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Differential Diagnosis and Aspects on Epidemiology and Pathogenesis of Equine Herpesviruses

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SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Abstract

Herpesviruses constitute a family of DNA viruses found in virtually all animal species. In horse five herpesviruses have been identified namely Equine herpesvirus (EHV) type 1, 3 and 4 belonging to the subfamily alphaherpesvirinae and Equine herpesvirus type 2 and 5 belonging to the gammaherpesvirinae. EHV-1 and 4 are the major causes of abortion and respiratory disease respectively and both cause large economical losses. EHV-2 has been associated with respiratory disease, immunosuppression and is a predisposing factor for more severe secondary infections. EHV-5 is a new virus first isolated in Australia from horses suffering from upper respiratory disease. This thesis is dealing with the diagnosis, epidemiology and pathogenesis of EHV-1, 2, 4 and 5.

EHV-2 is a predisposing factor for bacterial infection particularly with *Rhodococcus equi* and this role was studied by vaccination of foals with an EHV-2 subunit (ISCOM) vaccine. Foals vaccinated twice developed neutralising antibodies and were protected against the pneumonia while the non-vaccinated control group developed respiratory disease including pneumonia with abscesses containing *R.equi* which in some cases was fatal. Natural infection induce neutralising EHV-2 antibody as detected by a blocking ELISA. This ELISA recorded a much higher number of seropositive horses in a stable with annual EHV-2/*R.equi* infections compared to a stable with no such problems. Thus, the blocking ELISA was found to be a reliable tool for detecting recent EHV-2 infections. Likewise antibodies induced by a newly acquired EHV-2 infection in foals were also detected by the blocking ELISA and all foals tested were found to have experienced an EHV-2 infection by the age of 4 to 6 months.

The prevalence of EHV-2 and EHV-5 were studied by type-specific Polymerase chain reaction (PCR). Up to 68% of tested horses in Sweden were EHV-2 positive, i.e. had virus DNA in peripheral blood leukocytes, while 100% of the tested foals in Sweden and Hungary were positive before 2 to 8 months of age. No EHV-5 positive horses were found in Sweden while 4 out of 27 tested foals in Hungary had EHV-5 specific DNA by the age of 13 to 23 weeks showing that EHV-5 cause infections later in life.

Convalescent sera from horses contain EHV-1/EHV-4 cross-reactive antibody which up to recently made differentiation virtually impossible. A new indirect ELISA that differentiate between the two virus types showed that the epidemiology between EHV-1 and EHV-4 differ considerably. While almost 100% of the horses were seropositive to EHV-4, the number of EHV-1 seropositive horses varied between 9 and 56 % in different stables. Most foals had maternally-derived antibodies to EHV-4 until 4 to 6 months of age, after which they all became infected with EHV-4 and seroconverted. Only 2 out of 48 foals had maternal antibodies to EHV-1 and very few became infected.

Key words: EHV-1, -2, -4, -5, differential diagnosis, *R. equi*, ISCOM, ELISA, PCR,

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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 Nordengrahn A., Rusvai M., Merza M., Ekström J., Morein B. and Belák S. 1996. Equine herpesvirus type 2 (EHV-2) as a predisposing factor for *Rhodococcus equi* pneumonia in foals: prevention of the bifactorial disease with EHV-2 immunostimulating complexes. *Veterinary Microbiology 51, 55-68.*

II Nordengrahn A., Merza M., Svedlund G., Ronéus M., Treiberg Berndtsson L., Lindholm A., Drummer H.E.. Studdert M.J., Abusugra I., Gunnarsson E. and Klingeborn B. 1999. A field study of the application of a type-specific test distinguishing antibodies to Equine herpesvirus -4 and -1. *Equine Infectious Diseases VIII 125-128.*

III Nordengrahn A., Klingeborn B., Lindholm A. and Merza M. The use of a neutralising monoclonal antibody to detect infections of Equine herpesvirus type 2 (EHV-2). *Submitted.*

IV Nordengrahn A., Ros, C., Lindholm, A., Pálfi, V., Belák S. and Merza M. Studies on the prevalence of Equine herpesvirus type 2 and 5 DNA in horse populations by using type-specific PCR assays. *Manuscript to be submitted.*

Abbreviations

AGID	agar-gel immunodiffusion test
AHV-1	Asinine herpesvirus type 1
AHV-2	Asinine herpesvirus type 2
AHV-3	Asinine herpesvirus type 3
AMI	antibody mediated immunity
b ELISA	blocking ELISA
BHV-1	Bovine herpesvirus type 1
CD	cluster designation
CF	complement fixation
CMI	cell mediated immune response
CPE	cytopathic effect
CTL	cytotoxic T-cell leukocytes
DNA	deoxyribonucleic acid
E.coli	*Esherichia coli*
EHV-1	Equine herpesvirus type 1
EHV-2	Equine herpesvirus type 2
EHV-3	Equine herpesvirus type 3
EHV-4	Equine herpesvirus type 4
EHV-5	Equine herpesvirus type 5
ELISA	enzyme-linked immunosorbent assay
FMDV	Foot and mouth disease virus
gB	glycoprotein B
gp	glycoprotein
GST	gluthatione-S- transferase
G + C	guanine + cytosine
HHV-8	Human herpesvirus 8
HSV	Herpes simplexvirus
IE	immediate early
IF	immunofluorescence
IFN-γ	interferon- gamma
Ig	immunoglobulin
IL-2	interleukin-2
i.m.	intramuscular
i.v.	intravenous
IR_S	internal indirect repeat sequences
ISCOM	immuno stimulating complex
kD	kilodalton
kbp	kilobase pairs
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
NK	natural killer cells
NO	nitric oxide

OD	optical density
OG	1-O-n-octylglycopuranoside
ORF	open reading frame
PCR	polymerase chain reaction
PI	percent inhibition
PRV	Pseudorabiesvirus
Quil A	Quillaja saponaria
R. equi	*Rhodococcus equi*
S	short
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
Tc	cytotoxic T cell
Th	T helper
TK	thymidine kinase
TNF-α	tumour necrosis factor-alpha
TR_S	terminal indirect repeat sequences
U_L	unique long
VNT	virus neutralisation test
α	alpha
β	beta
γ	gamma

Introduction

Herpesviridae

Herpesviruses derive their name from the Greek herpes, ερπειν, meaning "creep". The term herpes was used for at least 25 centuries in medicine to describe a variety of skin disorders, but its meaning changed considerably during that time. Initially herpesviruses were named after the clinical conditions or the disease they cause. Herpes simplex virus, herpes zoster virus, pseudorabies virus (Aujeszky's disease) and Marek's disease virus are examples of such designations. Subsequently, herpes viruses have also been designated after their hosts (e.g., herpesvirus hominis), the cell pathology they cause (e.g., cytomegalovirus), or their discoverers (e.g., Epstein-Barr virus).

Today about one hundred herpesviruses have been to a greater or less extent been characterised. They are ubiquitous and are found in insects, reptiles, and amphibia as a matter of fact in virtually every species of bird and mammal that has been investigated. Members of the family Herpesviridae are assigned to one of three subfamilies: Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae. The classification is primarily based on biological criteria including the kind of disease produced, cell tropism, nature of latency and rate of growth (Roizman et al., 1992). With advances in molecular biology, including understanding of amino acid composition and genome architecture, the molecular and biological properties were generally found to be correlated. Some notable exceptions have been reported, however, such as the recognition that Marek's disease herpesvirus of chickens, previously considered a lymphotropic gammaherpesvirus, possesses a genome structure compatible with alphaherpesviruses, Also, the equine herpesviruses 2 and 5, previously classified as betaherpesviruses and referred to as equine cytomegaloviruses (because of perceived similarities to human betaherpesvirus or cytomegalovirus) have been shown to share molecular properties with gammaherpesviruses such as Saimirine herpesvirus 2 (herpesvirus saimiri) and human herpesvirus 4 (Epstein-Barr virus) also included in this group is the newly recognised human herpesvirus 8 (HHV-8) a probable cofactor in development of Kaposis Sarcoma. While the subfamily classification continues to be useful, herpesviruses will probably have to be reassigned on the basis of molecular criteria. It is also recognised that the subfamily designations do not suffice to reflect the molecular variability found among the >100 members of the family.

