virus replicates mainly in the nasal and bronchial epithelium, sometimes in bronchioli, but seldom in alveolar epithelium in ferrets (Husseini *et al.*, 1983; Renegar, 1992). The virus yield from the first replicative cycle, as well as the ability of progeny virus to start the second cycle, have been suggested to be the main limiting factors regulating ferret tissue susceptibility for influenza virus infection (Kingsman *et al.*, 1977). Virus production in ferret alveolar cells *in vitro* have had varying success, but the release of virus from these cells were constantly lower than from cells derived from the upper airways, a feature which has been suggested to contribute to the prevention of alveolar infection (Cavanagh *et al.*, 1979; Husseini *et al.*, 1983).

Mink is a species, closely related to the ferret, which seems to be rather susceptible, or particularly exposed, to human influenza viruses. Serological surveys have shown that farmed mink often have antibodies to concomitant human influenza virus subtypes (I) (Okazaki *et al.*, 1983a; Yagyu *et al.*, 1982). It has also been shown that certain avian and mammalian influenza strains can spread from experimentally infected mink to contact mink (Matsuura *et al.*, 1979; Okazaki *et al.*, 1983b). Mink have also been experimentally infected with several human and avian influenza strains and have reacted with antibody production to all mammalian strains and some avian but seldom shown any signs of disease (Okazaki *et al.*, 1983b; Yagyu *et al.*, 1981). Mild clinical symptoms, i.e. sneezing and nasal discharge, were seen in some mink infected with influenza virus H1N1 and H3N2 of human and swine origin (Matsuura *et al.*, 1979; Yagyu *et al.*, 1981). In contrast, severe rhinitis, respiratory distress, and pneumonia were consistent features following infection with the avian influenza virus, H10N4, isolated from Swedish mink in 1984 (I, III, IV).

History of human influenza

Outbreaks of influenza-like respiratory disease have occurred periodically in humans for more than 2000 years. As early as in 412 BC an epidemic was recorded by Hippocrates. During the devastating "Spanish Flu" pandemic in 1918-19, between 20 and 40 million people reportedly died from this acute, febrile respiratory disease or complications thereof (Webster, 1994). The Spanish Flu killed more people in a few months than the first World War did

in five years (Nicholson, 1992). This phenomenon was predicted as early as in 1767 by Carl von Linnaeus who, in his work *Mundus invisibilis,* stated (freely translated) "The tiniest creatures perhaps cause greater damage than the largest; yes, they may even take the life of more than all wars". The Spanish Flu was first observed in army camps in North America. When the US forces were transported to the front in Europe in 1918 the new influenza virus was introduced into a previously unexposed population at war, which initiated the pandemic.

It was not until 1933 that influenza A virus was first isolated from humans (Smith *et al.*, 1933). Recently, modern technology was used to amplify viral genetic information in fixed tissues from an American soldier who died from acute influenza pneumonia in 1918 (Taubenberger *et al.*, 1997). These analyses showed that the causative agent was an influenza A virus of subtype H1N1 and the name A/South Carolina/1/18 was proposed. The analyzed 1918 HA, NA and NP gene segments were more related to the corresponding mammalian than to avian influenza genes, and the HA sequence appeared closely related to the swine influenza circulating in the European swine at the time (Taubenberger *et al.*, 1997). Why this strain was so devastatingly pathogenic to humans remains a riddle to be solved.

Comparative studies of the viral genomes of influenza strains isolated from humans, animals and birds show that all analysed strains appear to have a common avian ancestor (Gorman *et al.*, 1991; Scholtissek *et al.*, 1993). Different theories have been put forward as to when this transition from avian to mammalian hosts took place (Gammelin *et al.*, 1990; Gorman *et al.*, 1991; Webster *et al.*, 1992), including the suggestion that the strain causing the 1918 pandemic was in fact the introduction of the ancestral strain into the human population (Gorman *et al.*, 1991). The recent findings, however, indicate that the transition probably happened some time before the 1918 pandemic (Taubenberger *et al.*, 1997). After 1919, other pandemic variants of influenza virus, none as pathogenic as the "Spanish influenza", have been introduced into the human population in China following antigenic shifts, i.e "Asian influenza" (H2N2) in 1957 and "Hong Kong influenza" (H3N2) in 1968 (Webster, 1994; Webster *et al.*, 1992; Wharton *et al.*, 1989).

It is generally believed that the Chinese farming practices (i.e. outdoor pigs in and around ponds where domestic ducks and geese are kept) provide the neccessary requirements for genetic assortment between mammalian and avian strains (Yasuda *et al.*, 1991). It has been shown that avian influenza viruses with a variety of HA subtypes (H2-H13) can replicate in the respiratory tract of pigs to a level equivalent to that seen for swine and human viruses (Kida *et al.*, 1994). Phylogenetic analyses of the NP genes also showed that swine isolates were scattered in both avian and human lineages, indicating that pigs may have a relatively low species barrier which in turn allows them to become

infected with both avian and human influenza strains (Scholtissek, 1996). If these strains infect the pig simultaneously, the prerequisite for reassortment is achieved. Pigs occasionally transmit influenza viruses to humans (Dacso *et al.*, 1984; O'Brien *et al.*, 1977; Wentworth *et al.*, 1994) which shows that they have the potential to start a new pandemic if a reassorted virus can replicate in the new host and is sufficiently different, antigenically, from pre-existing strains to evade any immunity in the population (Scholtissek, 1996; Wentworth *et al.*, 1994).

The reintroduction in 1977 of "Russian influenza" H1N1, a strain which had not been isolated since the antigenic shift in 1957, from an unknown reservoir (possibly a laboratory) also caused a pandemic, because preexisting antibodies were not present in humans born after 1957 (Smith & Palese, 1989; Webster, 1994; Wharton *et al.*, 1989). This virus subtype is now endemic in the human population together with the H3N2 influenza viruses (Webster, 1994). Antigenic drift within these subtypes occurs frequently and will provide the virus with a slightly altered antigenic structure, enabling it to escape the immune response triggered by previous infections with the same subtype (Webster, 1994; Wharton *et al.*, 1989).

Like pigs, mink can be infected with avian, human and other mammalian influenza A strains (Yagyu *et al.*, 1982; Yagyu *et al.*, 1981) and could, at least in theory, function as a "mixing vessel", similar to the pig, for new influenza variants which under certain conditions could spread to humans in close contact with the mink. The initial steps in such an adaptation of an avian virus, or reassortant, to a mammalian host involve successful infection and production of infectious virus in the new host species.

Aims of the present study

i) To investigate and describe a severe respiratory disease in farmed mink, seen for the first time in 1984.

ii) To characterize the influenza A virus shown to be the causative agent for this disease outbreak.

iii) To experimentally reproduce and study the course of the disease in mink.

iv) To compare the isolated virus, influenza virus A/mink/Sweden/84 (H10N4), to related influenza viruses, utilizing both genetical and biological models.

