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Leif Oxburgh

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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A PCR-based system for the amplification of viral genes directly from clinical material was developed. HA genes were amplified from clinical samples and compared with HA genes from virus which had been cultured in embryonated hens´ eggs. Nucleotide sequencing revealed that growth in eggs leads to amino acid substitutions at the RBS of HA of recent American lineage strains. Since HA is the most important target of the humoral immune response against influenza virus, this finding questions whether the embryonated hen´s egg is a suitable culture system for the isolation and propagation of recent virus strains.

Key words: equine influenza, antigenic drift, epidemiology, phylogeny, adaptation.

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Leif Oxburgh
Department of Veterinary Microbiology/Virology
Uppsala

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Author´s address: Leif Oxburgh, Department of Veterinary Microbiology/Virology, Swedish University of Agricultural Sciences, Biomedical Centre, Box 585, S-751 23, Uppsala, Sweden.

Contents

Appendix

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Oxburgh, L., Berg, M., Klingeborn, B., Emmoth, E. and Linné, T. (1993). Equine influenza from the 1991 Swedish epizootic shows major genetic and antigenic divergence from the prototype virus. Virus Research 28, 263-272

II Oxburgh, L., Berg, M., Klingeborn, B., Emmoth, E. and Linné, T. (1994). Evolution of H3N8 equine influenza virus from 1963 to 1991. Virus Research 34, 153-165.

III Oxburgh, L., L. Åkerblom, T. Fridberger, B. Klingeborn, and T. Linné. (1998). Identification of two antigenically and genetically distinct lineages of H3N8 equine influenza virus in Sweden. Epidemiology and Infection. 120:61-70.

IV Oxburgh, L. & Hagström, Å. A PCR based method for the characterisation of equine influenza strains from clinical samples. Manuscript.

V Oxburgh, L & Klingeborn, B. Cocirculation of two distinct lineages of H3N8 equine influenza virus in Sweden. Manuscript.

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Abbreviations

HA	haemagglutinin
NA	neuraminidase
NP	nucleoprotein
NS1	nonstructural protein 1
NS2	nonstructural protein 2
M	matrix
M2	M2 ion channel
PB1	basic polymerase protein 1
PB2	basic polymerase protein 2
PA	acidic polymerase protein
SAα2,3Gal	sialic acid α2,3-galactose
SAα2,6Gal	sialic acid α2,6-galactose
RBS	receptor binding site
HI	haemagglutination inhibition
ELISA	enzyme linked immunosorbent assay
mAb	monoclonal antibody

Introduction

The influenza viruses have, since their isolation and characterisation in the early 1930s, been intensely studied with respect to their ecology, epidemiology, immunology, and molecular biology. The great interest in this virus is at least in part due to its role as the cause of the 1918 Spanish flu, a worldwide epidemic (pandemic) during which 20 to 40 million people died. The virus has since this first great pandemic of the century continued to pose a significant threat to mankind, returning in many different guises (Asian influenza, Hong Kong influenza and Russian influenza) and circulating annually, mostly causing disease among the elderly and children. Less publicised, but significant outbreaks among both animals and birds have occurred repeatedly, and influenza is continually circulating amongst certain domesticated species such as the pig and the horse.

One of the major goals of influenza research has been to explain how it is possible that the virus returns to circulate in previously infected or vaccinated populations. A great deal of knowledge has accumulated, and influenza has become a model for the understanding of the interactions of other viruses with the immune system, both at the individual and at the population level. Much remains to be done, however, as our understanding of many of the mechanisms which lead to recirculation of the virus is incomplete. Most of the work done so far pertains to the human influenza viruses, and studies of other animal-virus interactions remain scarce in comparison. The aim of the work presented in this thesis has been to characterise the circulation of influenza virus H3N8 in the equine population in Sweden, and to shed some light on the immunological structure of this virus.

I General influenza biology

Virus nomenclature

The family orthomyxoviridae is divided into three genera; the Thogoto genus (influenza D), influenza genus A and B and influenza genus C (Lamb and Krug, 1996). The Thogoto genus and influenza genus C contain only one species each, whereas two species are present in influenza genus A and B. Thogoto is a tick-borne virus infecting humans and livestock in Asia, Africa and Europe with unknown pathogenic consequences (Portela et al., 1992) and will not be discussed in the text. Influenza viruses B and C are not regarded as pathogens in other species than man, and hence only influenza A will be discussed further. Species A is divided into a number of different subtypes or subspecies on the basis of serological reactivity of the surface glycoproteins haemagglutinin and neuraminidase (see *Virus structure* below). Since the genome is segmented, each individual type of haemagglutinin can combine with a variety of different types of neuraminidase giving rise to a great number of different subtypes. A total of 15 different subtype-specific haemagglutinins and 9 different subtype specific neuraminidases have so far been identified. Individual virus subtypes are named

on the basis of i) influenza species, ii) animal species from which the strain was isolated, iii) location of isolation, iv) strain number, and v) year of isolation, followed by the surface antigen combination, eg A/equine/Jilin/1/89 (H3N8). Strains of the same subtype are distinguished by location and year of isolation. Animal species is generally omitted for human isolates. Only a small fraction of the subtypes which have been characterised to date have been shown to be pathogenic for mammals (Claas et al., 1998; Subbarao et al., 1998; Webster et al., 1992). These are listed in Table 1.

Table 1. Pathogenic influenza viruses of mammalian species.

Horse	**Human**	**Swine**	**Mink**	**Seal**
H7N7	H1N1	H1H1	H10N4	H7N7
H3N8	H3N2			
	H2N2			
	H5N1			

Species infected by influenza and virus ecology

Influenza A viruses infect a number of avian and mammalian species, a situation which reflects a complex ecology, enabling this virus to survive in different reservoirs. Since all isolated subtypes of influenza are perpetuated in aquatic birds without causing significant pathology (Webster et al., 1992), influenza virus is regarded as originating in avian species.

In contrast to the situation in the migratory waterfowl, influenza A infections of mammalian species generally lead to disease. The epidemic influenza viruses of man (H2N2, H3N2 and H1N1), pigs (H1N1) and equines (H3N8 and H7N7) give acute upper and lower respiratory tract infections with rhinitis, coughing and febrile response. In uncomplicated cases virus is cleared from the respiratory tract within one to two weeks, and convalescence is uneventful (Frank et al., 1981; Murphy et al., 1973b). Several sequealae to influenza virus infection have been reported, most frequently secondary bacterial infection of the respiratory tract (Smith et al., 1976), but also myocarditis (Adams, 1959).

Apart from the endemic subtypes of virus which circulate in these mammalian populations, direct transmissions of virus from the avian pool to mammalian species have been reported. Some of these transmissions have been associated with extreme virulence, reflecting the low level of evolutionary adaptation of the viruses to their new hosts. Noteworthy cases are the outbreaks of avian H7N7 virus in seals in New England 1979 to 1980 (Webster et al., 1981), avian H10N4 virus in mink in Sweden in 1984 (Klingeborn et al., 1985), transmission of avian H3N8 virus to horses in China in 1989 (Guo et al., 1992) and the transmission of avian H5N1 virus to humans in Hong Kong in 1997 (Claas et al., 1998; Subbarao et al., 1998). All but the mink outbreak of 1984 were associated with high virulence (mortality 20% to 30%) and generalised symptoms such as enteritis and encephalitis were seen in all four outbreaks, suggesting more generalised tissue-tropisms of the infecting viruses, a distribution resembling that seen in fowl

plague of domestic poultry rather than that seen in endemic virus infections of mammals.

In domestic fowl, infection with pathogenic subtypes of influenza (some H5 and H7 subtypes) known as fowl plague results in extremely severe disease. In these cases the infection is not limited to an epithelial barrier, but becomes systemic, with viremia and spreading of the virus to multiple organs, including the central nervous system. Generalised symptoms and mortality rates approaching 100% can be seen (Webster et al., 1992). Many other subtypes of influenza virus give subclinical infections in domestic fowl. Fowl plague strains are transmitted from the aquatic bird pool, and do not circulate endemically among domestic fowl (Webster et al., 1992).

The antigenic and genetic properties of two strains circulating in man have been shown to originate from a mixture of human and avian viruses (Kawaoka et al., 1989b; Scholtissek et al., 1978). Reassortment of genes from a circulating human virus with those from an avian virus has the potential to create a strain with host adapted growth properties for the human, but with novel antigenic determinants. Such strains have been proposed to arise in the pig (Castrucci et al., 1993; Claas et al., 1994; Scholtissek et al., 1985), which can serve as a "mixing vessel" since the airways of this species support replication of both avian and human influenza viruses (Ito et al., 1998; Kida et al., 1994). This antigenic shift is the origin of the 1957 H2N2 Russian influenza, and the 1968 Hong Kong influenza (Kawaoka et al., 1989b; Scholtissek et al., 1978). No pathogenic influenza virus of lower animals has been shown to originate from such a reassortment event.