Herpesvirus particles are fragile and do not survive well outside the body. In general transmission requires close contact, particularly the kinds of physical

contact that brings mucosae into apposition, for example, coitus, or licking and nuzzling as between mother and offspring or between foals. In large, closely confined populations, such as those found in a cattle feedlot, multiple farrowing unit, cattery, or broiler chicken run, sneezing and short distance droplet spread are major modes of transmission. Collectively and individually herpesviruses are versatile pathogens. Many alpha-herpesviruses produce localised lesions, particularly in the skin or on the mucosal surfaces of the respiratory and genital tracts. These are characterised by the sequential production of vesicles, pustules, and shallow ulcers which become covered by a pseudomembrane and heal after 10-14 days, usually without scar formation. Generalised alphaherpesvirus infection (like Pseudorabiesvirus (PRV) and Herpes Simplexvirus (HSV), characterised by foci of necrosis in almost any organ or tissue, is seen, when animals less than 3 months of age are infected without the protection provided by maternal antibody. In pregnant animals a mononuclear cell-associated viremia may result in transfer of virus across the placenta, leading to abortion, focal necrotic lesions being found throughout the foetus. Betaherpesviruses are associated with respiratory and generalised disease, while gammaherpesviruses may produce systemic disease and tumours.

Herpesviruses can survive from one generation to the next by establishing latent infections. Latency, i.e. persistence of the virus probably throughout life, is a feature of all herpesviruses. Reactivation is usually intermittent and may be associated with stress such as is occasioned by intercurrent disease, shipping, cold , or crowding. Shedding of virus in nasal, oral, or genital secretions provides the virus source for transmission to other animals, including transfer from mother to offspring. Some betaherpesviruses and gammaherpesviruses cause a persistent, cell-associated viremia and appear to be shed continuously via epithelial surfaces. For many members the sites of latency have not yet been defined. Many alphaherpesviruses persist in neurones, probably as a circular episomal form of the genome. The beta-and gammaherpesviruses persist in white blood cells or epithelial cells. Alpha herpesviruses show rapid growth and produce acute lytic infections. The beta- and gammaherpesviruses are slowly growing, slowly lytic viruses which tend to remain highly cell-associated during replication and are associated with low-grade, chronic diseases processes. Epstein-Barr virus, the prototype lymphotropic gammaherpesvirus, is linked to the aetiology of Burkitts lymphoma and nasopharyngeal carcinoma; in vitro, Epstein-Barr virus transforms (immortalises) B-lymphocytes. Recently a new gammaherpesvirus was discovered in man, namely human herpesvirus 8 was identified by comparing DNA sequences in Kaposis Sarcoma lesions with those of normal skin. Identified unique DNA sequences, were found to be closely related to regions of the genome of herpesvirus saimiri (HVS) and Epstein- Barr virus. The virus infects lymphocytes and are associated with cell immortalisation (Levy, 1997).

In general protection against virus infection is considered to a great extent to be dependent on cell mediated immunity (CMI) but also on antibody mediated immunity (AMI). Cell mediated immune protections against intracellular parasites including viruses is anticipated to be exerted by CD4 T helper 1 cells (Th1) producing interleukin-2 (IL-2) and interferon-γ (IFN-γ). Both IL-2 and IFN-γ have regulatory effects on the immune system e.g. by upregulating cytotoxic T-cell lymphocytes (CTL). The CTL´s are bearing CD8 molecules which are important to destroy the virus infected cells. IFN-γ also acts as an effector mechanism by stimulating macrophages for phagocytosis, nitric oxide (NO) production and tumour necrosis factor- α (TNF-α) production with virus killing effect. Macrophages also produce cytokines stimulating natural killer cells (NK) and complement as well as antibody dependent cytotoxic T cells for cell lysis. For many viruses like polio and foot and mouth disease virus (FMDV) AMI is also important e.g. by virus neutralisation (VN) antibodies and in the first line of defence as IgA in mucosal surfaces (Roitt, 1990).

Herpesvirus cause latent infection which means that the virus stay hidden in the host cells for very long periods and have short "life span" extracellulary. Therefore cell mediated immune response is important to destroy herpesvirus infected cells. Herpesvirus stimulate Th1 and production of IFN-γ and enhance CTL. AMI response on the other hand is important for immune protection against extracellular herpesvirus and this may be directed towards glycoproteins. Specific VN antibodies which efficiently neutralises virus in plasma and tissue fluids can be measured in-vitro by VN-test. Also virus fusion activity can be blocked by antibodies. IgA antibodies are involved in other mechanisms by blocking virus in the mucosa. Finally complement activation and antibody mediated cytotoxic T cells are examples on immune protection exerted by AMI.

Properties of herpesviruses

Herpesviruses are about 150 nm in diameter. The core contains a linear double-stranded DNA genome. The core is enclosed in an icosadeltahedral capsid approximately 100 nm in diameter that is composed of 12 pentameric and 150 hexameric capsomers. An amorphous, sometimes asymmetric tegument layer (consisting of mainly unknown protein) surrounds the nucleocapsid which in turn is surrounded by a lipid-protein layer (envelope) i.e. about 10 amphipathic viral glycoprotein spikes (peplomers) anchored in a double lipid layer and projecting outward. The virus particle contains over 30 proteins, of which 6 are present in the nucleocapsid, 2 being DNA-associated, These have various biological functions including attachment to cellular receptors, fusion activity and Fc receptor activity i.e. binding normal IgG. The location of the remaining virion proteins is not well known but some other structural proteins are tegument associated. Antigenic relationships are complex. There are some shared antigens within the family, but different species have distinct envelope glycoproteins.

The large double-stranded DNA genome of at least one representative of each subfamily has been entirely sequenced. Genome size is characteristic for each virus species and varies between 124 and 235 kbp. The base composition varies from 32 to 75 G+C moles %. Repeat sequences are a feature of most herpesviruses and at least six different repeat arrangements have been defined (Roizman et al., 1992). The number of ORFs is a characteristic for each virus species and varies from approximately 68 for varicella-zoster virus to >200 for human cytomegalovirus. There are regions, which are particularly conserved within the subfamily showing colinearity. Departures from colinearity are recognised in that gene orders may be altered, and particular homologues are deleted in particular viruses.

Viral replication

Herpesvirus replication has been most extensively studied with herpes simplex virus. Betaherpesviruses and gammaherpesviruses probably follow a similar pattern, but replicate more slowly. Following adsorption via the peplomers of the envelope to host cell receptors the nucleocapsid penetrates to the cytoplasm either by fusion of the virion envelope to the cell membrane or via a phagocytic vacuole. A DNA-protein complex is freed from the nucleocapsid and enters the nucleus and shuts off host cell macromolecular synthesis.

Three classes of mRNA α, β, γ, are transcribed in sequence by the cellular RNA polymerase II. Thus α (immediate early) RNAs, when appropriately processed to mRNAs, are translated to α proteins which initiate transcription of β (early) mRNAs, whose translated product(s), β proteins, suppress transcription of further α mRNAs. Viral DNA replication then commences, utilising some of the α and β proteins as well as host cell proteins. The program of transcription then switches again, and the resulting γ (late) mRNAs which are transcribed from sequences situated throughout the genome, and structural proteins translated into the γ proteins. Over 70 virus-coded proteins are made during the cycle, many of the α and β proteins being enzymes and DNA-binding proteins whereas most of the γ proteins are structural. Intricate controls regulate expression at the level of both transcription and translation. Viral DNA is replicated in the nucleus, and newly synthesised DNA is spooled into preformed immature capsids. Maturation involves the encapsidation of DNA into nucleocapsids and the association of nucleocapsids with altered areas of the inner layer of the nuclear membrane of the host cell, followed by envelopment by budding from the nuclear membrane. Mature virions accumulate within vacuoles in the cytoplasm, and they may be released by exocytosis (discharge of virus from cell) or cytolysis (cell destruction). Virus-specific proteins are also found transported in the plasma membrane, where they are involved in cell fusion and may act as Fc receptors. Any viral protein, including nucleoproteins processed to peptide and presented by MHC class I molecules, in the plasma membrane are potential targets for

immune cytolysis. Intranuclear inclusion bodies are characteristic of herpesvirus infections and can usually be found both in cells from tissues of herpesvirus-infected animals and in appropriately fixed and stained cell cultures (for more details on Herpesvirus, Fenner et.al., 1993).