Comments on materials and methods

For a more detailed account of the materials and methods used in these studies, the reader is referred to papers I-IV.

Animals (I, III, IV)

Mink for research are only available from commercial herds kept for fur production and are therefore poorly documented from an experimental point of view. All previous experimental influenza infections of mink have been performed in Japan on sapphire mink (Matsuura *et al.*, 1979; Okazaki *et al.*, 1983b; Yagyu *et al.*, 1981), a colour type homozygotous for the aleutian gene (Nes *et al.*, 1988) which is known to cause an increased sensitivity to infections through a Chediak-Higashi-like syndrome (Prieur, 1996). According to the Japanese protocols these mink were also not tested for antibodies against Aleutian disease, a common persistent viral mink disease leading to accumulation of immune complexes and increased susceptibility to other infections (Hunter, 1996). Our mink were controlled for these factors, both of which would increase the susceptibility to infectious diseases such as influenza. Thus, the experimental animals were of a non-aleutian colour type (standard dark mink) and from herds free from aleutian disease, although this information was unfortunately omitted in papers I and II, allowing for unnecessary speculation.

The studies in papers I and III used adult mink (not stated in paper I), whereas the extensive experimental study in paper IV was carried out using eight-week-old mink kits. Younger animals were chosen for the last study for three main reasons: *i*) in using young animals, before the autumn months, we could minimize the risk of previous exposure to human influenza strains, *ii*) mink kits are more similar to rats in body size, which enabled us to use the available aerosol exposure system, designed for mice or rats, without major alterations and *iii*) virus exposure of unsedated animals required manual handling of the mink, something which is very difficult already when the mink are a few weeks older. We chose to use only male mink kits in paper IV, since one of the parameters we wanted to register was daily weight gain, and the growth rates and adult live weights are markedly higher for male than for female mink (Enggaard Hansen *et al.*, 1990; Nes *et al.*, 1988).

Detection of influenza virus (I, II, IV)

Virus isolation and identification

The primary virus isolations from the field material, and from the first experimentally infected mink (I, II) were carried out by inoculation of mink lung suspensions into the amniotic sacs of 11-day-old chicken embryos which were harvested after 48 hours incubation. Human influenza virus is more efficiently isolated in the amniotic, rather than the allantoic, sac because of a

recently described difference in sialic acid-containing receptors between these two sites. SAα2,6Gal receptors, the preferred receptor for human influenza strains, are found only in the amniotic cavity while SAα2,3Gal receptors, preferred by avian and equine strains are present in both amniotic and allantoic sacs (Ito *et al.*, 1997b). Thus, based on empirical experience, we routinely inoculated into the amniotic sac, thereby avoiding the initial virus isolations being influenced by receptor specificity. In paper IV we chose to inoculate the lung and brain suspensions into the allantoic sac, following standard routine procedures for egg passaged isolates, and found no differences in the isolation results as compared to amniotic inoculation, which is in accordance with the described receptor specificity for avian influenza strains.

The presence of virus in the harvests from the inoculated eggs was detected by the HA-test using chicken erythrocytes. The HI test, and in papers I and II also the neuraminidase-inhibition (NI)-test, was subsequently applied to identify the isolated influenza virus subtypes. In the first two papers HI and NI-tests were carried out using reference antisera to all 13 HA and 9 NA subtypes known at the time. In contrast, in paper IV, the two viruses used in the experimental study were identified in HI by use of monospecific ferret antisera to mink/84 and chicken/49 viruses, prepared in our laboratory (II).

Immunohistochemistry

Fixed tissues, mounted on glass slides, were treated with hydrogen peroxide which we empirically have found to reduce the unspecific background staining (IV). A commercially available primary goat anti-influenza A virion polyclonal antiserum (H1N1) was used to detect influenza proteins in the fixed tissues. After rinsing, to remove unbound antibodies, biotin-conjugated swine anti-goat/mouse/rabbit-Ig secondary antibodies were applied and allowed to attach to the primary antibody. This was followed by the addition of an avidin-labelled enzyme detection system (ABComplex/AP, Dako) which, utilizing the strong affinity between biotin and avidin, forms avidin-biotin complexes (ABC) with the secondary biotin-linked antibodies. The avidin-biotin complexes were finally visualised with a red enzyme substrate (Fast Red, Dako). This is a sensitive and influenza A-specific system giving a strong red signal and very little, if any, background staining. In our experiments we could, however, not use this method in brain tissues because of, probably cross-reactive, false positive staining of neurons and axon structures in all slides, similar to the staining observed for rabbit anti-influenza A antibodies in mouse and human brains (Yamada *et al.*, 1996). That staining was believed to be caused by antigenic mimicry between influenza A virus and cytoplasmic dynein. Further investigation into the reasons for the "false positive" staining in mink brains was not included in this study.

Detection of antibodies to influenza virus (I-IV)

Antibodies to mink/84 virus in paper I and to mink/84 and chicken/49 viruses, respectively (IV), were detected by hemagglutination-inhibition (HI) tests. An HI-test is used to detect and identify either antibodies or virus, depending on which component is the unknown sample and will discriminate between different HA subtypes. In paper I, two field isolates of mink/84 virus were used as antigens in the HI-test in an effort to detect any antigenic discrepancy between the hemagglutinins of the two isolates of the same subtype. In paper IV all mink sera were tested in HI-tests against both mink/84 and chicken/49 viruses to detect any antigenic differences between the two H10 hemagglutinins. The HI-test is particularly suitable when discrimination between antibodies to different virus strains of the same subtype is required but it is a manual test and too time-consuming for sceenings of large numbers of samples.

For screening purposes, when the target virus is known, an enzyme-linked immunosorbent assay (ELISA) is more appropriate. An antibody detecting indirect ELISA was developed for the epidemiological screening of almost 3000 mink serum samples (II) and for the comparative experimental study of four defined virus isolates (III). This test was carried out in microtitre wells coated with the appropriate whole virus antigens, purified by sucrose gradient centrifugation as described by Abusugra et al. (1985). The mink sera were tested at dilutions 1:40 and 1:200 (II) or 1:32 (III) and the absorbance was measured at 450 nm on a commercially available automatic reader (Titertek, Flow Lab., Irvine). Absorbance values above 0.5 were considered to be a positive titre in both studies.

