Interaction of equine herpesvirus 1 with equine peripheral blood mononuclear cells

Karen van der Meulen

Proefschrift voorgedragen tot het behalen van de graad van
Doctor in de Diergeneeskundige Wetenschappen,
2003

Promotoren: Prof. Dr. H. Nauwynck, Prof. Dr. M. Pensaert

Laboratorium voor Virologie
Vakgroep Virologie, Parasitologie en Immunologie
Faculteit Diergeneeskunde, Universiteit Gent

ISBN 90-5864-031-0

Omslag: tekening van een paardenhoofd door Randy Follis

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADCML	antibody-dependent complement-mediated lysis
BoHV-1	bovine herpesvirus 1
CF	complement-fixing
ConA	concanavalin A
CTL	cytotoxic T-lymphocytes
DNA	deoxyribonucleic acid
d pi	days post inoculation
EBV	Epstein-Barr virus
EBNA	Epstein-Barr nuclear antigen
EDTA	ethylenediamine tetra-acetic acid
EEL	equine embryonic lung
EHV-1	equine herpesvirus 1
EMA	ethidium monoazide bromide
ER	endoplasmatic reticulum
Fab	Fragment antigen binding
Fc	Fragment crystalizable
FITC	fluoresceine isothyocyanate
g or gp	glycoprotein
HCMV	human cytomegalovirus
h pi	hours post inoculation
HSV	herpes simplex virus
Ig	immunoglobulin
IONO	ionomycin
IPMA	immunoperoxidase monolayer assay
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK	natural killer
PBMC	peripheral blood mononuclear cells
PDB	phorbol dibutyrate

PHA	phytohaemagglutinin
SuHV-1	suid herpesvirus 1
PWM	pokeweed mitogen
RK13	rabbit kidney 13
SN	seroneutralization
TCID	tissue culture infectious dose
VN	virus-neutralizing
VZV	varicella-zoster virus

1. REVIEW OF THE LITERATURE

1.1 EQUINE HERPESVIRUS 1 (EHV-1): GENERAL CHARACTERISTICS

Equine herpesvirus 1 (EHV-1) is a major pathogen of horses, responsible for respiratory disorders, abortion, neonatal foal death and, less frequently, nervous system disorders. EHV-1 infection is one of the most important causes of infectious abortion and, therefore, brings about serious economic losses to the horse industry worldwide. The first chapter of this review will describe the characteristics of EHV-1. In the following chapters, the interaction of EHV-1 with its host cell as well as the interaction of the virus with the naive and the immune horse will be discussed. An important feature in the pathogenesis of an EHV-1 infection is the ability of the virus to be present inside peripheral blood mononuclear cells (PBMC) and to spread to internal organs without being recognized by the host's immune system. The last chapter will review several strategies that are exploited by herpesviruses to avoid elimination of infected PBMC by the immune system.

1.1.1 History of EHV-1

In 1932, a filterable virus that caused abortion in mares was isolated by Dimock and Edwards (Dimock and Edwards, 1933). The virus was first designated equine abortion virus or equine rhinopneumonitis virus and, later, it was called "equine herpesvirus 1" or "EHV-1". Until 1981, 2 subtypes of this virus were described. "EHV-1 subtype 1" was mainly associated with abortion and nervous system disorders and "EHV-1 subtype 2" was mainly responsible for respiratory disorders. Restriction endonuclease and nucleotide sequence analysis, however, indicated that "EHV-1 subtype 1" and "EHV-1 subtype 2" were 2 distinct viruses (Sabine *et al.*, 1981; Studdert *et al.*, 1981; Turtinen *et al.*, 1981) and it was proposed to designate them EHV-1 and EHV-4, respectively (Studdert *et al.*, 1981). In 1988, this nomenclature was adopted by the Herpesvirus Study Group of the International Committee on Taxonomy of Viruses.

1.1.2 Classification

EHV-1 is a member of the family *Herpesviridae*. *Herpesviridae* are widely disseminated in nature and infections are common in humans and most animal species. All members of the family *Herpesviridae* share four biological properties (Roizman, 1991). Firstly, they contain a large deoxyribonucleic acid (DNA) genome that encodes a variety of enzymes involved in nucleic acid metabolism and DNA synthesis. Secondly, transcription of the herpesviral genome proceeds in a co-ordinately regulated cascade manner. Thirdly, formation of the capsid occurs in the nucleus. Fourthly, all known herpesviruses are able to establish a latent infection in their natural host.