Herpesviruses in the horse

Five herpesviruses are known to infect the horse, equine herpesvirus type 1 or equine abortion virus (EHV-1), equine herpesvirus type 3 (equine coital exanthema, EHV-3) and equine herpesvirus type 4 (equine rhinopneumonitis virus, EHV-4), which all are members of the subfamily Alphaherpesvirinae, and equine herpesvirus type 2 (EHV-2) and equine herpesvirus type 5 (EHV-5), members of the Gammaherpesvirinae. Homologues of EHV-1, EHV-2 and EHV-3, designated asinine herpesvirus 3 (AHV-3), AHV-2 and AHV-1 respectively, have been isolated from donkeys (Browning and Agius. 1996).

Equine herpesvirus type 1 and 4

EHV-1 and EHV-4 are related alphaherpesviruses, which are major causes of abortion respectively respiratory disease and are of considerable economic importance all over the world (Campbell and Studdert, 1983, Allen and Bryans, 1986). Before 1981, EHV-1 and EHV-4 were considered to be the same virus and was named EHV-1. It was assumed that EHV-1 caused respiratory disease, established a latent infection and subsequently caused abortion by reactivation of latent virus. A similar series of events was implied for the occurrence of EHV-1 encephalitis. Restriction endonuclease DNA fingerprints of EHV-1 was the tool to separate what has been designated EHV-1 into two different viruses (Sabine et al., 1981, Studdert et al., 1981, Turtinen et al., 1981). In 1988 the viruses were designated EHV-1 and EHV-4 (Roizman et. al., 1992).

Epizootology

As with all herpesviruses, it is assumed that EHV-1 and EHV-4 establish persistent, life-long latent infections. Experimentally, both viruses can be reactivated with very high, prolonged doses of corticosteroids and mild nasal trauma (Edington et al., 1985; Browning et al., 1988a,b). Reactivation of latent EHV-4 probably causes recurrent disease accompanied by virus shedding and the opportunity for transmission to other horses, directly or indirectly. Usually, each year in the foal crop born on a farm there is a round of EHV-4 respiratory disease (rhinopneumonitis). Reactivation of latent EHV-1 in a horse may cause mild respiratory disease and transmission to other horses in contact. If virus is reactivated in a mare which has not previously aborted an EHV-1 infected foetus she may now abort. Abortion of an EHV-1 infected foetus provides a high risk for in-contact mares to become infected from the conceptus (foetus, foetus

membranes and foetal fluids). It, thus, sets the scene for an abortion storm whereby up to 80% of in-contact mares infected in this way may abort within 3 weeks of the first case. Where abortions occur, the index case may have at least three definitions: 1) it may be a carrier mare that after reactivation sheds virus from the respiratory tract only 2) it may be a mare that delivers an aborted conceptus, or 3) it may be a mare that initially shed EHV-1 from the respiratory tract and subsequently aborts. Mares appear to abort from EHV-1 only once. Following the first case of abortion, the virus is efficiently transmitted horizontally to other in-contact mares (Campbell and Studdert 1983; Allen and Bryans, 1986; Studdert et al., 1992). It has up to now not been possible to routinely identify EHV-1 carrier mares because of the extensive antigenic cross-reactivity between EHV-1 and EHV-4. However, an ELISA capable of distinguishing between EHV-1, EHV-4 and dually infected horses has now been developed (Crabb et al., 1995). Using this test in a broad seroepidemiological survey Crabb and Studdert (1993) showed that only 9% of the sera tested were positive for EHV-1 antibody, while all tested sera were positive for EHV-4 antibody. The sera were from randomly selected samples of Australian Thoroughbred horses obtained between 1967-1974, prior to the first confirmed case of abortion in Australia in 1977. More recently, the prevalence of EHV-1 specific antibody in a random sampling of 97 Australian Thoroughbred horses was 30% (Crabb et al., 1994) while the prevalence of EHV-4 specific antibody in these horses was 100%.

Disease signs

Respiratory disease

Acute EHV-4 respiratory disease, which is rarely fatal, occurs mainly in foals, weanlings and yearlings, characterised by fever, anorexia and profuse serous nasal discharge which later becomes mucopurulent. Extensive necrosis of the epithelial cells of the upper respiratory tract, especially in the nasal cavity, is accompanied by an acute inflammatory response. Virus may reach the lungs, especially in young horses, and cause bronchopneumonia; most damage is probably caused by secondary bacterial infection, especially with streptococci of Lancefield group C (Studdert, 1974; Burrows and Goodridge, 1978; Liu, 1978). The incidence of horses with antibodies to EHV-4 is greater than the reported incidence of acute disease, and mild or subclinical infection is presumably common. Respiratory disease after natural and experimental infection with EHV-1 resembles that produced by EHV-4 (Gleeson and Coggins. 1980; Allen and Bryans, 1986).

Abortion

The natural route of EHV-1 infection is presumed to be via the upper respiratory tract, followed by a cell-associated viremia, resulting in placental transfer and

infection of the foetus with subsequent abortion (Bryans and Pricket, 1970). EHV-1 is mostly recognised as a cause of abortion in mares with no other clinical signs. The mares usually abort at 6 to 11 month gestation (Bryans, 1978). Tissue from aborted foetuses shows a range of macroscopic and microscopic lesions. In abortions occurring up to 6 months of gestation, foetuses are usually autolyzed. In late abortions (after 7 months) necrotic foci are visible in about 25% of the cases, notably in the liver, lung, spleen and adrenal glands. Pregnant mares were reported to abort 14 to 120 days after exposure without showing any clinical signs (Doll and Bryans, 1962). However, if EHV-1 infection is followed by lifelong, latency then clearly a mare may abort months or years after primary infection as protective immunity to EHV-1 last probably for only a certain period of time.

Perinatal disease

The birth of weak and dying foals in EHV-1 abortions has been reported (McGee, 1970); also neonatal foal disease associated with prenatal EHV-1 infection without concurrent abortion or respiratory disease has been described (Dixon et al., 1978: Hartley and Dixon, 1979). The syndrome involved stillbirths, the birth of weak, depressed foals which died within 24 h, and foals appearing normal at birth but after which they developed severe respiratory distress within 18 to 24 h and died within 24 to 72 h of birth.

Genital disease

In spite of the fact that EHV-1 causes abortion it has rarely been isolated in connection with natural disease in the genital tract (Petzholdt, 1970). Also, lesions after experimental EHV-4 infection were rarely found (Turner et al., 1970).

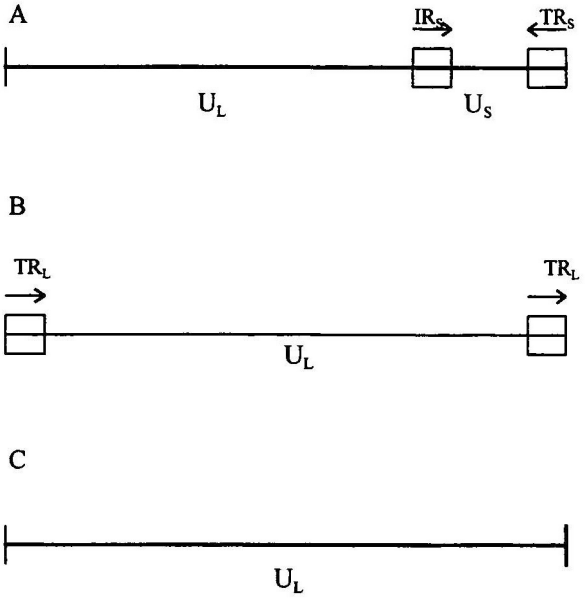
Myeloencephalitis

Neurologic disease associated with EHV-1 infection has been reported with increasing frequency (Charlton et al., 1976; Dinter and Klingeborn, 1976; Little and Thorsen, 1976; Thomson et al., 1979b; Greenwood and Simson, 1980; Crowhurst et al., 1981; Thein, 1981; Saxegaard, 1966; Britsch and Dam, 1971). Outbreaks of EHV-1 neurologic disease are usually associated with abortion and/or respiratory disease, but not invariably, as reported by Dinter and Klingeborn (1976) and Thein (1981). The clinical signs vary from a mild ataxia to complete recumbency with fore and hind limb paralysis.