Influenza virus strains (I-IV)

The main topic of this thesis, mink/84 virus (A/mink/Sweden/84, H10N4), was isolated from the lungs of Swedish farmed mink in 1984 (I). Five antigenically identical isolates of the same subtype were isolated from the field cases (II). All experimental infections in mink have been made with isolate No. 3900. Through oligonucleotide fingerprinting only minor differences of two to three paired changes were detected between the 3900 and one other isolate, 3876 (III). The two other isolates of this subtype used in this study, mallard/85 (A/mallard/Gloucestershire/374/85) and fowl/85 (A/fowl/Hampshire/378/85), were isolated from feral and domestic birds in Great Britain only a few months after the influenza outbreak in Swedish mink. The two strains were kindly donated by Dr D. Alexander, VLA, UK. Both these strains were similar to mink/84 with a genomic homology around 98%, when estimated by oligonucleotide fingerprinting (III). The observed differences were scattered in all gene segments. Thus, the mink/84 virus and the two concomitant avian derived stains were very closely related. The fourth influenza strain included in this study is chicken/49 (A/chicken/Germany/N/49, H10N7, also known as virus N). This virus was originally isolated from domestic fowl during the

investigation of an outbreak of Newcastle disease in Germany and was later found to be non-pathogenic for chicken, although in other respects similar to the virus causing fowl plague (Dinter, 1949). This virus was chosen for the experimental comparisons since it is a well-defined prototype strain for subtype H10 and apathogenic for chicken. Comparisons between the HAs of mink/84 and chicken/49 have shown that both have single arginins at the cleavage site and identical glycosylation patterns (Feldmann *et al.*, 1988). These strains were chosen to study whether mink were especially sensitive to H10 influenza strains, and if they therefore would respond to infection with avian-derived H10 strains with as severe a pneumonia as when infected with mink/84 virus.

Oligonucleotide fingerprinting (III)

The influenza strains were genetically compared by oligonucleotide fingerprinting. This method is based on the principle that, after treatment with a specific enzyme (T1 RNase) which cleaves bonds adjacent to guanosine residues, the viral RNA will divide into segments, oligonucleotides (ONs), all ending with guanosine 3'-phosphate. After a total digest, the number of segments will be one more than the total number of guanosine residues in the RNA (Kew *et al.*, 1984). These segments are radioactively labelled and separated in a two-dimensional polyacrylamide gel electrophoresis, thus forming a pattern which is characteristic for the viral RNA sequence. By comparing the ON patterns for two virus strains, equally processed, the level of sequence homology and thus the genetic relationship, can be assessed in a computer model which states that 25, 50 and 85% identity between the ON patterns represent 10, 5 and 1% sequence heterology between the two investigated virus strains, respectively (Aaronson *et al.*, 1982). The method is fast and sensitive but is useful only for strains with a base sequence homology above 90% and not for more divergent strains.

Experimental infection (I, III, IV)

Experimental infection of mink was made through either intranasal instillation (I, III) of virus suspension or exposure to virus aerosol (IV). Intranasal instillation is easy to perform, needs no special equipment, but a prerequisite (when used in mink) is that the animals are anaestethized. It was clear in our studies that, although the mink were given the same amount of anaesthetics per kg body weight, differences in their response to the intranasal instillation were frequently observed. The amount of sneezing or swallowing varied between none and constant. This influences the viral dose actually reaching the mink airways, although no such variation was registered by the fairly rough methods used in papers I and III. For the more detailed experimental studies of the sequential pathological changes in the airways (IV) we therefore chose a method of infection where the mink were unaffected by anaesthetics. The Battelle aerosol system used is a commercially available system used for mice and rats. Since the variables influencing the aerosol production and

distribution in this and similar systems are well documented (Dahlbäck *et al.*, 1989; Newman *et al.*, 1986; Raabe *et al.*, 1988; Schlesinger, 1985), the viral exposure could be calculated. Due to lack of data for mink we estimated the amount of virus deposited in the lungs using physiological data from ferrets and other species (Boyd & Mangos, 1981; Raabe *et al.*, 1988; Vinegar *et al.*, 1982). This system for virus exposure allows identical procedures for the two viruses as well as for all mink, which in turn made our sequential comparisons unbiased by differences between administered doses, although the exact deposited doses can only be estimated.

Pathology (I-IV)

Pathological investigations were used to characterize the field outbreak of mink influenza (I, II) as well as for studies of pathological lesions in experimentally infected mink (I, III, IV). The field cases consisted of animals found dead in the affected farms and submitted to us via ordinary mail service. The autolytic status, of these animals, was therefore variable. In the experimental studies, however, all necropsies were performed directly after euthanasia and tissue samples for histopathology were removed from the carcasses and immersed in fixative early during the procedures, thus minimizing the changes caused by autolysis. All tissues for histopathology were fixed in either 10% formaldehyde (I, II, III), which is the routine method in our laboratory, or PLPG (periodate-lysine-paraformaldehyde-glutaraldehyde) (Alexandersen *et al.*, 1987) which was recommended to us as a fixative more suitable to preserve the viral structures required for *in situ* viral detection (S. Alexandersen, 1994, pers. comm.).

To achieve a comparable and reasonably objective description of the extent of lung consolidation after experimental influenza infection (IV) we chose to measure the area density of pneumonia. Large, fixed tissue sections, one from each of the remaining five lung lobes, were randomly coded and photographed and all areas of consolidation were marked and controlled through a microscope. Consolidations caused by atelectasis without signs of inflammation were excluded. The areas of the remaining consolidations were measured, as well as the total area of each lung section. After the codes were broken the total consolidations for each mink lung were calculated as per cent of the total photographed lung area. This provided us with figures, unbiased by group affiliation, allowing statistical comparisons of the developments of lung lesions over time in the different experimental groups.

Results and general discussion

Characterization of the disease outbreak (I, II)

The predominant clinical symptoms observed in the affected mink during the disease outbreak in October 1984 were typical for upper respiratory disease, i.e. coughing, sneezing, ocular and nasal discharge, and loss of appetite. The disease also clearly had a contagious progress, spreading both within and between farms until 33 farms, housing approximately 100,000 mink, in the same area reported similar disease signs. These symptoms are among those seen in mink with distemper caused by morbillivirus. However, a classical outbreak of distemper in a mink farm usually displays a combination of symptoms from the central nervous system, respiratory tract, and later also digestive tract and skin, characteristic for the systemic infection caused by distemper virus (Bindrich *et al.*, 1959; Pearson & Gorham, 1987). No signs of convulsions, diarrhoea, swelled paws or hyperkeratosis were reported from the affected farms, and distemper has not been recorded in Swedish mink farms since 1968. Thus, there was little reason to believe that distemper virus had caused this outbreak. The most common respiratory disease in mink at the time was hemorrhagic pneumonia caused by *Pseudomonas aeruginosa* (Knox, 1953). This disease usually occurred during the late autumn months, causing peracute deaths with blood seeping from the mouth and nostrils of the dead animals, but was not associated with catarrhal symptoms like those seen during the outbreak in 1984. Apart from the two mentioned infections only one other infectious respiratory disease had been described in mink at the time: when infected with Aleutian disease virus at an early age, young mink kits had been shown to develop an acute, fatal interstitial pneumonia instead of the more well-known chronic infection characteristic for Aleutian disease (Alexandersen, 1986). No young mink kits exist in farms during October. It could thus be suspected, already from the clinical observations in the mink farms, that the reports in October 1984 described a previously unknown disease in farmed mink.