Other biological characteristics such as host cell range, replication cycle, ability to destroy cells and site of latency vary greatly among the different members of the *Herpesviridae*. Based on these biological characteristics, the *Herpesviridae* have been divided in three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Table 1). More recently, the subfamilies have been subdivided into genera based on DNA sequence homology, similarities in genome sequence arrangement and relatedness of major viral proteins (Roizmann *et al.*, 1992) (Table 1).

Table 1. Characteristics of the subfamilies of *Herpesviridae*

Characteristics	Herpesvirus subfamily		
	Alphaherpesvirinae	*Betaherpesvirinae*	*Gammaherpesvirinae*
Host cell range	wide	restricted	restricted
Replication cycle	short	long	long
Effect on host cell	lysis	cytomegaly	transformation, destruction
Site of latency	primarily but not exclusively in sensory ganglia	secretory glands, lymphoreticular cells, kidneys	lymphoid tissues
Genera	*Simplexvirus* *Varicellovirus* *Mardivirus* *Iltovirus*	*Cytomegalovirus* *Muromegalovirus* *Roseolovirus*	*Lymphocryptovirus* *Rhadinovirus*

EHV-1 is classified into the genus *Varicellovirus* within the subfamily of *Alphaherpesvirinae*. This classification is shared with bovine herpesvirus 1 (BoHV-1), suid herpesvirus 1 (SuHV-1) and varicella-zoster virus (VZV) (McGeoch and Cook, 1994).

Beside EHV-1, other equine herpesviruses are known to infect the horse as well. EHV-3, which causes equine coital exanthema, and EHV-4, which causes rhinopneumonitis, are members of the *Alphaherpesvirinae*. EHV-2 and EHV-5 are members of the *Gammaherpesvirinae*. Their role as pathogens for equines is still unclear. EHV-6, EHV-7 and EHV-8 infect donkeys and have, therefore, been redesignated as asinine herpesvirus 1, 2 and 3, respectively (Browning *et al.*, 1988). Their pathogenic role is comparable to EHV-3, EHV-2 and EHV-1, respectively. More recently, a new equine herpesvirus was isolated, EHV-9 (Fukushi *et al.*, 1997; Yanai *et al.*, 1998). EHV-9 seems to display only mild pathogenicity in horses (Taniguchi *et al.*, 2000).

1.1.3 Virus structure and genome organization

The EHV-1 virion has the typical architecture of a herpesvirus. The different components of the virion are schematically depicted in Figure 1.

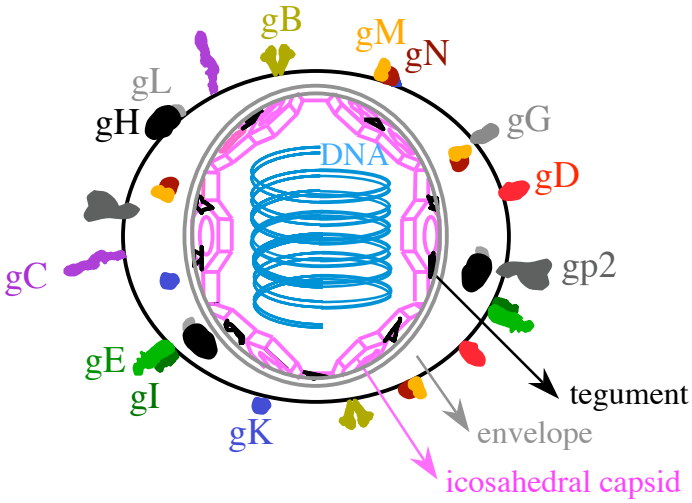

Figure 1. Schematic drawing of an EHV-1 virion (adapted from Favoreel, 1999)

The innermost core of an EHV-1 virion contains a linear double-stranded DNA genome. The core is enclosed in an icosahedral capsid of approximately 100 nm in diameter composed of 162 capsomeres (12 pentameric and 150 hexameric). Each capsomere has a cylindric shape with a channel running through the long axis. Core and capsid form the nucleocapsid, which is surrounded by a layer of electron-dense material known as the tegument. The tegument is enclosed by a lipoprotein envelope derived from the host cell, in which different viral glycoproteins are embedded. Up till now, 12 different envelope glycoproteins have been described for EHV-1: glycoprotein (g) B, gC, gD, gE, gG, gH, gI, gK, gL, gM, gN and gp2. Since the envelope glycoproteins play a major role during the EHV-1 replication cycle, they will be discussed more thoroughly in Chapter 1.2.2.