Genome

The genomes of EHV-1 and EHV-4 are linear dsDNA with a length of about 150 and 145 kbp respectively. The genomes are composed of a unique long (U_L) region and a short (S) region; S is flanked by an internal and terminal indirect repeat sequences (IR_S and TR_S) between which is located a unique short (U_S) region (see fig 1a). EHV-1 has been entirely sequenced and 80 ORFs likely to encode 76 proteins were identified (Telford et al., 1992). Only parts of the EHV-4 genome has been sequenced (Cullinane et al., 1988, Riggio et al., 1989, Nicolson et al., 1990b, Nicolson et al., 1990a, Nicolson and Onions, 1990, Nagesha et al., 1993, Riggio et al., 1993). The data strongly support the view that EHV-1 and EHV-4 are colinear, i.e., homologous genes are located in approximately the same positions in their respective genomes. However, some variations in the arrangement of the genes with respect to the repeat structures have been recognised (Nagesha et al., 1993).

Fig 1.
The genome structure of a) Equine herpesvirus type 1 and 4, b) Equine herpesvirus type 2 and c) Equine herpesvirus type 5.



Proteins and Immunity

Much of the current herpesvirus research is focused on delineating the functions of the 76 or so predicted gene products. For herpes simplexvirus 1(HSV-1) it is known that viral proteins and their precursor mRNA transcripts are synthesised in a co-ordinated regulated, and sequentially ordered cascade and are assigned to one of three classes of genes, α (immediate early; IE), β (early) or γ (late), based on the time they are expressed during replication (Roizman and Sears, 1990). Four high molecular weight IE proteins have been identified in EHV-1 infected cells. As with other alphaherpesviruses it is anticipated that production of these IE proteins is required for the expression of all other genes (Gray et al., 1987). In the case of HSV, 33 virion proteins have been identified so far, including seven capsid proteins, at least eight envelope glycoproteins and numerous tegument proteins (Roizman and Sears, 1990). The composition of the EHV-1 and EHV-4 genomes is probably similar, with 25-30 proteins resolved for EHV-1 and EHV-4 by SDS-PAGE. Comparative studies of the virion proteins, including glycoproteins of EHV-1 and EHV-4 have revealed similar but not identical overall electrophoretic profiles (Turtinen et al., 1981, Bridges et al., 1988, Meredith et al., 1989, Crabb and Studdert, 1990). The glycoproteins of herpesviruses like in all enveloped viruses play important roles in the infection process, mediating attachment and entry of the virion into the host cell and cell-to-cell spread (Spear, 1985). Because of their location in the viral envelope and on the surface of infected cells they are principle targets for host immune responses and hence the focus of much research into the development of vaccines and diagnostic reagents. Also, there is considerable evidence to show that many of the homologous glycoproteins of other alphaherpesviruses, in particular Herpes simplex 1 and 2 (HSV1 and HSV2), pseudorabies virus (PRV) and bovine Herpesvirus 1 (BHV-1), have the same or a similar function. EHV-1 homologues for 10 of the 11 recognised HSV1 glycoproteins have been identified from DNA sequence analyses; gB (Whalley et al., 1989, Guo, 1990), gC (Allen and Coogle, 1988, Guo et al., 1989), gD (Audonnet et al., 1990, Flowers et al., 1991), gE (Audonnet et al., 1990, Elton et al., 1991), gG (Colle et al., 1992, Telford et al., 1992), gH (Robertson et al., 1991), gI (Audonnet et al., 1990, Elton et al., 1991), gK (Zhao et al., 1992), gL (Telford et al., 1992) and gM (Baines and Roizman 1993, Telford et al., 1992,). Also EHV-1 possesses at least three other glycoproteins which have no HSV homologue, namely gp2, gp 21/22 and gp 10 (Allen and Yeargan, 1987, Whittaker et al., 1990). In contrast to EHV-1, EHV-4 has only been partially sequenced. Genes encoding the EHV-4 homologues of gB (Riggio et al., 1989), gC (Nicolson and Onions, 1990), gD (Cullinane et al., 1993), gG (Crabb et al., 1992) and gH (Nicholson et al., 1990a) have been obtained. It is assumed that the EHV-4 genome contains homologues of all the mentioned EHV-1 glycoproteins.

By aid of EHV-1 and EHV-4 specific monoclonal antibodies (mAbs) and monospecific postinfection EHV-1 and EHV-4 sera it has been shown that both

EHV-1 and EHV-4 glycoproteins possess both common and type-specific epitopes (Yeargan et al., 1985, Allen and Bryans, 1986, Allen et al., 1988, Whittaker et al., 1990, Crabb et al., 1991). There are two exceptions, EHV-1 gp21/22, which has not yet been studied in detail, and EHV-4 gG which elicits a type-specific response in the horse (Crabb et al., 1992). EHV-1 gG has also been shown to possess type-specific epitopes (Crabb and Studdert, 1993). Infection or immunisation with either virus elicits cross reactive antibody revealed by virus neutralisation, ELISA, western blotting and immunoprecipitation (Allen and Bryans, 1986, Crabb and Studdert, 1990).

Immune responses resulting from natural infection of the respiratory tract is of short duration. Despite persistence of VN antibody, the respiratory mucosa may be asymptomatically reinfected within 3-4 months (Doll, 1961). Protective immunity against abortion is more durable, but unpredictable. Only rarely do naturally infected mares abort in consecutive pregnancies i.e. with an interval of 7-11 months between abortions. Maternal antibodies does not cross the foetal placenta but the maternal immunity is solely obtained from colostrum (Kendrick and Stevenson, 1979). Natural infection with one of the viruses do not appear to give cross-protective immunity to the other virus type.

Diagnosis

Unambiguous diagnosis of EHV-1 and EHV-4 infection has traditionally required virus isolation, identification and typing. Specimen from a horse with respiratory disease are obtained by swabbing the nasopharynx. For virus isolation from an aborted foetus usually material from the lung, spleen or kidney is inoculated on equine or rabbit monolayer cell cultures. If virus is present, cytopathic effect (CPE) is seen and electronmicroscopy is used to identify the virus as herpesvirus. At this stage a diagnosis of EHV-4 for respiratory isolates, or EHV-1 for abortion isolates would be most probable. To determine the identity of the virus type-specific mAbs (Yeargan et al., 1985), DNA fingerprinting (Allen et al., 1983) or PCR (Ballagi et al., 1990; O'Keefe et al., 1991; Sharma et al., 1992: Kirisawa et al., 1993) are used.

Because of the high crossreactions between EHV-1 and EHV-4 serological determination has been difficult. Commonly used are virus-neutralisation (VN) tests and the complement fixation (CF) test that detect the acute phase of a EHV-1 or EHV-4 infection. Recently a type-specific ELISA test has been developed that allows the clear distinction between EHV-1, EHV-4 and dual-infected horses (Crabb et al. 1995). The antigens used are defined regions of the EHV-1 respectively EHV-4 glycoprotein G (gG) homologues expressed in *E.coli* as fusion proteins with regions of the gG that contain highly type-specific epitopes (Crabb and Studdert 1993). Another test, which utilises an EHV-1 specific monoclonal antibody, has successfully been used for the detection of EHV-1 seropositive horses (van de Moer et al., 1993).