The necropsies performed on the field cases showed pneumonia in 37 of the 46 mink suitable for pathological investigation. Some of these mink had a purulent or necrotic bronchopneumonia, indicative of secondary bacterial infection, whereas most had interstitial pneumonia with masses of macrophages and desquamated epithelial cells, and a marked epithelialization, in the alveoli. The lesions were very similar to those described for primary influenzal pneumonia in pigs and humans (Bachmann, 1989; Louria *et al.*, 1959; Nicholson, 1992). Such lesions are not pathognomonic for influenza virus infection but rather indicate a viral aethiology. However, five antigenically identical strains of influenza A virus of subtype H10N4 were isolated from lung tissues of mink from five of the affected farms, strongly indicating the aethiology.

Further evidence that this new influenza virus was in fact contagious for mink, and the cause of the observed outbreak, was gained from an experimental pilot study. In this experiment we showed that adult mink, when intranasally inoculated with the influenza H10N4 virus, developed a respiratory disease which clinically and pathologically mimicked the field cases. The virus could be reisolated from the lungs of experimentally infected animals, as well as from contact animals which developed identical lesions and disease signs. All infected animals also produced a detectable serum antibody response to H10N4 virus from four days after infection and, as expected, antibodies were detected a few days later in the contact mink. The new virus, A/mink/Sweden/84 (H10N4), referred to below as mink/84, thus met the specifications in Koch's postulate for a causative agent.

Several different bacterial isolates were recovered from the lungs of most of these field cases, which is expected when the cultures are made several days after death. However, no consistent finding was made to implicate that the outbreak was caused by a primary bacterial infection, which also corresponded with the observed lung lesions. It has been suggested that protease-producing bacteria, such as certain strains of *Staphylococcus aureus*, can play a synergistic role in a combined influenzal-bacterial pneumonia by activating the HA through proteolytic cleavage (Tashiro *et al.*, 1987). The HA of the H10 virus strain (A/chicken/Germany/49, H10N7, referred to below as chicken/49) used in that study was, however, insensitive to *S. aureus* protease which shows that sensitivity to bacterial proteases is not a common feature for all influenza hemagglutinins. The protease production of the bacteria found in the lungs of our dead mink was not analyzed, but the presence of protease producing bacteria cannot be excluded. The multitude of bacterial species isolated, however, made it less likely that bacterial proteases would be a prerequisite for mink/84 virus infectivity. Furthermore, growth of bacteria was not detected in the virus-infected mink with pneumonia in our subsequent experimental studies which were carried out under controlled hygienic conditions. Taken together, this strongly supported our initial suggestion that the virus itself, without assistance from a concomitant bacterial infection, could cause the severe respiratory disease seen in the mink, although as expected, secondary bacterial infection often followed under field conditions.

Classification and genetic comparisons of virus isolates (I, III)

The primary isolations of virus from the field cases were recovered from the amniotic cavity of chicken embryos. All five isolates were immunologically identical in HI and NI tests against all 13H and 9N reference antisera available at the time, by reacting with H10 and N4 antisera. This is standard procedure for the subtyping of new influenza strains but will only describe the immunogenic properties of the two surface glycoproteins, i.e. the two most variable influenza proteins. The H10 hemagglutinin has been isolated from

several bird species, including feral ducks, shorebirds and gulls and is mostly apathogenic (Dinter, 1949; Otsuki *et al.*, 1987; Ottis & Bachmann, 1983; Webster *et al.*, 1992), but has occasionally been associated with disease-outbreaks in turkeys (Karanukaran *et al.*, 1983). The N4 NA has occasionally also been isolated from bird species (Otsuki *et al.*, 1987; Webster *et al.*, 1992). However, neither the H10 nor the N4 subtypes appear to be very common in birds and none of these surface antigens have been described in viruses isolated from mammals before or after the disease outbreak in the Swedish mink (Murphy & Webster, 1990; Webster *et al.*, 1992).

We originally proposed that the mink/84 virus was of avian origin and that direct transmission from migrating birds might have been the source of infection in the mink farms. Another possible route of infection is through influenza virus contamination of the unheated chicken offal included in the standard mink feed. No isolations of H10N4 influenza virus from wild or domestic birds in Sweden have been made. However, a survey of avian influenza virus in Japan during the winter before the mink outbreak revealed several isolations of the H10N4 subtype from the faeces of migrating waterfowl (Otsuki *et al.*, 1987). Those findings also prompted the Japanese authors to speculate whether the Swedish mink might indeed have been infected by such fowl. Further support for migratory birds as the source of infection was found when the H10N4 subtype was isolated from both feral mallard and domestic fowl in two locations in England within six months after the disease outbreak in mink (Alexander & Gough, 1986).

Genetic studies in other laboratories have compared the H10 hemagglutinin (Feldmann *et al.*, 1988) and the nucleoprotein (Reinhardt & Scholtissek, 1988) genes of mink/84 virus and chicken/49 virus. The latter being the prototype H10 influenza strain, apathogenic for chicken (Dinter, 1949). The two NP genes were both closely related to the NPs of other avian strains, but differed distinctly from the NPs of human influenza strains (Reinhardt & Scholtissek, 1988). From phylogenetic studies of NPs of avian and mammalian influenza viruses, it appears that avian NPs are functionally optimized and thus undergo little change over time, whereas human strains have a high mutation rate most probably due to the necessity to overcome the immune response of the host (Scholtissek *et al.*, 1993; Gammelin *et al.*, 1990). In both these studies mink/84 NP fell within the avian lineage.

Feldmann *et al.* (1988) showed that the HAs of mink/84 and chicken/49 H10 viruses, isolated 35 years apart, were closely related with nucleotide and amino acid sequence homologies of 89 and 94%, respectively. Between isolates of a single HA subtype such differences in amino acid sequence are expected to be below 10%, whereas differences between subtypes can be as high as 75% (between H1 and H3) or as low as 20% (between H2 and H5) (Murphy & Webster, 1990). Both mink/84 and chicken/49 HA had oligosaccarides in the

same positions, with only one of them attached to the globular domain (an unusually low level of glycosylation) (Feldmann *et al.*, 1988). This means that the glycosylation pattern, which can bear a strong influence on HA cleavability (Deshpande *et al.*, 1987; Kawaoka *et al.*, 1984; Rott *et al.*, 1995), is probably not the reason for the observed difference in pathogenicity between the two viruses. The two HAs varied in only 35 amino acids of which only one difference, gly 218 in chicken/49 and arg 218 in mink/84, was located in a receptor binding site (Feldmann *et al.*, 1988). The same single amino acid exchange has, interestingly, been observed to alter the receptor binding properties of another influenza virus (Daniels *et al.*, 1987). The initial attachment and uptake of virus into the infected cells does not, however, seem less succesful for chicken/49 than for mink/84 virus in mink airways (IV), thus suggesting that receptor specificity is not a likely explanation for the difference in pathogenicity.