Virus structure

Influenza virus particles are pleomorphic in shape, but can be seen to be mainly spherical after repeated passages in cell culture or embryonated hens´ eggs (Horne et al., 1960). The virions are roughly 90-100nm in diameter and are coated by lipid envelopes. Influenza A virus particles are composed of eight individual proteins. The nucleoprotein (NP), the matrix (M), and the three proteins of the polymerase complex PB1, PB2 and PA are regarded as internal proteins, i.e. they are contained within the lipid envelope of the virus, and are not exposed in an intact virus particle. The haemagglutinin (HA) and neuraminidase (NA) glycoproteins, and the M2 protein are embedded in the lipid envelope of the virus, and are regarded as surface proteins. Recently, the NS2 protein of influenza virus was shown to be incorporated into the virus particle, a surprising finding since this polypeptide was previously thought only to be expressed in the infected cell (Richardson and Akkina, 1991; Yasuda et al., 1993). The genes of influenza virus are encoded on eight separate strands of single stranded negative sense RNA. These RNA segments are associated with nucleoprotein and the proteins of the polymerase complex to form ribonucleoproteins (RNPs).

An outline of the replicative cycle of influenza virus

The replicative cycle is initiated by the attachment of the virus to its cellular receptor sialic acid (see V Structural and functional studies of the haemagglutinin and its receptor,sialic acid, below) through interaction with the HA (Rogers et al., 1983; Weis et al., 1988). The aggregation of large numbers of HAs attached to sialic acid triggers internalisation of the viral particle in a clathrin coated pit (Ellens et al., 1990). After endosomal fusion, the pH within the vesicle decreases, initiating a conformational change within the HA which leads to fusion of the viral envelope with the cell membrane ((Carr and Kim, 1993), see V Structural and functional studies of the haemagglutinin and its receptor, sialic acid, below). The contents of the virion undergo conformational change when the endosomal pH is lowered, facilitating detachment of RNPs from M (Hay, 1992; Martin and Helenius, 1991). The acidification of the virion is mediated by M2, which functions as an ion channel (Pinto et al., 1992). After fusion of the virion and cell membranes, RNPs enter the cytoplasm of the cell and are transported to the nucleus (Martin and Helenius, 1991) where transcription is initiated. Association of viral polymerase proteins with the RNPs is essential for replication, since no cellular polymerases are capable of RNA templated RNA transcription (Huang et al., 1990; Ulmanen et al., 1981). The functions of the three sub-units of the polymerase are distinct, but only the functions of PB1 and PB2 have been elucidated in detail. In order to initiate transcription, the PB2 protein removes 5´sequences from cellular RNAs, and these are subsequently used as primers for the polymerisation reaction (Braam et al., 1983; Plotch et al., 1981). This process is known as "cap snatching". Elongation of the transcript is mediated by functions of the PB1 protein and presumably also the PA protein (Braam et al., 1983; Nakagawa et al., 1996). Initially, complementary RNA copies of the genes are transcribed (Hay et al., 1977; Shapiro et al., 1987; Smith and Hay, 1982). Complementary RNA is the template for synthesis of the genomic (viral) RNA. Transcription of cRNA to vRNA ensures that there is enough negative sense template for transcription of large amounts of mRNA for subsequent protein translation. The vRNA transcript contains packaging signals, which ensure that it is complexed with NP before export to the cytoplasm (Hay et al., 1977; Pons, 1971). By the time the nascent RNPs are being transported to the cytoplasm, sufficient amounts of the other viral proteins have accumulated to allow virion formation. The surface proteins are translated and subsequently modified in the endoplasmatic reticulum, where oligosaccharide and fatty acid modifications are added (Elder et al., 1979). Surface proteins are transported through the Golgi network, where further post-translational processing takes place, and subsequently transported to the cell membrane where their hydrophobic transmembrane regions ensure that they are embedded (Doms et al., 1993). The HA is translated as a continuous gene product (HA0), and is transported to the cell surface in this form. It is, however, not fully functional and must be proteolytically cleaved into sub-units HA1 and HA2 in order to gain fusion activity (Huang et al., 1981; Klenk et al., 1975; White et al., 1981). Proteolytic enzymes with this activity have been located in a number of different cellular

compartments, but the most common localisation for proteolytic cleavage of the HA of circulating mammalian strains is the cell surface, where Clara cell secreted tryptase has been shown to be capable of mediating cleavage (Kido et al., 1992). The M protein has been shown to be distributed both in the cytoplasm and in the nucleus, and performs functions in the transport of RNPs from the nucleus to the cytoplasm, complexing with the RNP and NS2 protein (Yasuda et al., 1993) in either the nuclear or the cytoplasmatic compartment, and migrating to the cytoplasmatic face of the cell membrane (Martin and Helenius, 1991). The surface proteins embedded in the host cell membrane bud off together with the RNP-M-NS2 complex forming virus particles (Compans and Dimmock, 1969).

The NS1 protein is found in infected cells, performing functions during viral transcription such as inhibition of splicing and inhibition of export of polyadenylated mRNAs from the nucleus (Alonso-Caplen et al., 1992; Fortes et al., 1994). These functions are presumably important in the shutoff of cellular translation seen after infection.

Influenza virus is genetically unstable

Early studies of influenza virus showed that variants of virus resistant to neutralisation by a particular anti-HA antibody (antibody escape mutants) could be isolated from a virus stock at a frequency of approximately one mutant in every $1x10^6$ infectious units of virus (Yewdell et al., 1979). This was interpreted to mean that a large number of variants with mutations in their genomes arise in each replication cycle, an assumption which was proven correct when the mutation rate of influenza A virus was shown to be approximately $1.5x10^{-5}$ mutations per base per infectious cycle (Parvin et al., 1986). This extreme mutability ensures that on average one mutation is incorporated into each genome every replicative cycle of the virus. A full selection of single amino acid mutants is thus present in a relatively small virus inoculum, providing a heterogeneous population, or quasispecies, from which variants with enhanced fitness can be selected.

II The protective immune response to influenza virus

Influenza infection has been used for a number of years as a model for the study of immune responses to a lytic virus. Several facets of the immune response have been explored, and both the innate (Ada and Jones, 1986; Neuzil and Graham, 1996) and acquired immune responses (Ada and Jones, 1986; Crowe, 1996; Karzon, 1996) have been shown to be important in protection against this pathogen. Innate immune mechanisms such as interferon release by the respiratory epithelium have been shown to confer protection in humans (Murphy et al., 1973a; Richman et al., 1976). Mediators of interferon induced protection such as the Mx proteins have been characterised in a number of animal species (Meier et al., 1988; Pavlovic et al., 1990; Staeheli et al., 1986), but their relation to protection in natural hosts of influenza virus remains unclear. The Mx proteins are specific inhibitors of influenza virus (Krug et al., 1985), acting through

inhibition of the viral polymerase (Huang et al., 1992).

The general paradigm which has emerged for the acquired immune response is that local and circulating antibody is required for protection from infection (Virelizier, 1975), but that cellular immune responses are important in viral clearance, and also ameliorate clinical signs when antibody levels are insufficient to prevent infection (Yap et al., 1978). This situation is, however, complicated by the constant adaptation of the virus to the humoral immune response through antigenic drift (see III The antigenic structure of the haemagglutinin, below). In a natural setting, individuals are seldom reinfected with the same strain with which they previously have been immunised. The efficacy of the humoral response is therefore difficult to predict, antigenically divergent strains frequently showing little or no cross-reactivity (Haaheim and Schild, 1980; Virelizier, 1975). Since the targets of the cellular response are highly conserved (to such an extent that cytotoxic cross-immunity can be seen between viruses of different subtypes), protective responses often rely on this arm of the immune response (Lukacher et al., 1984; Ulmer et al., 1993). Protection from clinical symptoms is attained, but virus shedding is not eliminated, implying that virus continues to circulate in the population, and new individuals can be infected. Vaccines stimulating cross-reactive cytotoxic responses show promise for clinical use.

III The antigenic structure of the haemagglutinin

The antigenic variability of influenza virus has been extensively studied, and the haemagglutinin has been the main focus of this work since it is the major target of humoral immunity (Ada and Jones, 1986). Two basic mechanisms of variability have been defined; antigenic shift and antigenic drift. The former implies exchange of the surface proteins of circulating endemic viruses for proteins with differing subtype specificities, thereby introducing a virus with a totally new antigenic structure into the population.