Vaccination

Several attempts have been done to develop EHV-1 and -4 inactivated vaccines (Campbell and Studdert, 1983). The inactivated EHV-1 vaccine of Shimizu et al. (1974b) elicited a VN antibody response only in horses previously infected with EHV-1. Mayr et al. (1978) demonstrated that acetylethyleneimine- inactivated EHV-1, adjuvanted with mineral oil or aluminium hydroxide induced satisfactory immune response in both seronegative and seropositive horses resulting in VN and CF antibody titres of similar levels to those after vaccination with a modified live virus vaccine ("Prevaccinol") or natural infection. The inactivated virus vaccine ("Pneumabort-K") developed by Bryans (1978) evoked VN antibodies in 94% of pregnant mares at levels purported to be protective against abortogenic infection during a 5 to 6 month period of pregnancy. Comparing control and vaccinated groups, the abortion incidence was decreased by 65% in the vaccinated group.

Attempts to prepare subunit vaccines based on one or more of the immunodominant membrane glycoproteins have been done. Affinity purified preparation of gC used to immunise hamsters induced VN antibodies and protected the animals against homologous challenge (Stokes et al., 1991).
EHV-1 gC and gB have been expressed in vaccinia virus vector and the recombinant virus elicited an immune response that protected hamsters against EHV-1 challenge (Guo et al., 1989, 1990). A third major glycoprotein, EHV-1 gD, when expressed by a recombinant baculovirus was shown to elicit neutralising antibodies in rabbits and mice (Love et al., 1993). Significantly, this antibody neutralised both EHV-1 and EHV-4. For man the HSV-1 and -2 causing genital tract disorders the general vaccine candidate is gB inducing VN antibody titres and cytotoxic T cell (Tc) response and gD inducing VN antibodies.
The use of new adjuvants, inducing right type of immunity in the host of interest, have high priority in the development of new vaccines. Immune stimulating complex (ISCOM) experimental vaccines have been prepared for different herpesviruses including HSV-1 and -2 (Erturk et al., 1989). ISCOMs contain selected proteins bound to the plant glycoside Quil A forming a cage-like formation (Morein et al., 1984). EHV-1 ISCOM vaccines, prepared from detergent-treated purified virions have been described (Cook et al., 1990). When tested in hamsters the vaccine provided full protection and was significantly better immunogen than a non-adjuvanted aqueous suspension of the virus. ISCOMs have also been prepared with EBV (γ- virus) and induced protection to tumourgenic virus challenge in cotton tap tamarins (Wilson et al., 1999).

An interesting area in vaccine development is engineered viruses where gene(s) have been deleted and/or inserted. The gene(s) of choice should reduce virulence to a level that does not cause disease but allows adequate virus replication so as to induce good antiviral immunity. An EHV-1 vaccine with a thymidine kinase (TK) deletion has been described which did not cause disease when administered i.m. or i.v.; its safety with respect to the induction of abortion was not assessed

(Cornick et al., 1990).

Glycoprotein genes are deleted to reduce virulence and for marker purposes e.g. in swine, so that vaccinated animals can be distinguished from nonvaccinated ones. So far no such vaccine has been developed for EHV-1 and EHV-4. The choice of gene for deletion is again important. Deletions in the PRV gE, gC and gG (van Oirschot et al., 1990) showed that the immunogenicity of the gE and gC deletion mutants was inferior to that of the parental virus, while the gG deletion mutant performed equally well. If EHV-1 and EHV-4 gG deletion mutants behave similarly to those of PRV, this together with the type-specific properties of the EHV-1 and EHV-4 gGs would make them ideal candidates for marker purposes in EHV1/4 recombinant vaccines (Crabb et al., 1992; Crabb and Studdert, 1993).

Equine herpesvirus type 2 and 5

EHV-2 has until recently been described as a cytomegalo- or betaherpesviruses. Studies on the genomic heterogeneity (by restriction enzyme cleavage) on equine betaherpesvirus isolates by Browning and Studdert (1987a) led to the proposal that there exists a group of betaherpesvirus which is distinct from, and yet related to EHV-2. This group of betaherpesvirus has been designated EHV-5. Further molecular studies have demonstrated that these two viruses belong actually to the Gammaherpesvirinae subfamily, which includes the human pathogen Epstein-Barr virus (EBV) (Browning and Studdert, 1987a, Telford et al., 1993).

Infections by EHV-2 are among the most common viral infections of the horse and has been isolated from healthy as well as horses with different clinical signs but proof of its role as a pathogen has been disputable. EHV-5 has so far only been reported in Australia, Switzerland and Germany (Browning and Studdert 1987b, Francini et al. 1997, Borchers et al 1999). In Australia 4 isolates have been obtained from horses with upper respiratory tract disease. In Germany EHV-5 has been isolated from a Przewalski's wild horse originating from a Zoo in Berlin. An extensive research on these viruses is now going and hopefully new insights on the pathogenesis and also a renewed search for links between infection and poor physical performance of the horse. Not only the role of EHV-2 and EHV-5 as primary pathogenic agents, but also in enhancing the pathogenicity of other infectious agents.

Epizootiology

EHV-2 has been isolated from horses throughout the world. A prevalence of VN antibodies up to 90% has been reported and the virus has been isolated from circulating leukocytes in 89% of adult horses (Kemeny and Pearson, 1970; Roeder and Scott, 1975).

In a study in New Zealand of the role of EHV-2 in respiratory disease of foals, virus was recovered from all animals intermittently over the first 8 months of life. Respiratory disease was prevalent over this period. ELISA antibody titres rose gradually between 1 and 6 month of age, then reaching a plateau which corresponded to a decline in isolation rates of EHV-2. In contrast EHV-1 titres did not rise until about 4 months of age or later (Fu et al., 1986).

Disease signs

EHV-2 has been associated with a variety of symptoms and has frequently been isolated from both foals and adults with upper respiratory disease, serous nasal discharge, swollen lymph nodes, conjunctivitis, anorexia, fever and pneumonia (Plummer and Waterson, 1963; Burrows 1968; Studdert et al., 1970; Turner et. al., 1970; Roberts et al., 1974; Dutta and Shipley, 1975; Rose et al., 1974; Horner et al., 1976; Sherman et al., 1977; Palfi et al., 1978: Sugiura et al., 1983; Fu et al., 1986). EHV-2 has been linked to poor physical performance of the horse (Rose et al., 1974). It has also been suggested that EHV-2 acts as predisposing factor for other infections such as bacterial *Rhodococcus equi* infection (Belák et al., 1980).The natural portal entry of EHV-2 is the upper respiratory tract (Bagust et al., 1972:; Sherman et al., 1977; Blakeslee et al., 1975). The virus can establish either a persistent or a latent infection and is latent in B lymphocytes (Turner and Studdert 1970; Blakeslee et al., 1975; Gleeson and Studdert, 1977; Drummer et al., 1995). EHV-2 have been isolated from peripheral mononuclear cells and mammary gland macrophages and organs as kidney, bone marrow and salivary glands (Dutta and Campbell, 1978; Dutta and Myrup, 1983; Gleeson and Coggins, 1985 Kono and Kobayashi, 1964; Harden et al., 1974) and is secreted from the nose, nasopharynx and vagina.

So far very little is known about EHV-5 infections, but the virus has been connected to upper respiratory disease (Agius et al 1992).

Genome

The entire genome of EHV-2 has been sequenced and comprises about 184 kbp (Telford et al. 1995). The genome is composed of a unique sequence (Us) flanked by terminal direct repeat structures, TRs (Fig 1b.). The 79 ORFs identified are predicted to encode 77 proteins. Amino acid sequence comparisons confirmed that EHV-2 is a gammaherpesvirus whose genome is colinear with that of herpesvirus saimiri (HSV, a gamma(2)herpesvirus) and EBV (a gamma(1)herpesvirus), with a closer relationship to the former. The EHV-2 genes are numbered in conformity with the system used for HSV genes (Albrecht et al., 1992). Only 42 kb of the EHV-5 genome has been sequenced and restriction enzyme analysis revealed that the genome is 179 kb long (Agius et al., 1992) and is composed of one unique sequence, Us (Fig 1c.). Restriction

endonuclease patterns of EHV-2 and EHV-5 are distinct from other equine herpesviruses and from each other (O'Callaghan et al., 1981; Studdert et al., 1981; Allen et al., 1983; Browning et al., 1988).