The genome of EHV-1 is organized as a linear double-stranded DNA molecule with a base composition of 56 to 57 % G + C (Darlington and Rendall, 1963; Soehner *et al.*, 1965; Telford *et al.*, 1992). The DNA molecule is approximately 150 kilobase pairs in size and can be divided into 2 covalently linked components, L and S (Henry *et al.*, 1981; Whalley *et al.*, 1981; Ruyechan *et al.*, 1982; Telford *et al.*, 1992) (Figure 2).

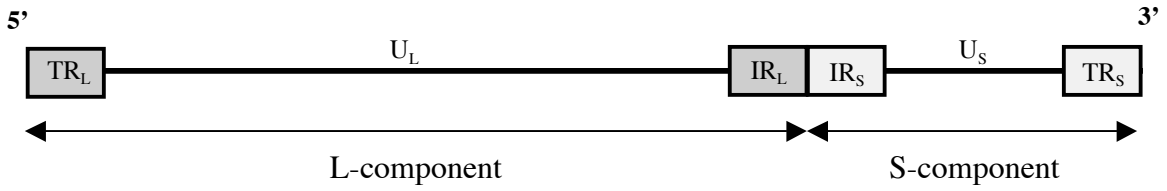

Figure 2. Genomic organization of EHV-1

Abbreviations: TR, terminal repeat; IR, internal repeat; U, unique

The S or short component consists of a unique (U_S) sequence which is flanked by an internal repeat (IR_S) and a terminal repeat (TR_S) sequence. The L or long region comprises a unique (U_L) sequence again flanked by an inverted repeat (IR_L) and a terminal repeat (TR_L) sequence (Chowdhury *et al.*, 1990; Yalamanchili and O'Callaghan, 1990). The complete genome sequence of EHV-1 has been elucidated in 1992 by Telford *et al.* (1992). It contains 76 genes, all likely to encode proteins involved in the viral replication cycle.

1.2 REPLICATION OF EHV-1 IN THE CELL

1.2.1 EHV-1 replication cycle

The replication cycle of EHV-1 is divided into several steps, including entry of the virus into the host cell, replication of the virus inside the host cell and release of the virus from the host cell. A schematic representation of the various steps of the EHV-1 replication cycle is shown in Figure 3.