When the whole genomes of three H10N4 viruses isolated less than six months apart, two from British birds and one from Swedish mink, were genetically compared by oligonucleotide mapping (III) they showed a very high (≈ 98%) sequence homology in all gene segments, confirming that the H10N4 virus isolated from mink was indeed of recent avian origin. So far, the gene segments from the two H10N4 isolates from bird species have not been sequenced. Thus the NP and HA genes, particularly the cleavage and receptor binding sites, of these viruses cannot be compared in any detail to the corresponding genes of mink/84 and chicken/49 viruses. However, when the large oligonucleotides (ONs) for these genes (genes 4 and 6) were compared for mink/84, mallard/85, and chicken/49, obvious differences were observed. The mallard and mink viruses had six out of seven ONs of gene 4, and five out of six ONs of gene 6 in common, whereas mink and chicken viruses only had two out of seven and one out of six ONs in common in the corresponding gene segments. In spite of the relatively few common oligonucleotides the nucleotide homology between the HA genes of chicken/49 and mink/84 was 89% (Feldmann *et al.*, 1988).

Antibody response (I-IV)

The presence of antibodies to the mink/84 virus was confirmed by HI-test in sera from convalescent mink during the outbreak (I) and by ELISA in more than half of 2,400 samples tested from the affected farms five months later (II). No antibodies to subtype H10N4 were detected, by HI-test, in a few samples from unaffected farms during the outbreak, or in 491 sera from mink in other parts of the country five months after the outbreak and tested in ELISA. The initial serological investigation, during the disease outbreak, also confirmed the results from other surveys (Okazaki *et al.*, 1983a; Yagyu *et al.*, 1982) that mink often have antibodies to concomittant human influenza strains, without any symptoms of disease. The farmed mink are presumably exposed to human influenza virus from their keepers during influenza

outbreaks in humans and susceptible enough to produce an antibody response, unlike the pet bear *Brunus edwardii* which, although in extremely close contact with humans, appears to be totally resistant to infection (Blackmore *et al.*, 1972).

Our results indicate that the new influenza virus was not present in all mink farms in the affected area or elsewhere. Occasional, limited serological screenings of mink sera from Swedish farms have been made during subsequent years but antibodies to influenza virus of subtype H10N4 have not been detected (unpublished results), nor have similar clinical or pathological disease signs been observed after 1984. Thus, it seems that the outbreak was an isolated event, although dramatic in appearance at the time.

When mink were first experimentally infected, intranasally, with the field isolate of mink/84 virus (I) they responded with a detectable antibody production (ELISA) from day 4 after infection. In the contact-mink, detectable antibodies first appeared on day 6-9. In the subsequent experimental infections, intranasally (i.n., III) as well as via aerosol (a. IV), all mink infected with mink/84 virus produced positive antibody titres detected by HI or ELISA on day 7, but were negative in samples collected on day 3 (III) or 4 (IV). Positive antibody responses were also seen by day 7 in all mink infected with fowl/85 i.n., three out of six mink infected with mallard/85 i.n., and in all mink infected with chicken/49 via aerosol. By 14 days after infection all mink, intranasally infected with any of the four viruses, were positive for antibodies to the homologous virus in ELISA. All experimentally infected mink, as well as the responding contact-mink, in paper III showed a titre increase by at least two titration steps in the five-fold dilutions used, reaching a final titre of 1:1250 or more. A simplified summary, compiled from papers III and IV, of the temporal appearance of detectable antibody, correlated to virus type and mode of infection is shown in Table 1.

The appearance of detectable antibodies to mink/84 as early as day 4 in the first experimental study and not until day 7 in the subsequent studies may have been influenced by a difference in the number of egg passages between the two inocula, i.e. one passage in paper I and 3-4 passages in papers III and IV. This can, however, only be subject to speculation since the mink groups were too small for any conclusions to be drawn. Both fowl/85 and mallard/85 spread to contact-mink as detected by seroconversion, whereas chicken/49 did not. The latter result corresponds with the findings that chicken/49 could not be reisolated from the lungs of infected mink and did not spread within the infected lungs (IV), thus making it unlikely that substantial amounts of infectious chicken/49 virus were produced in mink lung tissues.

Table 1. Serum antibody response after various modes of infection in mink exposed to the influenza viruses mink/84, fowl/85, mallard/85, or chicken/49. Results were compiled from papers III and IV

time after	mink/84			fowl/85		mallard/85		chicken/49		
exposure	i.n	a.	c.	i.n.	c.	i.n.	c.	i.n.	a.	c.
0	-	-	-	-	-	-	-	-	-	-
3 h	/	-	/	/	/	/	/	/	-	/
6 h	/	-	/	/	/	/	/	/	-	/
12 h	/	-	/	/	/	/	/	/	-	/
1 d	/	-	/	/	/	/	/	/	-	/
2 d	/	-	/	/	/	/	/	/	-	/
3 d	-	/	-	-	-	-	-	-	/	-
4 d	/	-	/	/	/	/	/	/	-	/
7 d	+	+	(+)	+	-	(+)	-	-	+	-
14 d	+	/	+	+	(+)	+	(+)	+	/	-
18 d	+	/	+	+	(+)	+	(+)	+	/	-
22 d	+	/	+	+	(+)	+	(+)	+	/	-

i.n. = intranasal inoculation, **a.** = aerosol, **c.** =contact
+ = ELISA titres ≥ 1:50 (i.n. and c.) or HI titre ≥ 1:32 (a.)
- = ELISA titre <1:10 or HI titre < 1:8
(+) = some animals were negative, **/** = samples not taken

Comparative experimental infection (III, IV)

In the experimental study in paper III, intranasal and contact infection of mink were used to compare the pathogenicity of mink/84, fowl/85, mallard/85, and chicken/49 viruses. All animals in this study were sacrificed 22 days after virus exposure. Pulmonary lesions could therefore be studied only three weeks after infection. The development of early lesions, during the first week after viral exposure were instead described in a subsequent experimental study (IV). In the latter study we chose to compare only the two virus strains, mink/84 and chicken/49, for which obvious differences in pathogenicity had been observed (III) and the whole nucleotide sequences of the HA genes were known (Feldmann *et al.*, 1988).