Proteins and immunity

EHV-2 has homologues of the glycoproteins gB, gH, gM, and gL found in other herpesviruses but appears to lack those of gC, gD, gE, gG, gI, gJ and gK (Browning and Agius 1996). Nine major capsid proteins have been identified (average molecular weights 148, 52, 49.5, 46, 43.5, 27, 20 and 18 kD), constituting 89% of total nucleocapsid protein in purified EHV-2 nucleocapsids using SDS-PAGE (Caughman et al., 1984). These appear similar in molecular weight and proportional composition to the major capsid proteins of other herpesviruses. Purified virions contain at least 37 proteins with molecular weights ranging from 14 kD to over 200 kD and out of which 7 are glycoproteins. Comparison of the virion proteins between EHV-2 and EHV-5 show differences, especially when comparing the profiles of the glycoproteins (Agius et al., 1994). EHV-2 have two major (87 and 72 kD) and four minor (126, 107, 52 and 31 kD) glycoproteins, while EHV-5 has one major (105 kD) and five minor (128, 84, 77, 73 and 39 kD) glycoproteins.

There is a high degree of serological cross reactivity between the two viruses (Agius et al., 1994). The glycoprotein B which appears to be the most immunodominant virus protein, is cross reactive between the viruses and have a size of approximately 64 kD in EHV-2 and 66 kD in EHV-5 (Agius et al., 1994). No antigenic relationship has been established between EHV-2 and any of the alphaherpesvirus, including the three equine representatives, by cross VN, CF, immuno fluorescence (IF) or agar-gel immunodiffusion (AGID) (Plummer and Waterson, 1963; Plummer, 1964; Karpas, 1966; Hsiung et al., 1969; Plummer et al., 1969a). Also, no antigenic relationship has been found between EHV-2 and the human cytomegalovirus (a betaherpesvirus) by CF, nor with the malignant catarrhal fever virus, (a gammaherpesvirus), by CF or IF. Although antigens common to all EHV-2 isolates can be detected by CF and indirect IF, considerable heterogeneity is apparent in VN tests (Kono and Kobayashi, 1964b; Erasmus, 1970; Turner et al., 1970; Plummer et al., 1973; Harden et al., 1974; Mumford and Thomson, 1978).

Diagnosis

Diagnosis of EHV-2 and EHV-5 infections can be made by virus isolation on equine foetal kidney cell cultures. Differentiation of the two equine gammaherpesviruses can be done by genomic restriction endonuclease pattern (Browning and Studdert 1987) or by PCR (Reubel et al. 1995). Tests in use for serological diagnoses are virus neutralisation test and different ELISAs which often suffers from poor specificity.

Immune protection

In the only partly successful attempt at prevention of disease associated with EHV-2 infection, Belák et al. (1980) were able to protect foals from clinical disease for 3 weeks with a single injection of hyperimmune serum, while conventional formalin killed vaccine failed to evoke detectable antibodies and protection (S Belák, pers. communication).

Aims of the study

1.
To study the role of Equine herpesvirus type 2 (EHV-2) as a predisposing factor for bacterial infections caused by *Rhodococcus equi* by using specific EHV-2 immunostimulating complexes (ISCOMs) for immunoprophylaxis to prevent the disease complex.

2.
To study the prevalence of Equine herpesvirus type 1 and Equine herpesvirus type 4 in Sweden by the use of recombinant proteins homologous to type specific regions of glycoprotein G of EHV-1 and EHV-4 in an indirect ELISA.

3.
I -To detect infections of Equine herpesvirus type 2 in Swedish horses by the use of a type-specific monoclonal antibody in a blocking ELISA.

II -To differentiate between Equine herpesvirus type 2 and 5 by using a PCR system.

4.
To investigate the prevalence of Equine herpesvirus type 2 and Equine herpesvirus type 5 DNA in horses in Sweden and Hungary by the use of PCR.

Comments on methods

An indirect ELISA has been developed to differentiate between EHV-1 and -4 (Crabb et al. 1995). This ELISA was used to study the prevalence of EHV-1 and EHV-4 in Sweden utilising EHV-1 and EHV-4 glycoprotein G gene products ligated in a pGEX-3X vector expressed in *E.coli* as GST fusion proteins. The expressed proteins comprise corresponding regions of the gG molecules that are highly divergent and encompass strongly antigenic, typespecific epitopes. Serum samples are added to wells in microtitre strips coated separately with EHV-1 or EHV-4 antigens complemented with a well coated with control antigen i.e. GST alone. The detection system used was a peroxidase anti-horse conjugate. Before interpretation of results, OD values in the wells coated with EHV-1 or EHV-4 antigen are corrected by subtracting the OD value of the corresponding well containing the control antigen.

A blocking ELISA (bELISA) was developed by the author to monitor infections of EHV-2 in Sweden detecting antibodies to EHV-2. It is based on an EHV-2 specific monoclonal antibody that posses neutralising activity. Gradient purified EHV-2 virus was coated on the solid phase. Serum samples were added and allowed to bind to the antigen before adding the monoclonal antibody. Detection of the bound mAb was done by addition of peroxidase conjugated anti-mouse antibodies. The degree of positivity of the samples was measured in percent inhibition (PI), i.e. by the ability of antibodies in the sera to block out the binding of the mAb to the antigen in the well according to the formula; PI = neg control OD - sample OD/ neg control OD x 100. Establishment of a cut-off PI was done on sera from foals tested over a period of 6 months where all of them went through a EHV-2 infection. All sera with a PI over 30 were considered positive.

The EHV-2 virus neutralisation (VN) test detects neutralising antibodies to EHV-2. Two-fold dilutions of sera were incubated with EHV-2 virus and, thereafter, inoculated on cell cultures grown in 96-well plates. The VN test was read when complete cytopathic effect (CPE) was seen in control wells. The VN test was used "as gold standard" to compare results obtained by the EHV-2 b ELISA.

A polymerase chain reaction (PCR) was used to study the prevalence of EHV-2 and EHV-5 infections of horses in Sweden and Hungary. PCR is a technique which amplifies selected DNA sequences. It encompass repetitive cycles, each involving three steps, denaturation, annealing and extension. At the first step the DNA is denatured by heat, resulting in single-stranded molecules. In

the annealing step two oligonucleotide primers specifically hybridise to the opposite strands (templates) flanking the target region of the DNA to be amplified. The last step is the extension, in which the annealed primers are extended by a DNA polymerase. The newly synthesised strands serve as templates in the next cycle. This cycle was in this study repeated 35 times, resulting in an exponential accumulation of the target sequences. Thereafter the target sequences are being visualised with electrophoresis on agarose gel.

Primers for the EHV-2 and EHV-5 PCR were designed to amplify type-specific parts of glycoprotein H (gH). These primers were selected from the nucleotide sequence of gH in order to be type-specific and easy to use in a routine diagnostic laboratory.

The ISCOM technology is a delivery system for antigens which was used to present EHV-2 envelope proteins. This construct was used as an experimental vaccine against EHV-2. Envelope proteins were prepared by solubilisation of EHV-2 particles with the detergent 1-O-n- octylglycopuranoside (OG) being a non-ionic mild detergent with minimal risk to denaturing the conformational functional epitopes e.g. important for virus neutralisation (Merza et. al. 1988). Detergent treated material was mixed with Quil A and allowed to interact by the gradual removal of the detergent with dialysis. Unincorporated material in the iscom preparation was separated from iscom particles by ultracentrifugation through a 10% sucrose layer. Thereafter, the iscoms were filtered through 0.45μ filter and dispensed into sterile glass bottles. The ISCOM preparation was used to immunise foals in order to study its protective effect against natural EHV-2/*R.equi* infection.