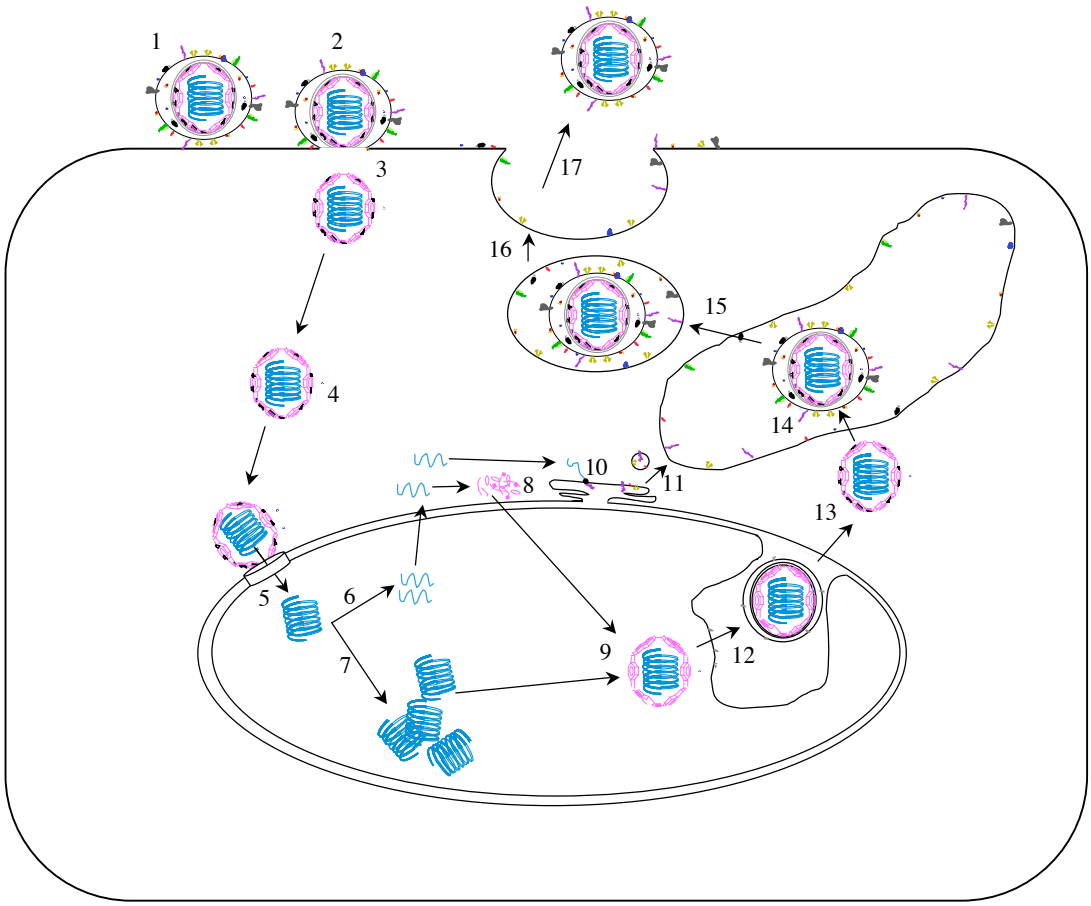

Figure 3. The EHV-1 replication cycle (adapted from Favoreel, 1999)

The first step in the replication cycle of EHV-1 is the attachment of free virions to the cell surface (1). After fusion of the viral envelope with the plasma membrane (2), the nucleocaspid is released into the cytoplasm (3) and transported into the nucleus (4-5). DNA transcription (6) and replication (7) occur and RNA molecules are transported to the cytoplasm and translated to proteins (8, 10). Capsid proteins are redirected into the nucleus where encapsidation occurs (9). Viral envelope proteins are co-translationally transported into the endoplasmatic reticulum (ER) (10) and subsequently incorporated into various cellular membranes, while passing through the secretory pathway (11, 15-16). The nucleocapsid leaves the nucleus via budding

through the inner leaflet of the nuclear membrane (12) and subsequent fusion with the outer leaflet of the nuclear membrane (13). The nucleocapsid acquires its secondary envelope at the Golgi apparatus (14) and then leaves the cell via vesicle-mediated exocytosis (15-17).

The replication cycle of EHV-1, and *Alphaherpesvirinae* in general, starts with the attachment of free virions to the surface of a target cell. The first interaction between the virion and the cell occurs via heparan-sulfate containing glycosaminoglycans present on the cell surface and is relatively unstable (WuDunn and Spear, 1989; Mettenleiter *et al.*, 1990; Osterrieder, 1999). Subsequently, the interaction is converted to a stable binding (Karger and Mettenleiter, 1993). Once stable binding of the virion to the cell is achieved, the viral envelope and the plasma membrane will fuse and the nucleocapsid will be released into the cytoplasm, a process called penetration (Spear, 1993). After release in the cytoplasm, nucleocapsids are then transported to the nucleus. Here the DNA leaves the capsid and enters the nucleus via nuclear pores (Roizman, 1991).

The transcription of viral DNA takes place inside the nucleus. As for all herpesviruses, transcription of the EHV-1 genome is typically divided into distinct phases. First, the immediate-early gene, called IR1, is expressed (Gray *et al.*, 1987a, 1987b). IR1 is transcribed into a single mRNA (Harty *et al.*, 1989, 1991), but multiple immediate-early polypeptides are observed (Caughman *et al.*, 1985, 1988; Robertson *et al.*, 1988). The major immediate-early polypeptide (IE1) functions as a regulatory protein that activates expression of the next sets of genes, the early and late genes. Early genes are expressed before DNA replication and encode proteins required for DNA replication such as DNA polymerase and other enzymes such as thymidine kinase. The late genes are expressed after the onset of DNA replication and mostly encode viral structural components like capsid proteins and envelope glycoproteins.