It was obvious from the study in paper III that all three H10N4 influenza viruses, irrespective of species of origin, caused clinical disease signs, antibody response, and lung lesions, i.e. bronchitis, bronchiolitis and interstitial pneumonia. These manifestations were very similar to those observed in association with the original outbreak in the mink farms. All three viruses were also transmitted from mink to mink via contact, although minor differences in contact transmission were observed. The chicken/49 virus, of the same H10

subtype but with a different NA (N7), also induced antibody response and lung lesions. The lung lesions were, however, less pronounced than those caused by the H10N4 viruses. No disease signs or indications of contact transmission were, however, observed for the H10N7 virus.

A more detailed picture of the sequential development of the lesions in the respiratory tract could be drawn when mink were studied during the first week after aerosol exposure to either mink/84 or chicken/49 virus (IV). The results from virus isolations and immunohistochemical detection of viral antigens are summarized in Table 2. During the first three observation points, 3, 6 and 12 hours after virus exposure, similar lesions and locations of viral antigens were observed for both viruses. During the first 1-2 days the infected mink reacted with a rapid inflammatory response characterized by destruction of epithelial cells and influx of macrophages and neutrophils in the nasal mucosa, bronchi, bronchioli, and alveoli. However, as early as the second day post exposure (PE), marked differences between the two groups were recorded both histopathologically, morphometrically and immunohistochemically. In the mink infected with mink/84 virus the initial focal lesions gradually spread to virtually all parts of the lungs during the week they were studied. The lesions were best described as a severe progressive bronchointerstitial pneumonia. Viral antigens were detected in substantial numbers in the lungs and in the nasal mucosa, as well as in the regional lymph node and in the tracheal epithelium. One mink actually died 7 days PE from having the severe pneumonia covering 100% of the lung tissues examined. The viral antigens detected in the tracheal epithelium did not appear until 2 days PE, which indicated that they were the result of infectious virus spreading from either nasal or lower respiratory epithelium. Virus was recovered from the lungs of all mink, except one, as well as from the brains of six mink 12 hours to 7 days PE. In contrast, in the mink infected with chicken/49 virus, the pulmonary lesions remained focal, and presence of viral antigens sparse, all through the week and the nasal mucosa rapidly recovered from the initial viral infection. Reparative processes were also observed in the lungs from day 4 PE. No spread of virus to the tracheal epithelium was observed, but the presence of viral antigen in the regional lymph node was similar in time and location to that of the mink/84 mink.

The difference in severity of pneumonia between the mink/84 and the chicken/49 groups were obvious, but difficult to quantify and compare objectively by histopathological examination alone. By also utilizing morphometry on coded lung sections, we obtained a relatively unbiased asessment of the area density of pneumonia (ADP) for each mink. When the ADP-values are plotted (Figure 1) it is clearly seen that the two viruses cause pulmonary lesions of distinctly different spatial distributions. The observed difference was also statistically significant on a 5% level (III).

Table 2: Virus presence, as detected by ABC-immunohistochemistry or virus culture, in various tissues of mink after experimental infection via aerosol with mink/84 or chicken/49 influenza viruses. PE refers to time after exposure to virus. Extracted from paper IV

time PE	mink/84					chicken/49				
	nose	trachea	lung	l.n	brain	nose	trachea	lung	l.n	brain
3 h	/ -	/ -	+ -	/ -	- /	/ -	/ -	- -	/ -	- /
6 h	/ (*)	/ -	+ (*)	/ -	- /	/ (*)	/ -	- (*)	/ -	- /
12 h	/ (*)	/ -	+ (*)	/ -	+ /	/ (*)	/ -	- (*)	/ -	- /
1 d	/ *	/ -	+ *	/ (*)	+ /	/ (*)	/ -	- *	/ (*)	- /
2 d	/ *	/ (*)	+ *	/ (*)	+ /	/ (*)	/ -	- (*)	/ (*)	- /
4 d	/ *	/ *	+ *	/ -	+ /	/ (*)	/ -	- (*)	/ -	- /
7 d	/ (*)	/ -	+ *	/ -	+ /	/ -	/ -	- -	/ -	- /

nose = nasal mucosa, **l.n.** = mediastinal lymph node
+ (left) = virus reisolated in eggs
* (right) = virus demonstrated by immunohistochemistry, (*) in scattered cells only
- = if to the left; virus not reisolated, if to the right; immunohistochemistry negative
/ = not done

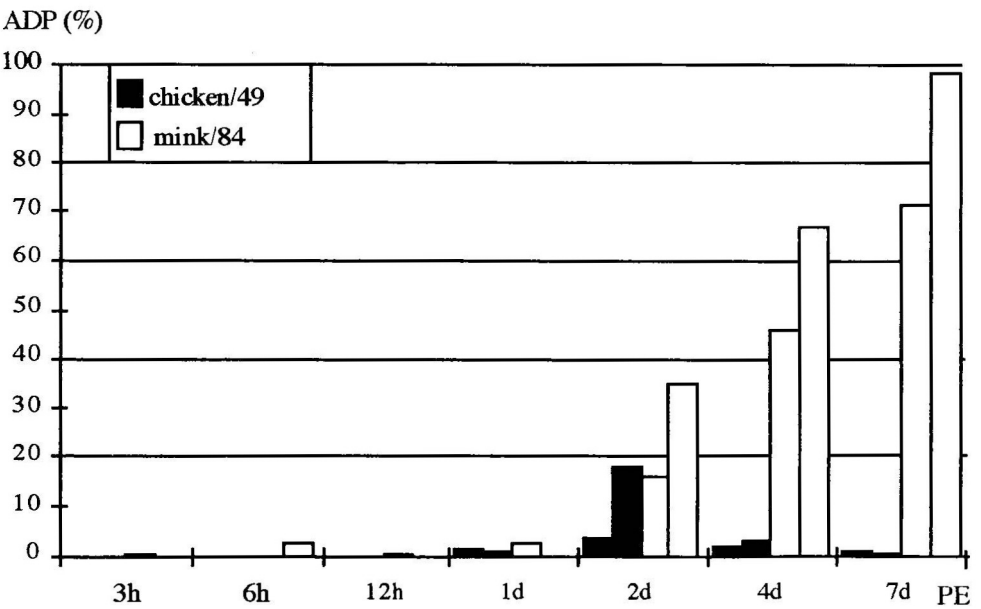

Figure 1: Area density of pneumonia (ADP) for each mink exposed to mink/84 (open bars) or chicken/49 (filled bars). PE refers to time after exposure to virus. From paper IV

When the morphometrical (Figure 1) and histopatological results are considered in combination with the results from virus isolations and immunohistochemistry (Table 2), we propose that the recorded difference in pathogenicity between chicken/49 and mink/84 viruses is not caused by factors influencing virus adherence or entry, such as receptor specificity. It is more likely that the explanation is found among factors influencing virus replication and spread, such as sensitivity to proteolytic cleavage.