Less dramatic changes in antigenicity of the HA are seen as a result of antigenic drift. The high mutability of the influenza virus genome ensures that variants which are neutralised less efficiently by circulating antibody are selected in the population. Several studies employing monoclonal antibodies and polyclonal sera raised against a variety of virus strains have demonstrated this antigenic evolution of the virus to be of biological significance (Haaheim, 1980; Haaheim and Schild, 1979; Haaheim and Schild, 1980; Schild, 1970; Underwood, 1982). Analyses of natural antigenic variants have shown that the antigenic changes can be correlated with amino acid substitutions in the HA of the variant (Laver et al., 1980; Laver et al., 1981; Laver et al., 1979). This conclusion was based on chemical determination of the amino acid composition of HA. Later work correlated antigenic variation with changes in the nucleotide and deduced amino acid sequences of HA (Both and Sleigh, 1980; Both and Sleigh, 1981; Both et al., 1983; Sleigh et al., 1981). Nucleotide sequencing of HA from viruses which have been serologically characterised with polyclonal or monoclonal antibodies has since become the method of choice for the characterisation of

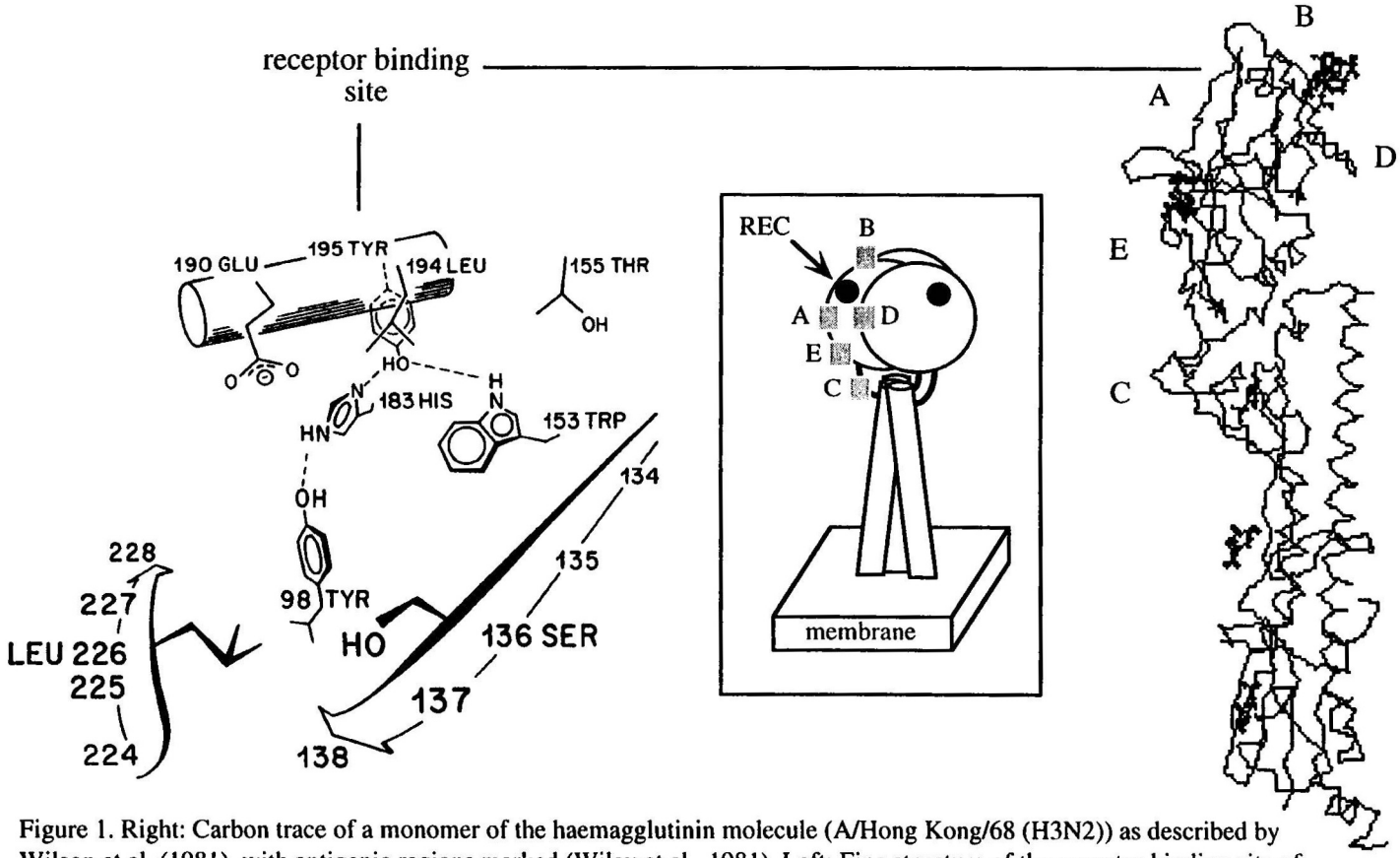

Figure 1. Right: Carbon trace of a monomer of the haemagglutinin molecule (A/Hong Kong/68 (H3N2)) as described by Wilson et al. (1981), with antigenic regions marked (Wiley et al., 1981). Left: Fine structure of the receptor binding site of haemagglutinin as described by Weis et al. (1988), with amino acid positions involved in interactions with the cellular receptor sialic acid marked. Inset is a schematic drawing of the haemagglutinin trimer with receptor binding sites and antigenic regions labelled.

antigenic variability. A number of studies employing antibodies for the selection of antigenic variants *in vitro* pinpointed the presence of distinct, non-overlapping antigenic regions on the surface of the HA molecule (Gerhard and Webster, 1978; Haaheim and Schild, 1976; Laver et al., 1979; Natali et al., 1981; Yewdell et al., 1979). The structural basis for this was elucidated by Wiley and colleagues, who defined the localisation of several amino acid substitutions at antigenic regions on the three dimensional model of the HA (Wiley et al., 1981). The five antigenic regions which they proposed in this work, A to E (see Figure 1), provide a functionally complete model for the humoral immune response to the H3 strains, and are also used in the study of HAs of other strains (Caton et al., 1982; Gibson et al., 1992; Okuno et al., 1993). The antigenic map of HA is based on the sequences both of natural antigenic variants and *in vitro* cultivated antibody escape mutants. Therefore, the antigenic regions represent positions on the molecule at which binding of an antibody is neutralising, and not the total spectrum of possible antibody binding sites.

Many mechanisms of neutralisation of virus through antibody binding to HA have been defined. Since HA carries the receptor-binding moiety of the virus, one obvious mechanism is inhibition of docking of the virus with its receptor at the cell surface. Another function of HA, fusion of virus and cell membranes, is a target for neutralisation. Antibody may block this important step in the virus infective cycle, so preventing viral replication. Other mechanisms such as aggregation of virus particles due to multimeric binding by antibody, complement activation by bound antibody, inhibition of uncoating of the virus, and even inhibition of viral transcription have also been reported (Dimmock, 1993; Dimmock, 1995). From this summary of neutralisation mechanisms it is obvious that a full explanation for the locations of antigenic sites on HA is not yet possible. All of this work has been performed using monoclonal antibodies. The mechanism of neutralisation of virus *in vivo* is complex, and thus the selective pressure driving the evolution of HA antigenicity remains obscure. The extreme variability seen in natural virus variants around the receptor binding site of HA (Both et al., 1983; Wiley et al., 1981) however, indicates that steric inhibition of attachment could constitute a major selective force.

IV The mechanism of antigenic drift

The HA can be seen in terms of antigen as a collection of five individual neutralising regions (Wiley et al., 1981), and it is therefore improbable that a change at one or two of these could yield a virus variant which is not neutralised by the antibody response to the original virus. It would be expected that antibody directed against one or more of the constant regions would neutralise the variant. It has, however, been shown that antibody responses often are skewed (Lambkin and Dimmock, 1995; Natali et al., 1981; Patera et al., 1995), resulting in strong responses against one or two regions, and weak or absent responses against the others. Human sera often exhibit a limited antibody repertoire to HA (Wang et al., 1986), and this finding has also been made for rabbits (Lambkin and Dimmock,

1995). This suggests that antigenic variants could be selected in such individuals (Cleveland et al., 1997; Lambkin et al., 1994), and subsequently spread to the rest of the population. However, if the majority of the population had a more diverse anti-HA repertoire, these single antigenic region mutants would be neutralised. It is conceivable that this slight antigenic difference might be enough to allow the virus to go through one or a few replication cycles, and be perpetuated without causing disease in the population at large. Eventually, after a number of passages in individuals with selective repertoires, a variant may arise with a sufficient number of mutations at antigenic regions to enable it to productively infect the entire population. This argument relies on the presence of individuals in the population with repertoires skewed to each of the antigenic regions. Studies in mice and rabbits have addressed this question, and have concluded that repertoires often are skewed towards recognition of sites A and B (Cleveland et al., 1997; Lambkin et al., 1994; Patera et al., 1995). Other specificities have not been noted. In conclusion, the basic mechanism behind antigenic drift has not yet been fully explained. Future studies with larger numbers of immunised individuals will yield further information regarding this mechanism.

V Structural and functional studies of the haemagglutinin and its receptor, sialic acid

The three dimensional structure of the HA glycoprotein of the A/Hong Kong/68 H3N2 influenza virus was determined by Wilson and colleagues in the early 1980s (Wilson et al., 1981), and has since become a paradigm for the correlation of structure and function of membrane proteins. The HA is a trimer, each monomer being composed of two sub-units, the globular head domain, containing exclusively the HA1 sub-unit, and the stem region, containing parts of HA1 and the entire HA2 region (see Figure 1). The HA1 and HA2 sub-units are held together covalently by a disulphide bond, and the individual monomers form trimers through non-covalent interactions. The molecule is fixed to the envelope of the virus through an anchor of hydrophobic amino acids at the carboxy terminus of HA2. Seven carbohydrate modifications were found in the structure of A/Hong Kong/68, but the glycosylation pattern of influenza HA is strain-specific since the number of potential glycosylation sites varies between different strains. Notable structural features of the molecule include a hydrophobic peptide buried in the intermolecular interface, the fusion peptide, and a shallow pocket of conserved amino acids at the membrane-distal end, the receptor-binding site.