Viral DNA replication, like transcription, takes place in the nucleus, resulting in multiple copies of the EHV-1 genome. These newly synthesized DNA copies have to assemble with the capsid proteins to form nucleocapsids. Upon translation in the cytoplasm, capsid proteins enter the nucleus, most likely via a nuclear targeting signal, after which capsid assembly and incorporation of viral DNA molecules occurs. Unlike capsid proteins, viral envelope proteins are co-translationally transported into the

endoplasmatic reticulum (ER) and are subsequently incorporated into various cellular membranes while passing through the secretory pathway.

Although still not unanimously accepted, there is increasing evidence for a two-step envelopment process during the maturation of *Alphaherpesvirus* virions, including EHV-1 (Granzow *et al.*, 2001; Mettenleiter, 2002). First, newly formed nucleocapsids bud through the inner leaflet of the nuclear membrane, thereby acquiring a primary envelope. For herpes simplex virus (HSV-1) (Roller *et al.*, 2000) and SuHV-1 (Klupp *et al.*, 2000) it has been demonstrated that the UL34 protein is necessary for primary envelopment. For EHV-1, it is not yet known which protein(s) is(are) involved. Primary envelopment is followed by fusion of the primary envelope with the outer nuclear membrane, resulting in the release of naked nucleocapsids into the cytoplasm. Inside the cytoplasm, final tegumentation of the nucleocapsids occurs. A secondary envelopment takes place at the *trans*-Golgi network. The nucleocapsids bud into lamellae and vesicles of the *trans*-Golgi network, which contain newly synthesized and fully glycosylated viral envelope proteins, and obtain their secondary envelope. The resulting complete virions then leave the cell via vesicle-mediated exocytosis.

1.2.2 Role of viral envelope glycoproteins

EHV-1 encodes 12 different envelope glycoproteins which are involved in virus attachment, penetration, egress and cell-to-cell spread. The function of each of the glycoproteins will be briefly reviewed.

Glycoprotein B is one of the so-called essential envelope glycoproteins, meaning that it is indispensable for virus replication. This glycoprotein is involved in the penetration of the virus into the host cell and the cell-associated spread of the virus from infected to uninfected neighbouring cells (Wellington *et al.*, 1996a; Neubauer *et al.*, 1997).

Glycoprotein D is another essential envelope glycoprotein of EHV-1. It converts the first unstable binding of the virion to the cell surface into a stable binding and is also involved in cell-to-cell spread of the virus (Wellington *et al.*, 1996b; Csellner *et al.*, 2000).

Glycoprotein C is a non-essential envelope glycoprotein. Thus, a mutant EHV-1 lacking gC is still able to replicate in the host cell. The penetration of the mutant virus is, however, delayed, most likely as a result of an impaired attachment, and the production of virus progeny is greatly reduced (Osterrieder, 1999). Extracellular virus titres are 5- to 10-fold lower when the mutant virus is grown in continuous cell lines and 48- to 210-fold lower when it is grown in primary equine cells. Osterrieder (1999) concluded that gC is involved in the first unstable interaction of the virion with the glycosaminoglycans on the surface of the target cell and in the process of egress.

The non-essential EHV-1 glycoproteins gE and gI (Flowers and O'Callaghan, 1992; Matsumura *et al.*, 1996, 1998) as well as gM (Osterrieder *et al.*, 1996) contribute to the cell-to-cell spread of the virus. Glycoprotein M is also involved in virus penetration (Osterrieder *et al.*, 1996). Processing of gM inside the infected cell depends on the expression of gN (Rudolph *et al.*, 2002).

Although expression of gG (Colle *et al.*, 1992), gH (Robertson *et al.*, 1991; Stokes *et al.*, 1996), gK (Zhao *et al.*, 1992) and gL (Stokes *et al.*, 1996) has been demonstrated in infected cells, their functions in the replication cycle of EHV-1 are still unclear.

All genes encoding the above-mentioned glycoproteins have a sequence homologue in other *Alphaherpesviruses*. In contrast, gp2 has only been described in the equine *Alphaherpesviruses* EHV-1 and EHV-4. The functional significance of gp2 is not yet fully known. Studies using a gp2-negative deletion mutant, demonstrated that gp2 is not essential for virus growth in cell culture (Sun *et al.*, 1994) and may be involved in entry and in egress (Sun *et al.*, 1996; Rudolph and Osterrieder, 2002).