Thus, in response to the question initiating the experimental studies in papers III and IV, mink do not respond to infection with all avian-derived H10 influenza viruses with as severe a pneumonia as when infected with mink/84. However, the factors in the mink or the virus causing this increased pathogenicity for mink of A/mink/Sweden/84 (H10N4), and to some extent other H10N4 viruses, remain to be identified.

Addendum: *in vitro* study

Induction of interferon

Influenza virus infection can induce varying levels of interferon α and β (type I IFN) production in infected cells (Bender & Small, 1992), but the type I IFN-response to influenza infection varies between different cell types (Ronni *et al.*, 1997). The ability of IFN to render cells resistant to subsequent infection with the same virus, or another IFN-sensitive virus, is commonly applied in bioassays for type I IFN, measuring antiviral effects. In brief, dilutions of a sample with unknown content of IFN are incubated on monolayers of cells. These cell-cultures are subsequently inoculated with a cytopathic virus, such as vesicular stomatitis virus (VSV), in an amount known to cause 100% cytopathic effect (CPE) in the same cell cultures within 24 hours. An inhibition of the viral-induced CPE indicates that the cells are protected by IFN or IFN-like substances present in the sample.

Our samples were collected from mink lung-cell monolayers (MiLu-cells), inoculated with mink/84 or chicken/49 viruses with and without trypsin added to the medium. Supernatants collected 1, 6, 12, 24, 48, and 72 hours after infection were analyzed for their content of IFN by a bioassay carried out according to Ojo-Amaize et al. (1981). Briefly, each sample was diluted 1:2 and 1:10 in Eagle's MEM and added to microtitre wells precoated with baby hamster kidney (BHK)-cells. Control wells were prepared with samples from non-infected cell-cultures. After 24 hours' incubation (5% CO_2, 37°C) the supernatants were removed and VSV in Eagle's MEM was added, in an amount known to cause 100% CPE in untreated BHK cell cultures within 24 hours. After a further 24-hour incubation CPE was assessed by microscopical examination.

Surprisingly, no IFN-activity was observed in samples from mink/84 virus-infected MiLu-cells. Neither did the supernatants from MiLu-cells infected with chicken/49 virus without trypsin show signs of IFN-activity. In contrast, IFN-activity was seen, as inhibtion of CPE, when the BHK-cells had been pretreated with supernatants collected from MiLu-cells infected 48 and 72 hours previously with chicken/49 virus in the presence of trypsin. Thus, the results from this preliminary study indicate that chicken/49 and mink/84 viruses may differ in their ability to induce IFN-production in MiLu-cells.

Type I IFN contributes to the antiviral mechanisms by inhibition of viral replication, increase of MHC I-molecule expression, and increased NK-cell-activity. Thus, a poor induction of type I IFN by mink/84 virus *in vivo* could benefit virus spread in the mink tissues. The results were, however, obtained from an indirect assay and identification of the VSV-inhibiting factor was not done. The antiviral activity in the supernatant can be caused by a variety of cytokines, IFN being one of them. A more specific assay in which induced IFN, or IFN-like substances, can be identified directly in the MiLu-cell supernatants is necessary to further explain these preliminary results.

Influenza virus growth in cell culture with and without trypsin

To provide additional information about the two influenza A virus isolates (mink/84 and chicken/49) used in paper IV, comparisons with regard to growth patterns and interferon-production were made in mink lung-cell (MiLu) cultures. This cell line was originally developed in our laboratory but was kindly returned to us by Dr A. Uttenthal, SVS, Denmark, for this study. Most influenza A viruses are cleaved extracellularly and normally require an addition of trypsin to the medium for multicycle growth in cell cultures (Webster, 1994). Among the few exceptions are some of the most pathogenic avian viruses, subtypes H5 och H7 (Rott, 1979), and one human H1 strain (Boycott *et al.*, 1994) which are all cleaved by intracellular proteases and will produce infectious virus in cell culture without added trypsin.

Monolayers of MiLu cells were grown in petri dishes (Nunc) and infected with either mink/84 or chicken/49 virus. The virus was allowed to adsorb for 30 minutes. Five μg/ml of trypsin was added to the medium in three out of six dishes for each virus isolate. All cultures were incubated in 5% CO_2 at 37°C. Samples of the supernatants were taken 1, 6, 24, 48 and 72 hours after infection. All samples were tested for hemagglutinin activity by standard hemagglutination (HA) test. Samples from the 72-hour cultures were then diluted 10^{-1}-10^{-7} and inoculated in the allantoic cavities of 11-day-old embryonated chicken eggs. The virus yields were harvested after 48 hours and again tested in HA.

Both viruses have previously been grown in MDCK, MDBK and CE cells without producing cleaved hemagglutinin and both viruses have nucleotide

sequences characteristic for restricted cleavability (Feldmann *et al.*, 1988). However, our observations from the experimental study (IV) suggested that the different spread of the two viruses may be associated with differences in their replicative patterns in mink lung tissues. Such differences were indeed observed *in vitro* in the MiLu cell culture. The difference between the isolates was clearcut, since mink/84 virus growth was sustained in MiLu cells without added trypsin, reaching a HA titre of 1:32 at 72 hours after infection. No virus growth (HA<1:2) was detected by HA-test for chicken/49 virus without trypsin. When trypsin was included in the culture medium, both viruses reached a HA-titre of 1:256 at 72 hours after infection.

Reisolations of both viruses were made from the eggs infected with supernatants from the 72-hour-cell cultures with, as well as without, trypsin in the medium. However, a marked difference in virus yield was observed. In the supernatant from petri dishes without trypsin, chicken/49 virus only reached a virus titre of $5x10^2$ EID_{50}/ml, whereas a 100-fold more mink/84 virus was recovered, i.e. $5x10^4$ EID_{50}/ml. The limited yield of chicken/49 virus represents a virus content in the harvested supernatant which is not detectable in HA. Both viruses were recovered at $5x10^6$ EID_{50}/ml from the trypsin-treated cell cultures.

As could be expected, both viruses produced substantial amounts of infectious virus at 72 hours when trypsin was included in the culture medium and the virus yield was seen to increase by 128 HA units/ml (HAU/ml) from 6 to 72 hours after inoculation, as detected by HA-test. In contrast to these expected results, mink/84 produced increasing amounts of progeny virus in MiLu cells without trypsin being added to the medium. The virus yield, as detected by HA, was less than the amount produced in the trypsin-enriched cultures, but still showed an increase by 16 HAU/ml from 6 to 72 hours after inoculation. If an increase of at least 8 HAU/ml within 72 hours is used to indicate production of infectious virus, as proposed by Tashiro et al. (1987), our results suggest an HA-cleavage of the mink/84 virus in MiLu cells by proteases other than exogenously added trypsin (Figure 2).