Studies of the structure of the receptor binding site of HA

The membrane-distal pocket comprising amino acids Tyr98, His193, Glu190, Trp153 and Leu194 (see Figure 1) was tentatively designated as the receptor binding site of the virus in the original study by Wilson and colleagues based on its high degree of conservation between strains, and its resemblance to the sialic acid binding site of wheat germ agglutinin. Studies of natural and laboratory grown antigenic variants have demonstrated that the majority of antigenic

changes provoked by growth in the presence of neutralising antibodies cluster around the pocket (Wiley et al., 1981), indicating that the neutralising antibody response is directed mainly against this region, suggesting that it is of importance in attachment and/or entry of virus into cells. The role of this region was confirmed by the finding by Rogers and colleagues that an amino acid substitution at residue 226, in the vicinity of the proposed binding site, changed the receptor specificity of the molecule (Rogers et al., 1983). Several studies have confirmed the involvement of amino acids in and around the putative binding site in receptor binding (Daniels et al., 1987; Underwood et al., 1987; Yewdell et al., 1986). The definitive structure of the receptor binding site was proposed by Weis and colleagues (Weis et al., 1988), who determined the structure of HA in complex with its receptor, sialic acid, thus defining all of the amino acid residues with potential interactions with the receptor (see Figure 1). Comparison of the structural data with studies of receptor binding variants reveals that not only amino acids of the HA which directly interact with the receptor influence specificity and affinity of the interaction, but a large number of residues which are not in direct contact with the receptor, and in some cases are distantly located with respect to the binding site in the so-called second layer of the pocket can have fundamental effects on this interaction (Daniels et al., 1987; Underwood et al., 1987; Yewdell et al., 1986). In a recent study by Martin and colleagues, the contribution of some of the residues affecting receptor binding in both the pocket and the second layer were assessed by mutagenesis and subsequent assay of affinity and specificity of the mutants (Martin et al., 1998). They concluded that changes at residues 98, 183, 194 and 153 severely restricted binding to the receptor, whereas mutations at residues 190 and 228 increased the binding affinity slightly. Changes at residues 136, 195, 225 and 226 were shown to have intermediate effects on affinity. Assays of specificity for the receptor revealed that changes at positions 225, 226, 136, 190 and 228 could change the specificity of binding. Although this study is by no means exhaustive, since only one substitution per position was analysed, it gives a picture of the hierarchy of importance of the individual residues involved in receptor binding for affinity and specificity respectively.

Studies of the structure and localisation of the cellular receptor, sialic acid

Sialic acid has for a number of years been known to be the cell surface receptor for influenza (Gottschalk, 1959), and it is the presence of this carbohydrate on the surface of erythrocytes which has facilitated studies of this interaction. The sialyl oligosaccharide structures present on glycoproteins and glycolipids of the cell membrane are diverse, and early studies of the affinities of influenza viruses for various sialyloligosaccharide sequences on erythrocytes showed that the viruses discriminate between variants of sialic acid (Carroll et al., 1981), raising the question of whether this differentiation could have biological significance. It was later shown that three major receptor specificities exist among influenza viruses; i) the sialic acid α2,6-galactose (SAα2,6Gal) specificity of human H3 strains, ii) the sialic acid α2,3-galactose (SAα2,3Gal) specificity of equine H3 strains and

also avian strains of the H7 subtype, and iii) the specificity of PR8, a laboratory adapted H1 human strain, for both SAα2,6Gal and SAα2,3Gal (Rogers and Paulson, 1983). It has thus been concluded that human viruses preferentially bind to SAα2,6Gal and avian and equine viruses preferentially bind to SAα2,3Gal. This basic tenet has been confirmed in more recent studies (Carroll, 1985; Connor et al., 1994), and has been proposed to be a determining factor in the host-range restriction of the influenza viruses (Ito et al., 1998; Vines et al., 1998). Modifications other than linkage of the penultimate galactose residue have been proposed to account for additional receptor-binding phenotypes. One such example is the differential recognition of N-acetyl sialic acid and N-glycolyl sialic acid shown by human influenza viruses (Suzuki et al., 1985; Suzuki et al., 1992).

Studies concerning the localisation of receptors for the attachment of influenza virus have been conducted on a number of cell types from several animal species. The presence of different sialyloligosaccharide structures on human, pig, duck and ferret epithelial cells has been mapped using labelled lectins (Couceiro et al., 1993; Ito et al., 1998; Leigh et al., 1995). Human and mink tracheal sections exhibit binding of SAα2,6Gal specific lectin, and very little or no binding of a lectin recognising SAα2,3Gal, whereas the opposite is true of the colon epithelium of ducks (the colorectal epithelium of the duck is the primary site of replication of influenza virus in this species). The tracheal epithelium of the pig binds lectins specific for both structures, endorsing the idea that the respiratory tract of this animal species supports the replication of influenza virus of differing receptor specificities. These studies have mapped the presence of receptors in the tracheal and intestinal epithelia, but the finding that viruses both with specificities for SAα2,6Gal and SAα2,3Gal replicate well in the upper respiratory tract of ferrets highlights the fact that only small fractions of the target organs for influenza virus have been examined, and that a different spectrum of sialyloligosaccharide structures is presumably expressed at each anatomical location, raising the question of how specific each sialyloligosaccharide is for each animal species.

Receptor-binding glycoproteins can be found naturally in the serum and secretions of a variety of animal species (Krizanova and Rathova, 1969). These substances have the capacity to bind to and neutralise influenza virus of subtypes H2 and H3, and are thus known as natural inhibitors. Inhibiting activities of varying strengths have been identified in serum from a number of species (Pritchett and Paulson, 1989), and the α2-macroglobulin fraction of horse, guinea pig, pig and rabbit sera have been characterised as the strongest of these (Pritchett and Paulson, 1989; Ryan-Poirier and Kawaoka, 1991). Human α2-macroglobulin is known to have extremely low virus-inhibiting activity, despite its biochemical similarities with the proteins of the stronger inhibitors (horse and guinea pig). Comparisons of chemical compositions of the glycoproteins of horse, guinea pig, human and pig reveal that all are fundamentally similar, being composed of a high molecular weight protein with SAα2,6Gal modifications (Pritchett and Paulson, 1989; Ryan-Poirier and Kawaoka, 1993). SAα2,3Gal was not seen to be

present on any of the glycoproteins. Apart from the presence of a 4-O-acetylated form of sialic acid on the equine α2-macroglobulin comprising 30-50% of the sialic acid structures attached to the protein, no obvious differences could be seen between strong and weak inhibitors, indicating that the difference in inhibiting activity was due to valency and/or distribution of carbohydrate modifications on the proteins (Pritchett and Paulson, 1989).

Other factors which influence the interaction of influenza virus with its receptor

From the studies summarised above it can be concluded that the HA molecules of influenza virus interact in a sterically complicated manner with cell-surface sialic acid. One fact which has not been mentioned in this context is the contribution of the NA to this interaction. Influenza inhibitors are generally classified on the basis of their sensitivity to the NA of the virus being assayed. This classification has been seen to vary, since the NA activities of various (sometimes closely related) viruses differ (Gottschalk et al., 1972). It is thus conceivable that the interaction of the HA with its cellular receptor might be influenced by the NA, and that the NA may play an important role in receptor specificity and/or affinity. Another potentially important determinant of receptor binding is the glycosylation of the HA. The effect of this modification has been studied mainly in H1 viruses of human origin, in which number, position and structure of the carbohydrate modifications of the HA have been shown to be of importance (Deom et al., 1986). A recent study by Gambaryan and colleagues demonstrated that glycosylation of amino acid 131 strongly influenced the binding of virus to a variety of receptor analogs (Gambaryan et al., 1998). Since the binding of differentially glycosylated virus particles to small carbohydrate molecules was indistinguishable, and differences were seen only in binding to larger glycoproteins, it can be postulated that glycosylations do not change the interaction between the terminal sialic acid and the receptor binding site, but rather sterically hinder the apposition of virus and glycoprotein or glycolipid receptor.

The binding of virus to receptor depends on a number of interactions apart from the sialic acid-receptor binding site interaction. Adaptation of the virus to growth in a cell type with different receptor characteristics from that in which the virus was propagated will lead to selection of a mutant with potential changes in a number of receptor binding determinants, such as receptor binding pocket, glycosylation pattern and/or NA. It follows that there are a great number of different mechanisms for blocking of receptor binding to cells by antibodies, making the interpretation of sequence data from natural antibody escape variants of virus difficult.