Results and discussion

Protection against *Rhodococcus equi* infection by vaccination with an EHV-2 iscom vaccine (paper I)

EHV-2 has been associated with a number of clinical signs including keratoconjunctivitis and respiratory disease in foals. Perhaps the most striking evidence linking EHV-2 to respiratory disease was provided in large studs in Hungary which indicated the capacity of EHV-2 to initiate a two-phase respiratory disease in young foals. The first (viral phase) was characterised by occasional appearance of nasal discharge and slight increase in body temperature. Subsequently, the foals apparently recovered but two to three weeks later they developed a serious respiratory disease, characterised by pneumonia, high fever, pulmonary abscesses, and death. The second phase was caused by bacterial complications, above all invasion of the respiratory tract with *Rhodococcus equi (R.equi)*. (Pálfi et al., 1978). The role of EHV-2 as a predisposing factor for the invasion of the lungs by *R.equi* was first indicated by the protective effect of EHV-2 hyperimmune serum and/or gammaglobulin (Belák et al., 1980). The respiratory disease syndrome has also been observed in Swedish studs. EHV-2 has been isolated during the early phase of the disease and *R. equi* was cultivated from serious cases of pneumonia during the second phase (Nordengrahn and Merza, unpublished data). These observations provide further evidence that the "trigger mechanism" of EHV-2 has to be considered in the two-phase respiratory disease complex of young foals. There are no earlier reports on immunoprofylaxis that protect against the *R.equi* infection apart from the passive immunisation by Belák et al, 1980. The first experiments, using inactivated EHV-2 vaccines failed to protect foals (Belák, pers. communication).

A vaccination experiment was designed with the dual purpose to both demonstrate the predisposing role of the virus and to have a vaccine that protects against disease after an initiative infection with EHV-2 and a subsequent infection with *R. equi.* The iscom technology was chosen to prepare experimental vaccine containing envelope proteins of EHV-2 to immunise foals at a farm with a history of consistent annual occurrence of EHV-2 and *R.equi* infections. Five foals were immunised once at an age of two weeks (group I) , 19 foals were immunised twice, first at two weeks of age and boostered two weeks later (group II) and 5 foals were used as non-immunised controls (group III). Out of the 5 foals immunised once, one foal had severe respiratory signs and fever at around two months of age. Two foals had slight respiratory signs for 6 days and the other two foals remained clinically healthy. 18 out of the 19 foals immunised twice remained clinically healthy throughout the entire observation period. The remaining foal had a fever for about a week, exhibited slight respiratory signs and then recovered. Four of the five non-immunised foals had severe respiratory disease and fever and three of them died. The remaining foal was symptomless during the observation period. EHV-2 was isolated from the nasal specimens

during the first days of the respiratory disease of the affected foals. *R. equi* was detected in the nasal swabs collected during the second phase of the respiratory disease and also by cultivation of samples from the lungs of each animal succumbed to the disease.

The results of the present study supports the hypothesis that infection with EHV-2 trigger secondary bacterial e.g. *R. equi* infection of the respiratory tract e.g. by affecting the mucosal epithelial or by infecting lymphocytes resulting in immunosuppression.

The results demonstrate that high VN antibodies are induced and overcome levels of maternally derived antibodies. It is likely that virus neutralisation is of protective value, probably in concert with cell mediated immunity. It is well established that iscoms induce Th1 type of immune response and CTL (for reference see Morein et al. 1999) being likely components in protective immunity against herpesviruses. However, the cell-mediated immunity in young foals has to be further studied. In a work by Johansson et al (1999) it is shown that iscoms induce cell mediated immunity in new-born mice immunised with iscoms prepared with envelope proteins from Sendai virus.

In conclusion, the results of the present study indicate that the entire two-phase respiratory disease complex, including *R.equi* pneumonia, was prevented by the two dose regimen of the EHV-2 iscom vaccine and that EHV-2 is a predisposing factor for a respiratory disease complex in foals .

Investigation of the prevalence of EHV-1 and EHV-4 in Sweden by the use of a differential ELISA (paper II)

EHV-4 and EHV-1 are the major cause of respiratory disease in young horses and abortion in pregnant mares, respectively. Both viruses have an ubiquitous distribution and are of considerable economic importance to the equine industry. Until recently there was no discriminative tests between the two herpesviruses which also made it difficult to distinguish differences in their pathogenicity. Convalescent sera from horses contain EHV-4/EHV-1 cross-reactive antibody which makes a routine specific serodiagnosis difficult. Recently a type-specific test was developed (Crabb et al. 1995) that allows the clear distinction between EHV-4 and EHV-1 as well as dually-infected horses. This ELISA was used to study the prevalence and incidence of EHV-1/EHV-4 infections in 334 Standardbred Trotters originating from 4 different breeding studs/racing stables in Sweden. The horses (foals, yearlings and 2 year or older horses) were sampled every second month for one year. For epidemiological comparison 207 horses from Iceland, which represent a closed population, and 191 horses from Sudan were also included in the study. The later two groups of horses were sampled once.

Prevalence and incidence of EHV-4: Most of the 34 foals showed levels of maternally-derived antibodies at the first sampling (4 months of age). At the next sampling, 2 months later only 13 foals showed a weak antibody reaction. At the third sampling, 2 months later, all foals had seroconverted indicating that the foals had gone through an active infection with EHV-4. All the yearlings , except one, were positive to EHV-4 throughout the sampling period. Of the older horses all except 2 (which seroconverted after 4 months) were positive throughout the sampling period indicating a prevalence of almost 100%. The prevalence of EHV-4 infection was 99.5% in the Icelandic horses and 95% in the Sudanese horses.

Prevalence and incidence of EHV-1: Only 6 of the 43 foals investigated sero-converted to EHV-1 throughout the study period. These foals were infected during the winter. Only 2 of the 5 foals in one of the stables had maternal antibodies which declined and at the age of 6 months they scored negative. None of the foals in the 3 other stables had maternal antibodies. The yearlings showed an increasing incidence of infection in the autumn after being at pasture. Older horses in two of the stables showed a gradually increasing incidence of EHV-1. The prevalence of EHV-1 infection was 8% in the Icelandic horses and 23% in the Sudanese horses.

This study confirmed the observation from previous investigations that the epidemiology of EHV-4 and EHV-1 infections differs. The high prevalence of antibodies to EHV-4 suggests that all horses included in this study were regularly boostered via reactivation of and /or reinfection with EHV-4. Vaccinations with combined EHV-1/EHV-4 vaccines are a common feature in many countries. It should be questioned whether immunisation with EHV-4 containing vaccines will lower the incidence of EHV-4 infection. It seems quite difficult to implement an efficient vaccination schedule to protect foals and young horses against infection as all foals were infected almost immediately with EHV-4 when maternal immunity had waned.

EHV-1 showed a much lower prevalence than that of EHV-4, not only in the Swedish studs and racing stables, but also in the samples of horses from Iceland and Sudan. However, seasonal differences in the prevalence of EHV-1 within the studs/stables (9% to 56%) were seen. A potential area for the use of this test would be measuring the efficacy of EHV-1 vaccination, but that requires studies to correlate levels of ELISA EHV-1 antibody versus immunity to abortion/infection. The low prevalence in foals, initially in yearlings as well as older horses and in closed populations as the Icelandic one, opens for the possibility of eradication of EHV-1 infection from countries like Iceland and from regions or single studs by aid of ELISA monitoring and segregation of seropositive horses. However, the movement of racing horses within and between countries all over the world is an obstacle to overcome.

It is evident that the type-specific EHV-4/EHV-1 ELISA provides a valuable tool for discriminating between horses that are infected with either EHV-4 or EHV-1 or both of these viruses. It provides a clear picture of the epizootiological status of EHV-1 in the area and provides a base for the segregation of mares into EHV-1 positive and negative groups, a useful tool for the management of outbreaks of EHV-1 abortion. The possibility of using this test system to screen for the maternal antibodies will provide the information for vaccine strategies to achieve a good relevant immune response and avoid unnecessary vaccination of older horses.