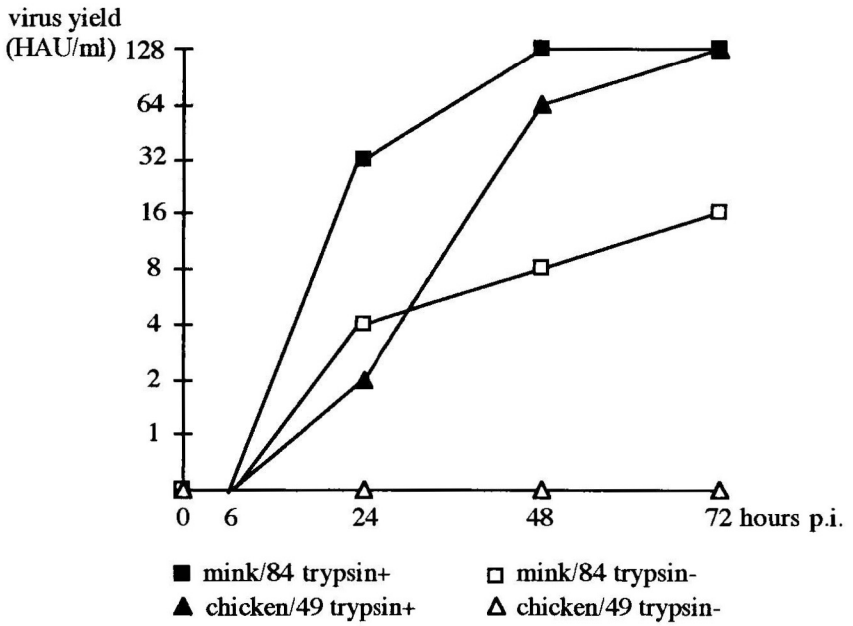

Figure 2: Virus yields in MiLu cells after infection with mink/84 and chicken/49 viruses with and without trypsin added to the medium.

The HAs of most avian and mammalian influenza viruses are cleaved extracellularly by proteases secreted by a restricted number of cell types (Boycott *et al.*, 1994; Rott *et al.*, 1995; Webster, 1994). Highly pathogenic avian strains of subtypes H5 or H7 are cleaved intracellularly by ubiquitous proteases, but in contrast to mink/84, such viruses have an amino acid sequence at the cleavage site which predisposes for the increased cleavability (Rott *et al.*, 1995). One mammalian influenza strain (A/WSW/33, H1N1) has also been shown to be cleaved intracellularly, at the stage of viral entry (Boycott *et al.*, 1994).

The NA has been implicated to play a role in intracellular cleavage of the A/WSW/33 virus (Boycott *et al.*, 1994). NA has also been suggested to be important for the release of progeny virus from infected cells by preventing aggregation of budding virus particles on the host cell surface (Liu *et al.*, 1995). The two influenza viruses we compared, mink/84 (H10N4) and chicken/49 (H10N7), have very similar HAs on the basis of theoretical assessment of cleavability (Feldmann *et al.*, 1988), but different subtypes of NA. One can thus speculate, whether a specific cleavage of mink/84 HA, but not of chicken/49 HA, is caused by proteases present in the mink lung cells. The influence of NA could also be different for N4 and N7, in such a way that the former but not the latter contributes to the production of infectious virus in MiLu cells.

We have not yet investigated if the MiLu-cells secrete any proteolytic enzymes into the medium or if they contain such proteases. Nor have we investigated where the activation of mink/84 HA takes place, if there are signs of progeny virus aggregation on the MiLu-cell surface, or if similar growth patterns in MiLu-cells are seen for H10N4 viruses of direct avian origin. These are indeed interesting subjects for future studies!

Concluding remarks

Influenza viruses are likely to have developed parallel to humanity for thousands of years. The influenza virus is well suited for survival, and evasion of the human immune response, through the ability to create virus variants which are not recognized by a previously infected population. This can basically be done in three ways:

- Antigenic drift. Mutations in the genes coding for the surface glycoproteins, predominantly HA, creating alterations in the antigenic properties, thus diminishing the effect of the human immune response. Antigenic drift is the main reason why the influenza vaccines have to be modified regularly, even if the target virus is still of the same subtype.

- Antigenic shift. Replacement of the HA gene alone or more genes creates an antigenic shift. The result can be a virus well adapted to growth in human tissues, but with a HA not previously encountered by the population. Such antigenically new viruses can cause a pandemic if other circumstances are right for viral spread. This reassortment of genes can happen in nature only when a cell in the host is simultaneously infected by two or more influenza viruses of different origins. Such reassortment can occur in pigs and perhaps also in other mammalian species. Reassortants can also be experimentally created *in vitro*.

- A direct invasion of the human population by an avian virus. It is generally believed that an initial adaptation of the virus to pigs, which later can transfer the virus to humans, is the most likely way for this rare event to happen.

However, a new influenza virus of subtype H5N1, previously only isolated from birds, has recently been isolated from a young boy dying from pneumonia in Hong Kong (de Jong *et al.*, 1997). The human isolate is closely related to an H5N1-virus which recently caused outbreaks of avian influenza in chickens is the same area (A.D.M.E. Osterhaus, 1997, pers. comm.). This

may be the first direct transmission of influenza virus from birds to humans. It remains to be investigated if this virus will spread in the human population.

Mink are naturally infected with concomitant human influenza viruses (I) (Okazaki *et al.*, 1983a; Yagyu *et al.*, 1982) and will occasionally transmit human influenza virus through contact under experimental conditions (Matsuura *et al.*, 1979; Yagyu *et al.*, 1981). We have shown that mink can also be naturally infected with a highly pathogenic influenza virus, A/mink/Sweden/84 (H10N4), presumably directly transmitted from birds. This virus causes a substantial production of infectious virus in the infected mink and spreads between mink through contact.

The fact that mink can be naturally infected with both human and avian influenza strains defines them as possible mixing vessels for reassortants and suitable hosts for adaptation of avian strains to mammalian hosts. The farming practices, obviously exposing mink to human viruses could theoretically equally well expose humans to viruses from mink. The same farming practices, however, probably prevent establishment of endemic influenza among farmed mink through the low contact rates between farms, a biased age distribution throughout most of the year, as well as very limited contact between farmed mink and aquatic fowl or migratory birds in most parts of the country.

Therefore, the likelihood of simultaneous infection of mink with avian and human influenza is low. Thus, even if the possibility of reassortment is there, the risk that it should occur is minimal. But still -?

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