Studies of the fusion activity of HA

The HA is responsible for the membrane fusing activity required by influenza virus for entry into the cell. The requirement for cleavage of the HA into HA1 and HA2 for fusion activity (Huang et al., 1981; White et al., 1981) has been

demonstrated, and a large body of work on the HA in its two pH-induced conformations provides an explanation for this finding. At low pH (approximately 5.5), the trimeric structure undergoes a conformational change such that the proteolysis resistant native form becomes sensitive to proteolysis by trypsin, chymotrypsin, bromelain, thermolysin and proteinase K (Daniels et al., 1982; Doms et al., 1985; Skehel et al., 1982). Thus, a substantial conformational change takes place in the HA at the acidic pH of the endosome. Liberation of low-pH conformation HA trimers yields rosettes of molecules, attached by the hydrophobic amino terminal of HA2 (Daniels et al., 1982; Ruigrok et al., 1988; Ruigrok et al., 1986; Skehel et al., 1982), indicating that the fusion peptide is extended at the pH of fusion. Recently, a model of the structural changes of the HA which preempt membrane fusion has been proposed (Carr et al., 1997; Carr and Kim, 1993). In this model, the globular domains of each of the monomers become relaxed and move apart, allowing protrusion of the hydrophobic amino terminal of HA2. The fusion peptide is embedded in the trimer interface at neutral pH, and is propelled towards the cell membrane by the formation of a long (88 amino acid) alpha helix, running the entire length of the HA molecule at low pH. This allows the fusion peptide to insert itself into the cell membrane and initiate fusion.

A number of virus mutants displaying elevated pH of fusion have been described (Daniels et al., 1985a; Daniels et al., 1987; Doms et al., 1986; Rott et al., 1985). Mutants can be divided into two categories, those with mutations in or around the hydrophobic amino terminal of HA2, and those with mutations along the trimer interface. The former destabilise the fusion peptide, allowing it to be liberated more easily. The latter generally facilitate relaxation of the trimeric structure. Different forms of selective pressure have been used to generate these mutants. The treatment of cells with amantadine elevates the endosomal pH, thus selecting mutants with high pH fusion capability (Daniels et al., 1985a). Alternatively, selection of virus mutants which are able to propagate without the addition of trypsin in MDCK cell culture yields high pH fusion mutants (Rott et al., 1985). A natural mutant has been isolated (Doms et al., 1986), and it has been shown that mutants can be isolated by antibody selection (Daniels et al., 1987).

Previous studies of H3N8 equine influenza virus

The H3N8 influenza virus of equines was first isolated in the United States in 1963 (Wadell et al., 1963). Since that time, a considerable amount of work has been done on the biology of this virus, and much work has concentrated on defining antigenic properties of circulating strains. Clinically, disease signs are similar to those seen in other mammalian species, i.e. respiratory symptoms, muscle soreness, depression and hyperthermia (Gerber, 1969). Sequelae can occasionally be seen in the form of secondary bacterial infections and myocarditis (Gerber, 1969). An increased incidence of chronic obstructive pulmonary disease (COPD) can be seen following influenza virus infection (Gerber, 1969). Virus replication is limited to the upper and lower respiratory tract, causing denudation of ciliated epithelia and focal erosions (Hannant and Mumford, 1996). The immune response of the horse to influenza virus has been characterised, and is discussed below.

Natural outbreaks of equine influenza

H3N8 equine influenza has circulated widely amongst horses on the American continent (both North and South) and in Europe after its first isolation in 1963 (Burrows et al., 1982; Gerber, 1969; Higgins et al., 1986; Hinshaw et al., 1983; Klingeborn et al., 1980; Mumford and Wood, 1993; Oirschot et al., 1981). Outbreaks in India (Gupta et al., 1993), South Africa (Kawaoka and Webster, 1989), Nigeria (Adeyefa and McCauley, 1994), China (Lai et al., 1994) and Hong Kong (Powell et al., 1995) have been reported, suggesting that the virus at this time is widely disseminated throughout the Eurasian continent, the Americas and also Africa. Only Australia, New Zealand and Iceland remain free from the disease. The ubiquitous nature of H3N8 equine influenza virus, and the frequent similarity of strains isolated in different countries have been attributed mainly to the extensive transport of racing horses.

Characterisation of equine H3N8 viruses isolated from outbreaks

Serological characterisation of epidemic strains of H3N8 virus by the haemagglutination inhibition assay have demonstrated that substantial differences are exhibited within the H3N8 subtype. Assays using post infection ferret antisera (Burrows and Denyer, 1982; Daly et al., 1996; Hinshaw et al., 1983), post infection horse sera (Burrows and Denyer, 1982; Daly et al., 1996) and monoclonal antibodies (Appleton et al., 1987; Hinshaw et al., 1983; Kawaoka et al., 1989a) have all indicated that this virus is antigenically variable. Sequence and oligonucleotide fingerprinting studies of the HA gene of selected strains has revealed a relatively high degree of genetic variability (Adeyefa et al., 1996; Berg et al., 1990; Binns et al., 1993; Daly et al., 1996; Daniels et al., 1985b; Endo et al., 1992; Guo et al., 1992; Gupta et al., 1993; Kawaoka et al., 1989a; Kawaoka and Webster, 1989; Lai et al., 1994), indicating that the virus is under immune selective pressure similar to the human H3N2 virus.

The outbreak of highly pathogenic influenza virus of the H3N8 subtype in

China in 1989 differed fundamentally from the pattern of epidemic strains seen previously, causing widespread morbidity and mortality (Guo et al., 1992). On analysis of the genes of this virus it was found that all were closely related to genes of avian origin, and it was concluded that this outbreak was caused by the introduction of an avian H3N8 influenza virus into the equine population. This strain circulated in China for two epidemic seasons, and has subsequently not been isolated despite intensified surveillance efforts (Guo et al., 1992; Lai et al., 1994).

Like other mammalian species, the horse can be infected both with circulating, host adapted epidemic strains causing high morbidity and low mortality, but also with non-adapted avian strains which cause substantial morbidity and mortality in the population.

Isolation and cultivation of H3N8 equine influenza virus

The culture system of choice for the isolation of equine influenza virus is the embryonated hen's egg (Hannant and Mumford, 1996). Embryonated hens` eggs have been shown to yield virus with homogeneous antigenic properties, as opposed to MDCK cells (Ilobi et al., 1994), indicating that host-cell mediated selection of virus variants occurs in cell culture. However, sequence comparisons between HA genes from clinical specimens and egg-grown and MDCK cell grown virus show that host adaptation occurs in both culture systems (Ilobi et al., 1998). The difficulty of isolation of equine influenza virus in MDCK cell culture suggests that this culture system is not fully permissive for virus replication, and that variants are selected on the basis of growth advantage, possibly by mutation in HA (Ilobi et al., 1998).

The immune response of the horse to infection

Studies of protection in horses after natural or experimental infection and vaccination have led to the conclusion that circulating antibody is required for protection from reinfection (Mumford et al., 1983; Rouse and Ditchfield, 1970).

The development of a model for natural infection of horses has enabled the study of both humoral and cell mediated immune responses against H3N8 (Mumford et al., 1990). Following infection, circulating antibody titres diminish to sub-protective levels after approximately one year (Hannant et al., 1988). However, animals are still protected from clinical infection, suggesting that the cell-mediated immune response in itself is enough for protection. Studies of cellular immune responses have concluded that cytotoxic T-cells are produced upon infection with H3N8, and that these have the capacity to lyse virally infected cells (Hannant and Mumford, 1989).

Qualitative aspects of the humoral immune response have been studied by Appleton and colleagues (Appleton and Gagliardo, 1992). Culture of peripheral B lymphocytes sampled from a convalescent pony showed that antibodies were directed against at least four discrete antigenic sites of the HA.

In all, the limited data available for H3N8 infection of the equine suggests that the protective immune response is similar to that seen in studies of human H3N2

virus infections.

Vaccination against equine influenza

Protection against influenza infection can be correlated with circulating antibody specific for the HA. Traditional vaccine strategies have therefore aimed at stimulating humoral immune responses through the use of inactivated virus preparations or sub-unit vaccines. As discussed above, the variability of the HA of the virus often limits cross-protection between epidemic strains. Vaccine breakthroughs seen during the 1989-1992 outbreaks of equine influenza have been attributed to this fact (Mumford and Wood, 1993), and it has become apparent that vaccines eliciting more cross-reactive immune responses should be employed. Vaccine concepts which have been applied to this end are; i) sub-unit ISCOM vaccines (Mumford et al., 1994; Sundquist et al., 1988), which have been shown to stimulate cellular immune responses in other species, ii) DNA vaccines (Olsen et al., 1997), which induce de novo synthesis of viral proteins in the host, and therefore elicit cellular responses, and iii) live temperature sensitive vaccines (Holmes et al., 1992). ISCOM based vaccine has been licensed for use in horses for a number of years in Sweden, whereas DNA vaccines and live temperature sensitive vaccines are still at the experimental level.

Aims of the study

The major goal of the work presented in this thesis has been to understand the basis for recirculation of equine H3N8 viruses in the equine population. The main questions which I have chosen to ask are:

1) What is the extent of antigenic variability of the haemagglutinin protein?

2) What is the extent of genetic variability of the haemagglutinin gene?