Detection of EHV-2 infections by the use of a neutralising mAb (paper III)

As mentioned earlier (paper I) EHV-2 causes keratoconjunctivitis and mild respiratory symptoms in foals. It has also been suggested that EHV-2 acts as a predisposing factor for a secondary bacterial infections, above all the invasion of the respiratory tract with *R.equi*. In order to further investigate the pathogenesis of EHV-2 and to demonstrate newly acquired infections, a specific and sensitive test is in need. A blocking ELISA was developed using an EHV-2 specific neutralising monoclonal antibody. The capability of sera from horses to block this neutralising mAb gives a more profound information about the immune status when the act of blocking shows that the horse has antibody to the specific neutralising epitope important for protection. In contrast, an indirect ELISA detects antibodies against virtually all antigens and parts of antigens generated at an earlier EHV-2 infection with or without neutralising activity. Also the specificity of this mAb is of great importance to differentiate the EHV-2 infection from that of the antigenically related EHV-5.

Field sera from 197 horses aged one year or older originated from two stables, one with constant problems of EHV-2/*R.equi* and one with no such problems. These samples were collected once over a period of 4 years and tested in the blocking ELISA. For comparison with a "gold standard" 91 of the sera were also tested in VN test. From 4 stables 11 foals, age 2 to 4 months were sampled three times i.e. with an interval of 2 months over a period of 6 months. The results in the bELISA shows that by the age of 6 months all the foals had gone trough an active infection of EHV-2. PI values raised from 10-20 to over 30. The PI value of the 197 horses tested varied between 0 and 71. In stable 1 (having constant EHV-2/*R.equi* problems), 84% of the horses had a PI over 30 i.e. considered positive, while only 34% of the horses in stable 2 had a PI over 30.

The big difference in the number of seropositive horses between the two stables tested indicate that the bELISA will be reliable for testing newly acquired infections in contrast to indirect ELISA which showed similar frequence of seropositivity. The antibody response measured by the blocking ELISA does not seem to last in the horse for long periods (years). All foals had antibodies

measured by the bELISA at the age of six months indicating that foals in general get the infection in an early stage of their life. Levels of antibodies in older horses (one year of age or older) differed significantly indicating a recurrent infection every year. This observation was noticed by the use of the bELISA.

Detection and differentiation of EHV-2 and EHV-5 DNA (paper IV)

Equine herpesvirus type 2 and 5 (EHV-2, -5) have recently become members of the Gammaherpesvirinae subfamily (Telford et al., 1993). EHV-2 is widely spread in horse populations establishing persistent infections. The virus has been isolated from healthy as well as from horses with different clinical signs such as respiratory disease (Browning and Studdert. 1987b).

Studies on the genomic heterogeneity of EHV-2 by Browning and Studdert (1987a) led to the proposal that there exists a group of gammaherpesvirus which is distinct from, and yet related to EHV-2. This group of gammaherpesvirus has been designated as EHV-5. The four initial isolates were obtained from cultures of buffy coat and cells from nasal cavity of 3 Australian horses and 1 horse imported to Australia from the United Kingdom all suffering from upper respiratory tract disease. Recently EHV-5 has also been reported from Switzerland (Francini et al 1997) and Germany (Borchers et al 1999). To carry out comparative studies on two closely related viruses such as EHV-2 and EHV-5 the choice of method is important. By the use of immunofluorescence, in studies of EHV-2 and EHV-5 carried out in Germany as many as 93% of the horses tested had antibodies to both virus types, suggesting that this test did not discriminate between the two viruses due to a strong serological cross-reactivity.

To overcome such problems in our studies on the pathogenicity and the prevalence of EHV-2 and EHV-5 we used the Polymerase chain reaction technique (PCR). Primers were selected from the glycoprotein H (gH) gene because they were specific for EHV-2 respectively EHV-5. Whole blood were collected from 81 Swedish Standardbred trotters (age one year or older) and 33 foals aged five to eight months. The horses originated from two stables. In Stable 1 the horses had suffered from respiratory disease which had been connected to EHV-2 and *R.equi.* Horses in Stable 2 had not suffered from such problems. Whole blood and nasal specimen were also collected from 27 Arabian thoroughbred foals in Hungary. The foals were sampled at the age of 2, 4, 6, 8, 13, 18 and 23 weeks. DNA was prepared and EHV-2 PCR was run on all samples. EHV-5 PCR was performed on samples from all Hungarian foals and on samples from 40 horses (20 from each stable) and 10 of the foals from Sweden.

Detection of EHV-2 DNA in Swedish horses: 68% of the horses in stable 1 and 56% in stable 2 were positive in the EHV-2 PCR, i.e. the two stables showed

similar prevalence of EHV-2. However, differences were seen between samples collected during the summer compared to the winter, i.e. 81 respectively 53% in stable 1 and 80 respectively 53% in Stable 2. All 33 foals tested were positive in the EHV-2 PCR.

Detection of EHV-2 DNA in Hungarian horses: All 27 foals were positive in the EHV-2 PCR by the age of 8 weeks. EHV-2 DNA was also found in the nasal specimen in 25 of the 27 foals.

Detection of EHV-5 DNA in Swedish horses: EHV-5 DNA was not detected in any of the 40 horses or 10 foals tested.

Detection of EHV-5 in Hungarian horses: Four of the 27 foals were positive in the EHV-5 PCR being detected by the age of 13 to 23 weeks. EHV-5 DNA was also detected in the nasal specimen in two of these four foals.

The prevalence of EHV-2 in the Swedish horses were high and all foals tested in Sweden and Hungary were positive in the PCR demonstrating infection at an early age of life. The prevalence in the two stables tested where almost the same, though the horses from stable 1 suffered from annual EHV-2 infections. The higher prevalence of EHV-2 positive horses during the summer than in winter may reflect that EHV-2 infections usually occurs in the spring-summer time and active virus with high copy number increase the possibility to detect DNA by PCR.

The difference in prevalence between EHV-2 and 5 indicate a difference in the epidemiological pattern between the two viruses, though, the low number of EHV-5 positive horses in Hungary and no positive case in Sweden complicate the epidemiological evaluation. It was seen that EHV-5 probably is much less spread than EHV-2 in both Sweden and Hungary but a larger number of samples need to be tested in order to make a complete study of the epidemiology in the two countries. The low number of EHV-5 positive cases could also be that the infection usually occurs in foals older than 6 months. The Hungarian foals were only sampled until the age of 6 months and the Swedish foals only once at the age of 5 to 8 months, on the other hand none of the 40 Swedish horses were positive. It could also be the case that the EHV-5 PCR is not sensitive enough detecting most cases of EHV-5. It is also not known if peripheral blood cells are the main reservoir of EHV-5.

Concluding remarks

EHV-2 seem to be involved in a two-phase respiratory disease complex characterised by initially mild respiratory signs, recovery and secondly a severe respiratory diseases including *R.equi* pneumonia. This disease complex was prevented by a two dose regimen of the EHV-2 iscom vaccine. The possibility to prevent the disease by a specific immune prophylaxis is a proof for the hypothesis that EHV-2 acts a predisposing factor for the *R.equi* invasion of the respiratory tract and the subsequent disease development.

The type-specific enzyme-linked immunosorbent assay (ELISA) based on *Esherichia coli*-expressed glycoprotein G (gG) fusion proteins that differentiate between EHV-1 and EHV-4 was successfully used to distinguish between antibodies to the two viruses. The epidemiological studies show a high prevalence of EHV-4 of almost 100% and lower frequency of EHV-1 with a range of 9-56% percent in the studied Swedish equine population.

An EHV-2 specific neutralising monoclonal antibody was successfully used in a blocking ELISA to detect recently acquired infections of EHV-2. The results of the bELISA were in agreement with the history of the tested horses i.e. a much higher prevalence of positive horses from a stable with a history of constant EHV-2/*R.equi* problems than in horses from stable with no problem.

The PCR technique is an excellent tool to distinguish between two very closely related viruses as EHV-2 and EHV-5. In this study it was used as a research tool to determine persistence and prevalence of these two virus types in horses from Sweden and Hungary. It was also successfully used to determine at what age the foal get infected which is valuable information to establish vaccination strategy.

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