The major functions of the EHV-1 envelope glycoproteins during the viral life cycle are summarized in Table 2.

Table 2. Functions of the envelope glycoproteins of EHV-1

Glycoprotein	(former name)	Essential	Function
gB	(gp14)	yes	penetration, cell-to-cell spread
gC	(gp13)	no	attachment, egress
gD	(gp17/18 or gp60)	yes	penetration, cell-to-cell spread
gE	(none)	no	cell-to-cell spread
gG	(none)	no	unclear
gH	(none)	most likely	unclear
gI	(none)	no	cell-to-cell spread
gK	(none)	most likely	unclear
gL	(none)	most likely	unclear
gM	(gp21/22a or gp45)	no	penetration, cell-to-cell spread
gN	(none)	no	processing of gM
gp2	gp300	no	unclear

1.2.3 Interaction of EHV-1 with the peripheral blood mononuclear cell (PBMC), the natural carrier cell of the virus

Despite the fact that PBMC form the natural carrier cells for EHV-1 in the blood and play a crucial role in the pathogenesis of an EHV-1 infection, studies on the interaction between EHV-1 and equine PBMC are limited and date from the early 1980's. The identity of EHV-1-infected PBMC *in vivo* was addressed by Scott *et al.* in 1983. They experimentally inoculated pony foals and collected PBMC as well as physically separated subpopulations of T-lymphocytes, B-lymphocytes and monocytes to detect and quantify the presence of virus within these cells. Infected PBMC were present between 2 and 14 days post inoculation, as determined by co-cultivation. The maximum number of infected cells was observed at 4 days post inoculation (43.2 infected cells per 2 x 10^6 PBMC). At all time points, T-lymphocytes were the predominant cell type that harboured virus. B-lymphocytes and monocytes also harboured virus, but to a much lesser extent. The maximum number of infected B-lymphocytes was 5-fold lower than that of infected T-lymphocytes and the maximum number of infected monocytes was 8.5-fold lower. Interestingly, when PBMC from infected ponies were co-cultivated in the presence of pokeweed mitogen (PWM), virus was detected in a 10-fold higher number of PBMC,

suggesting that mitogen in some way promotes viral replication in these cells (Scott *et al.*, 1983). Dutta and Myrup (1983) performed a similar study on PBMC isolated from EHV-1-inoculated ponies and extended it with *in vitro* infection of PBMC. Their results concerning *in vivo*-infected PBMC closely resembled those of Scott *et al.* (1983). *In vitro* infected, non-stimulated PBMC gave a higher number of plaques than mitogen-stimulated PBMC, in contrast to the results obtained *in vivo*. Until now, it has not been elucidated how mitogens affect the replication of EHV-1 in equine PBMC.

To our knowledge, no studies have been performed on the replication kinetics of EHV-1 in PBMC. In Chapter 3 of the present thesis, a closer look is taken at the replication kinetics of EHV-1 in freshly isolated as well as in mitogen-stimulated equine PBMC.

1.3 INTERACTION OF EHV-1 WITH THE HORSE

1.3.1 EHV-1 infection in the naive horse

EHV-1 causes a systemic infection in horses, which is characterized by mild respiratory disorders, abortion, neonatal foal death and, less frequently, nervous system disorders. A schematic drawing showing the events of an EHV-1 infection in the naive horse is presented in Figure 4.