3) Can this genetic variability be correlated with phenotypic change in the form of antigenic drift?

4) Is the haemagglutinin protein from cultured virus which is generally used for assays of viral genotype and phenotype identical to that of the virus infecting horses?

Comments on methods

Most of the methods which have been used in the studies presented in this thesis are standard techniques which are described in laboratory manuals (Harlow and Lane, 1988; Sambrook et al., 1989). Some details pertaining to individual experiments are given below:

Monoclonal antibodies and serological methods
BALB/c mice have been used consistently for the generation of monoclonal antibodies (mAbs). ISCOM particles containing surface glycoproteins from influenza viruses of different strains were prepared and used as immunogens (Sundquist et al., 1988). Subsequent screening for positive clones was performed using an indirect ELISA with whole virus as target antigen. Supernatants from positive hybridoma clones were tested in haemagglutination inhibition (HI) assays with strains of equine influenza virus homologous to those used for immunisation. Clones producing antibodies with HI activity were selected for further use.

Nucleotide sequencing
Two fundamentally different approaches to the sequencing of viral genes have been taken. In the first approach, reverse transcribed copies of the viral haemagglutinin genes were cloned into plasmid vectors, and subsequently sequenced. In the second approach, reverse transcribed copies of the gene were amplified by the polymerase chain reaction, and this DNA was sequenced directly.

In general, it is preferable to sequence viral genes directly from amplification products of the PCR reaction, since selection of individual clones can lead to the selection of variants of the gene which are not representative of the so-called master sequence. This is partly due to the genomic instability of the influenza virus, as has previously been discussed. The enzyme utilised in the PCR is another source of variability, since it lacks proof-reading capacity. Enzymes capable of proof-reading are now available, and the use of these reduces the risk of selecting variants. At the time when our first sequencing experiments were performed, these enzymes were not available, and we chose instead to sequence three individual clones derived from each strain in order to verify our sequence data. In later studies, some of the sequences generated from clones have been verified by direct sequencing of PCR amplification products (unpublished results). A complete consensus can be observed, arguing for the integrity of our early sequence data.

Phylogenetic studies
All of the phylogenetic trees in this study have been calculated using the distance matrix method developed by Fitch and Margoliash (Fitch and Margoliash, 1967). Maximum parsimony is thought by evolutionary biologists to be the method of choice for analysing sequences which are as closely related as those used in these

studies. Unfortunately, this method is extremely slow, and demands the use of extremely powerful computers in order to analyse the number of taxons used in our studies. Since we have previously observed identical topology using maximum parsimony (Swofford, 1991) and the Fitch algorithm for calculating phylogenies from distance matrices (Felsenstein, 1993), we have continued using the latter, and rely on the robustness of this method.

Extrapolation of sequence data to molecular structure

Comparisons of equine H3 virus HAs with the three-dimensional structure determined for the human H3 haemagglutinin (Wilson et al., 1981) have been undertaken in this work. The relatively conserved nature of the equine and human H3 HAs (approximately 80%) (Daniels et al., 1985b) and the similar distribution of potential glycosylation sites (Daniels et al., 1985b) indicate that this approach is feasible. Also, the conservation between equine influenza virus strains of amino acids predicted to comprise the receptor binding site and the variability seen at antigenic regions of the human H3 (Paper III, Figure 1) provide an indication that the structure is fundamentally conserved between human and equine H3. The correlation of variability in the equine H3 with the antigenic regions is not absolute however, raising the question of whether the structure is slightly different, allowing the exposure of different amino acids at the molecular surface, or whether a different epitope selection is made by the horse (Paper III).

Results and discussion

At the outset of these studies, the Swedish horse population had just experienced a severe epidemic of H3N8 influenza, affecting both unvaccinated and vaccinated animals. Vaccination of race horses had, for the previous six years been obligatory, and our studies were initiated in order try to understand the causes of the outbreak within this well-vaccinated animal population.

Serological studies indicate extensive antigenic drift of HA

Serological studies of H3N8 strains have been undertaken in these studies using both polyclonal sera and mAbs. In order to obtain a picture of the extent of antigenic drift of the whole HA, polyclonal sera from ferret and horse were used in Papers I and II in haemagglutination inhibition (HI) assays. Monospecific ferret antiserum raised against the MIA/63 prototype strain does not recognise later strains of virus, a finding which contradicts the demonstration of cross-reactive epitopes by mAb analysis (Paper III). This has previously been noted by other workers using ferret sera (Burrows and Denyer, 1982), and indicates that the ferret selectively recognises an epitope unique to this strain. HI assay using ferret sera raised against FON/79 and SOL/79 reveal the presence of a common epitope on all post-1963 strains assayed in Papers I and II. An equine post infection serum (collected during the 1991 epidemic) showing selective reactivity with the 1991 strains indicates the presence of a unique epitope on post-1988 viruses (Paper I). The same pattern is seen with ferret sera raised against VIS/90 and TBY/91 (Paper II). Strains can be divided into three antigenic groups on the basis of the serological pattern reviewed above; i) Pre-1979 type, recognised by ferret serum raised against MIA/63, ii) 1979-1988 type, recognised by ferret sera raised against FON/79 and SOL/79, but not by ferret sera raised against VIS/90 and TBY/91 or post-infection horse serum, and iii) Post 1988 type, recognised by ferret serum raised against FON/79 and SOL/79, by ferret sera raised against VIS/90 and TBY/91 and by post-infection horse serum. This shows that strains isolated over a number of years display antigenic heterogeneity suggesting antigenic drift, and that it is presumably of clinical significance, since it leads to the selective recognition of viruses by post-infection horse serum.

More detailed analysis of the antigenic structure of equine H3 HA was undertaken using mAbs in HI analyses (Paper III Table 2). Conserved epitopes could be distinguished by two mAbs (2ISCA2:2 and MS23). These epitopes are obviously accessible to antibody, since they can be detected in HI, but are not targets of antibody production by the ferret or the horse. Three major antigenic types can be recognised amongst the virus strains assayed in this study, pre-1990 strains, 1990-1993 strains, and post-1993 strains, a pattern which can be correlated to the phylogenetic distribution of the virus strains (see below).

In conclusion, the studies showed that antigenic drift is a significant factor in the epidemiology of equine H3N8 influenza virus. To gather more detailed information on the changes occurring in the HA protein of the virus during its

evolution, we chose to sequence the HA gene for a number of Swedish strains.

Variability within the haemagglutinin gene of H3N8 equine influenza viruses

In the studies presented in this thesis the haemagglutinin genes of 5 viral strains have been nucleotide sequenced in their entirety, 8 have been sequenced over the regions encoding the entire HA1 sub-unit of the protein, and a further 7 strains have been partially sequenced, yielding information on regions of the protein involved in receptor binding and antibody interaction (see Table 2).

Table 2. Summary of the genes sequenced in this study, and their years of origin.

Strain	**Year of origin**	**Nucleotides seq.**	**Paper**
SOL/79	1979	1-1060	II
ABY/84	1984	1-1060	II
SKA/88	1988	1-1060	II
VIS/90	1990	1-1060	II
TAB/91*	1991	1-1755	I
AVE/93	1993	1-1060	III
SOD/94	1994	1-1060	III
BOL/96	1996	1-1060	III
AVE/96	1996	1-1060	III
CS1	1997	1-1755	V
CS5	1997	1-1755	V
CS9	1997	1-1755	V
CS10	1997	1-1755	V
CS2	1997	322-842	V
CS3	1997	322-842	V
CS4	1997	322-842	V
CS6	1997	322-842	V
CS7	1997	322-842	V
CS8	1997	322-842	V

* The TAB/91 strain is designated TBY/91 in papers I and II

Table 3 summarises the evolutionary rate of the HA gene for equine H3 viruses. It is surprising to note that the rate of substitutions per year is almost the same for HA1 as it is for HA2. Studies of the evolutionary rates of human H3 viruses have shown that HA1 evolves over twice as fast as HA2, and that the rate for the entire HA gene is approximately 4.5×10^{-3} (from sequence data over an 18 year period). The question of whether rates of evolution for equine HA indicate positive selection pressure is answered by the proportion of coding to noncoding substitutions seen in the two sub-units. Over half of the substitutions in HA1 lead to amino acid substitutions whereas only one quarter of the substitutions in HA2 do so. The ratio of coding to noncoding substitutions in HA2 is similar to that seen in studies of the nucleoprotein of human viruses (Gorman et al., 1991), and presumably reflects either i) that only limited selective pressure is operating on this segment of the gene, perhaps because it is "buried" beneath the globular head

or, ii) that structural constraints on HA2 make the majority of changes deleterious. The ratio for HA1 is slightly higher than that determined for the human H3 (Bean et al., 1992), indicating that it is subjected to substantial selective pressure. The total (coding and noncoding) evolutionary rate calculated for HA1 is low in comparison with that previously reported (Daniels et al., 1985b). The estimate of $3.6x10^{-3}$ substitutions per site per year was made on the basis of comparison of MIA/63 and FON/79 strains. Studies of larger groups of viruses, however, show that the rate of evolution is erratic (see Paper II, Table 2), making the estimation of evolutionary rate variable depending on the strains used for comparison. Phylogenetic analysis has been employed to obtain a clearer picture of the evolutionary relationships between the various strains, and is discussed below.

Table 3. Calculation of the rates of coding and noncoding substitutions in the HA1 and HA2 sub-units of the equine H3 haemagglutinin gene.

	Nucleotide evolutionary rate* (substitutions per site per year)		
	Total	Coding	Noncoding
HA1	$1.6.10^{-3}$	$0.85.10^{-3}$	$0.75.10^{-3}$
HA2	$1.5.10^{-3}$	$0.35.10^{-3}$	$1.15.10^{-3}$

*Rates of change were calculated using sequence data from a selection of strains of equine H3 influenza; MIA/63, URG/63, ALG/72, FON/79, ROM/80, KEN/81, SAN/85, KEN/86, KEN/87, TAB/91, NS1/97 and NS2/97.

Variability at antigenic regions of the HA protein

The variability of antigenic regions is summarised for all HA1 genes of H3 equine influenza sequenced up to 1997 in Figure 1 of Paper III. Variability of strains sequenced in this study after this date is shown in Paper V, Figures 1 and 2. The main conclusions which can be drawn from these comparisons are i) that a great number of amino acid substitutions have become fixed in the genotype of the virus, indicating that they are beneficial to the virus, ii) that the majority of fixed substitutions are conserved, and that sequential change at a position is unusual, and iii) that the majority of substitutions are seen at or close to the antigenic regions determined for human H3. At least one centre of variability can be seen outside these regions (positions 260 to 268). This could depend either on slight differences in structure between human and equine H3 HA molecules, or differences in the recognition of epitopes by these different species. There is also the possibility that these substitutions may represent adaptation of the virus to some selective pressure other than immune selection.

All of the antigenic regions show variability in the collection of strains analysed in this study. Regions B, C and D, however, seem to be under the most intense selective pressure during recent years. The proximity of regions B and D

to the receptor binding site suggests that selection of mutants in these regions may be due to the selective pressure imposed by antibody-mediated inhibition of receptor binding. The selection of mutants at region C, however, must be explained in other terms, since this site is distant from the receptor binding pocket of the molecule. No mechanism has been suggested for neutralisation through binding of antibody to this region, but natural variation of this site has been detected in epidemic strains of human H3 influenza virus, indicating that it is of functional importance (Wiley et al., 1981). Sites A and E comprise fewer amino acids, and show more limited variability. Site A is placed in proximity to the receptor binding site, and a similar mechanism can be proposed for this region as for B and D. Site E is however adjacent to site C, and an explanation for the ability of antibody to neutralise through binding to this region has not yet been proposed.

Variability at the receptor binding site of the HA protein

Close examination of the sequence alignment in Paper III reveals that variability can be seen at four positions which have in earlier studies been implicated in receptor binding of the HA. Positions 137, 190, 227 and 228 show variability in natural isolates. Positions 137 and 190 have been shown to make up part of the receptor binding pocket, position 190 forming the membrane-distal ceiling of the site and position 137 forming the lateral limitation of the pocket (Weis et al., 1988) (see Figure 1). The other two positions have been shown to have the potential to affect the interaction with the receptor, but only the role of position 228 is clear, mutagenesis studies having revealed that changes of amino acid at this position can change receptor specificity of the HA (Martin et al., 1998). Of the positions found to be variable, only two have mutations which are fixed in the genome (positions 137 and 190). The other mutations can be seen in only a few strains, suggesting that they do not confer enough selective advantage on the virus to be conserved. The mutation at positions 137 appeared in the 1976 strain and is conserved in many later strains. It is impossible to say whether this change primarily reflects a change in receptor binding specificity or antigenic variation since this amino acid forms part of the exposed loop of antigenic region A. Mutagenesis studies must be carried out in order to ascertain the functional importance of this residue for receptor binding in equine H3.

The mutation at position 190 in recent strains of equine influenza virus has been studied in detail (Paper V). From comparisons of HA sequences from egg-adapted strains (Paper III, Figure 1) with sequences from strains amplified directly from clinical material, it can be concluded that the variability seen in Paper III, Figure 1 is due to mutation of position 190 during egg-adaptation of these strains. This indicates that the receptor-specificity of naturally circulating strains precludes growth to high titres in embryonated hens` eggs, and that variants with a mutation at position 190 have a growth advantage. The amino acid at position 190 constitutes the membrane-distal limit of the pocket and, in the structure of human H3, forms a hydrogen bond with the sialic acid. Replacing this glutamic acid with a lysine changes both the charge (negative to positive) and the

size of the side group at position 190. Hydrogen bonding with sialic acid would presumably be abrogated through replacement of the carboxy group of glutamic acid with the amino group of the lysine. Also, the changes in charge and size present different steric requirements for the binding of sialic acid.

From the sequencing studies it is possible to conclude that there is a strong correlation between changes in antigenicity and evolution of the gene encoding haemagglutinin. In order to further clarify the nature of evolution of the HA protein, we chose to study the inter-relationships of HA from a number of individual strains by inferring phylogenies based on these sequences.

Phylogenetic studies

Three individual phylogenetic analyses have been carried out in this study. The first is based on nucleotide sequence data, and demonstrates that two patterns of genetic drift of haemagglutinin can be seen up to 1991. Considerable heterogeneity can be seen between viral strains isolated from 1963 to 1976, with the TK2/71 and ALG/72 strains forming a disparate branch. The divergence of these two isolates represents an evolutionary dead-end, since virus belonging to this lineage has not been isolated since 1972. Instead, a mainstream lineage was established comprising viruses isolated both from Europe and the United States. These findings correspond well with those of Kawaoka and colleagues (Kawaoka et al., 1989a) who found the same linear evolution amongst post-1972 virus isolates. The phylogenetic relationship defined by Endo and colleagues (Endo et al., 1992) differs. In their paper they conclude that the HA genes have evolved in two fundamentally different lineages, one originating prior to 1963, containing the TK2/71 and ALG/72 strains, as well as the MIA/63 and URG/63 strains, and the other originating prior to 1967, containing all other strains. This difference in the interpretation of phylogenetic data is probably due to the use of different methods for the generation of phylogenetic trees. The topology of the three trees is basically identical, but the assumption by Endo and colleagues that two ancestral roots exist differs. This implies that virus was introduced into the equine population at two separate times, once around 1963, and a second time around 1967. The fact that a great number of coding substitutions appeared in the URG/63 isolate and subsequently became fixed in the genotypes of viruses belonging to both of the presumptive lineages argues strongly against this contention, and suggests that virus was introduced once, presumably from a non-equine host, and subsequently evolved in an increasingly linear fashion until 1991.

In order to infer a phylogeny based solely on phenotypic change of virus, we chose to calculate the second tree on the basis of amino acid sequences (Paper III, Figure 2). This tree encompasses over 40 taxons, the entire complement of sequences available for the equine H3 HA1 in the EMBL database at the time of the study. The topology of the tree up to 1988 correlates well with that seen in the first study. After this time, however, divergence into two separate lineages can be seen. These have been named European and American lineages because of the

origins of the majority of isolates belonging to each branch. The branching pattern seen in this phylogeny is similar to that which was seen by Daly and colleagues (Daly et al., 1996), who examined 38 sequences using the same method. All strains isolated after 1993 belong to the American lineage, raising the question of whether European-lineage virus has been superceded by American-lineage virus. The phylogeny presented in Paper V demonstrates that this is not the case, and that representatives of the American and European lineages cocirculate during epidemics. Cocirculation of variants of equine H3 influenza has previously been noted by several authors (Adeyefa et al., 1996; Daly et al., 1996; Gupta et al., 1993; Lindstrom et al., 1994). The studies by Adeyefa and colleagues and Daly and colleagues both demonstrated the cocirculation of American and European lineage viruses, whereas the studies by Lindstrom and colleagues and Gupta and colleagues showed that virus which was extremely similar to the original MIA/63 strain cocirculated with modern strains. Several explanations for the latter finding have been suggested, such as insufficient inactivation of virus used for vaccine manufacture, release of virus from a laboratory and so-called frozen evolution (Gupta et al., 1993; Lindstrom et al., 1994).

The basis for the European/American dichotomy, and circulation of members of both lineages in the population

The cocirculation of virus from American and European lineages suggests that the two variants may have adapted to immune selective pressure in different ways, differing in such a way that virus from one lineage could replicate in animals with immunity against virus from the other lineage, ensuring the survival of both lineages. Sequence comparison of the two variants, which are shown in Paper V to cocirculate, reveals multiple differences at antigenic regions B and C, indicating that the two strains differ antigenically. Serological studies of representative strains (Paper III) reveals that there are epitopes specific for each lineage. Differences in HI can thus be detected using both mAbs and polyclonal sera. Cross-protection studies have indicated that horses immunised with an American lineage strain (ARU/91) are protected against clinical symptoms upon challenge with a European lineage strain (NEW/2/93), but continue secreting virus for several days after the challenge (Mumford, J. A., personal communication). The use of homologous strains for vaccination and challenge leads to elimination of virus secretion within one to two days of the challenge. This provides support for the idea that two lineages are cocirculating because of inadequate cross-protection of infected individuals. As will be discussed below, alterations of receptor-binding site such as that shown in Paper V have been associated with escape from immune selective pressure. Only recent strains of the American lineage can be assumed to contain changes of this type and this could potentially contribute greatly to antigenic differences between the two lineages.

The possibility of utilisation of changes in receptor-binding site by the virus for immune escape

Naturally occurring receptor binding variants have been seen for human H1N1 and human H3N2 viruses (Morishita et al., 1997; Morishita et al., 1996; Nobusawa et al., 1996). The position responsible for alteration of the specificity of binding of recent H3N2 viruses isolated in Japan was shown be position 190 (Nobusawa et al., 1996), and the amino acid substitution was glutamine to asparagine. Receptor binding specificity has been assayed in these studies indirectly through the comparison of agglutination of erythrocytes from various animal species. Since the erythrocytes from the species used express terminal sialic acids of both the SAα2,6Gal and SAα2,3Gal linkages (Suzuki et al., 1985), differences in specificities for these structures are not detected. The differences in binding must therefore be due to other determinants of receptor binding, such as the presence of the N-glycolyl form of sialic acid on equine erythrocytes as opposed to the N-acetyl form expressed on human and chick red blood cells (Suzuki et al., 1985).

Position 190 has also been implicated in change in receptor binding specificity in an *in vitro* study. Temoltzin-Palacios and colleagues demonstrated that sequential mutation of human H3 influenza virus through selection of laboratory escape mutants eventually yielded variants with altered receptor binding properties to the parent virus, among others at position 190 (Temoltzin-Palacios and Thomas, 1994). Studies of antibody binding revealed that these variants were recognised by the selecting antibody in ELISA, but that the selecting antibody was unable to neutralise the virus, presumably due to the new receptor binding site configuration of HA. This result suggests that variability in specificity of receptor binding can be used as an immune evasion mechanism by the virus as an alternative to variation of antibody binding sites. A number of other workers have shown that viruses with changed receptor-binding phenotypes can be selected by growth of virus in the presence of anti-HA antibody (Daniels et al., 1987; Fazekas de St. Groth, 1977; Laeeq et al., 1997; Underwood et al., 1987; Yewdell et al., 1986). Taken together, these studies suggest that variability in receptor binding is a mechanism for the evolution of influenza virus, and the finding of receptor-binding site variability amongst circulating equine influenza viruses of the H3N8 subtype indicates that selective pressure in the equine population is selecting for such mutants in the American lineage. This has not been the case for European-lineage strains, which continue to circulate with unaltered receptor binding site, an unexpected relationship since immunity to European lineage virus is more widely spread in the equine population due to vaccination with TAB/91-like virus strains. It has been shown that selection of receptor binding variants is not a simple function of antibody neutralisation, but rather a complex response to low-affinity or sub-neutralising doses of antibody. The immune responses provoked in the horse by different viral strains have not been studied, and the basis for this difference in selection remains obscure.

An alternative explanation to the selection of variants at position 190 is that it is an effect linked to the substitution of position 189 during antigenic drift.

Position 189 comprises part of antigenic region B, and has been shown to be variable in epidemic strains of human H3N2 influenza. In all strains of equine H3N8 for which there is a substitution at position 190 (NEW/1/93, KEN/94, SOD/94, BOL/96, ALV/96, NS1-9/97), a substitution can also be seen at position 189. One could envisage a change at position 189 in response to neutralising antibody pressure, thus changing the local protein conformation somewhat. This could result in a compensatory change at position 190 leading to a slight alteration of the receptor binding site. However, one epidemic strain of equine H3N8 influenza has a substitution at position 189, but none at position 190 (FLO/93), arguing against this explanation. The fact that variant sequences at these positions are compared for egg-adapted strains of virus (Paper III, Figure 2) makes it difficult to draw clear conclusions, and a wider selection of genes directly amplified from clinical material must be analysed in order to draw any conclusions regarding the mechanism causing this mutation.

A comparison of sequences of haemagglutinin derived directly from clinical samples and from egg-grown virus stocks

The amplification of HA genes directly from clinical material (Paper IV) provides a method for analysing the wild type virus genotype, i.e. the so-called master sequence of the virus variants (or viral quasispecies) present in the airways of infected animals. Although the sequence generated in this type of analysis reflects the consensus at each position, and not the proportions of variants with different genotypes in the stock, it must be considered as representative of the infecting agent. Any passaging of the virus in cell culture or embryonated eggs after isolation will select a particular genotype. The isolation and propagation of circulating viruses is necessary in order to analyse antigenic properties of the virus, but sequence analyses of these stocks should be interpreted with caution. Paper V demonstrates through direct amplification of serial passages of virus from clinical sample egg-adapted laboratory stock that a mutation is introduced at position 190 in the HA gene of recently circulating strains BOL/96 and ALV/96. Ilobi and colleagues have previously reported that no consensus substitutions could be seen in the HA genes of egg adapted strains when compared to sequences amplified directly from clinical material (Ilobi et al., 1998). This discrepancy is presumably due to the choice of virus strains used in the study. It is clear that the necessity for adaptation to the culture system must be related to the receptor-binding specificity of the original virus. Only recent strains of the American lineage have been shown to be mutated at position 190, and it is presumably only these strains which require adaptation through mutation at this position. No definitive conclusion can be drawn regarding strains NEW/1/93, KEN/84 and SOD/84, but the similarities with BOL/96 at positions 189 and 190 suggest that these, too, represent egg-adapted virus strains. The fastidiousness of recent virus strains in their growth requirements indicate that the changed receptor specificity of circulating strains is making isolation in embryonated hens` eggs difficult. Alternative culture systems for the isolation of virus should be explored to facilitate the isolation of circulating strains, and also to prevent the

adaptation demonstrated in Paper V. As has been discussed above, it is a distinct possibility that the change in receptor binding site seen among circulating viruses could be an immune escape mechanism, in which case the incorporation of virus with the wild-type specificity in vaccines is important in order to elicit a neutralising humoral response.

Concluding remarks

From the studies presented in this thesis, it is possible to draw a number of conclusions regarding the epidemiology of equine influenza H3N8 virus.

Serological studies support antigenic drift for equine influenza virus
Studies using both mAbs and polyclonal sera show that discrete epitopes have changed during the evolution of the virus from 1963 to 1996. Polyclonal sera from ferret and horse demonstrate that sera raised against one variant has limiting cross-neutralisation capacity with other strains, showing in some cases inability to recognise heterologous strains. H3N8 equine influenza virus thus displays antigenic drift, and the extent of this drift may be such that cross-protection with different strains is limited or abrogated.

Sequence analyses of virus strains demonstrates that the HA1 component of the haemagglutinin evolves through immunological selective pressure
The high ratio of coding to noncoding substitutions seen in HA1 indicates that this protein is under strong positive selection pressure. On close examination of the localisation of substitutions, it is found that the majority of conserved substitutions, i.e. those presumed to be beneficial to the virus, are localised in parts of the protein previously shown to comprise antigenic regions in human H3 influenza virus.

Phylogenetic studies reveal the cocirculation of two divergent evolutionary lineages of the virus
Studies based on the amino acid sequences of equine influenza strains conclude that these viruses have split into two divergent lineages after 1988, and that strains belonging to both lineages are cocirculating. This finding is significant since it raises the question of why one lineage has not superseded the other because of immune pressure. Studies by other groups indicate immunity elicited against a member of one lineage is insufficient to eliminate virus replication and shedding by a member of the other lineage. This, together with the serological differences seen between representatives of both lineages, suggests that each lineage could circulate among horses which are previously immunised with a member of the other lineage.

RT-PCR facilitates amplification and sequencing of viral genes directly from clinical samples
An RT-PCR method was modified for use with nasal swab samples from horses infected with equine influenza virus of both subtype H3N8 and subtype H7N7. DNA copies of viral genes are generated which can be used for sequencing studies, thus gaining information on viral genotypes prior to egg adaptation. Since the isolation of virus has become problematic during recent years, use of this system in routine diagnostics will allow characterisation of virus even from

samples which will not grow in culture.

Adaptation to culture in embryonated hens` eggs through mutation in the haemagglutinin is seen in recent strains of H3N8 equine influenza
Sequence analysis of serial passages of virus from clinical sample to egg stock show that a mutation is introduced at position 190 of the haemagglutinin. This finding can be generalised only to include the most recently isolated strains.

Practical aspects
The regular updating of vaccines for equine influenza is important, but the finding of cocirculation of lineages suggests that representatives from each lineage should be incorporated in vaccines. The finding that variation occurs in the receptor binding site of American lineage virus emphasises this point. However, since virus of the wild type specificity adapts to growth in embryonated hens` eggs through a change in this phenotype, other methods for generation of an immunogen must be found. Either an alternative culture system should be sought or alternative vaccination methods, such as DNA vaccination, might be explored.

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