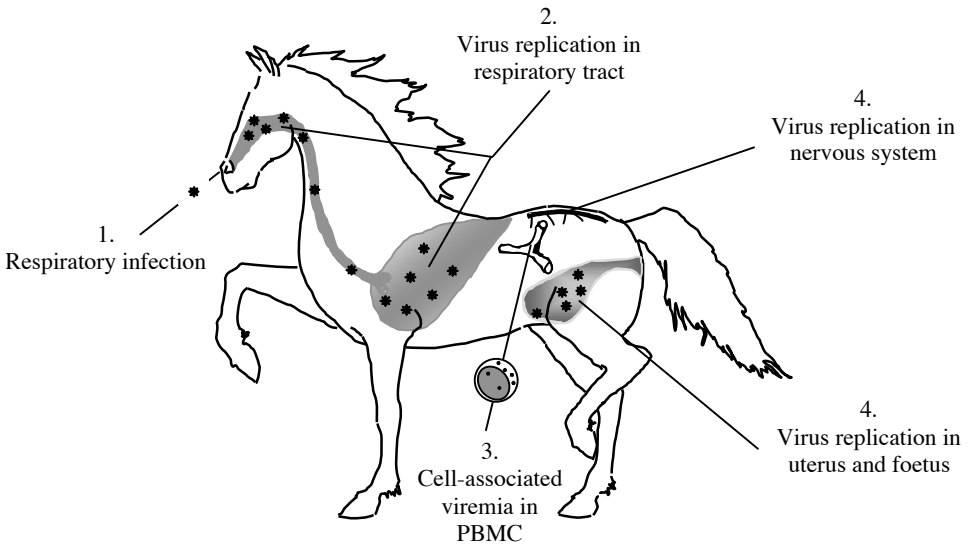

Figure 4. Pathogenesis of an EHV-1 infection in the naive horse

Respiratory infection

Infection with EHV-1 occurs via contact with infectious secretions or via inhalation of infectious aerosols. The primary site of replication is located in the epithelia of the upper respiratory tract, including turbinates, pharynx, soft palatinum and trachea (Kydd *et al.*, 1994a). Subsequently, the virus penetrates the epithelial barrier to replicate in deeper tissues of the respiratory tract and in the draining lymph nodes. From 12 hours post infection, replication of EHV-1 can be demonstrated in mononuclear cells of the retropharyngeal and submandibular lymph nodes (Patel *et al.*, 1982; Kydd *et al.*, 1994b) and from 1 day after infection, viral replication is observed in the cranial and caudal parts of the lungs (Kydd *et al.*, 1994a). Replication in the respiratory tract of immunologically naive horses can be extensive (Gibson *et al.*, 1992a) and is accompanied by vasculitis, thrombosis and development of herpetic lesions (Patel *et al.*, 1982; Kydd *et al.*, 1994a). Nasal shedding of virus is observed from 1 day up till 7 to 14 days after infection (Gibson *et al.*, 1992a).

EHV-1 respiratory disease in naive horses is characterized by mild, transient clinical signs, which are self-limiting within 9 days following infection (Gibson *et al.*, 1992a). Clinical signs include fever, anorexia, depression, swelling of the submandibular lymph nodes, ocular discharge and profuse serous nasal discharge, which may become mucopurulent. Virus replication in the lungs may cause bronchopneumonia. Secondary bacterial infections enhance the severity of the disease.

Viremia

Four to 6 days after respiratory infection, EHV-1-infected mononuclear cells enter the blood, resulting in a cell-associated viremia. Carried by infected PBMC, EHV-1 spreads throughout the body and, finally, reaches internal organs such as the uterus and the nervous system (Allen and Bryans, 1986). As mentioned in Chapter 1.2.3, T-lymphocytes are the predominant PBMC that harbour virus during viremia (Scott *et al.*, 1983). Viremia generally lasts until 9 to 14 days post infection (Thein and Brown, 1988; Gibson *et al.*, 1992a), although McCartan *et al.* (1995) reported viremia lasting for 27 days.

Abortion

When EHV-1-infected PBMC reach the pregnant uterus, this may result in abortion. A key factor in the pathogenesis of EHV-1-induced abortion is the infection of endothelial cells in the endometrium, resulting in vascular damage and subsequent dissemination of the virus into the foetus (Jackson *et al.*, 1977; Edington *et al.*, 1991; Smith *et al.*, 1992, 1993; Smith and Borchers, 2001). Uterine endothelial cells become infected by transfer of EHV-1 from infected PBMC. *In vitro* studies performed by Smith *et al.* (2002), indicated that activation of adhesion molecules on endothelial cells and leukocytes may be a key step in this transfer of virus. This indication was strengthened by the finding that endothelial cells of the pregnant equine reproductive tract showed an activated expression of adhesion molecules (Smith *et al.*, 2001). Interestingly, viral replication and vascular damage are more extensive in the late pregnant uterus (Smith *et al.*, 1992, 1993) than in the early pregnant uterus (Smith *et al.*, 1996) and 95 % of EHV-1-induced abortions occur in the last 4 months of gestation (Doll, 1952; Doll and Bryans, 1963). The reasons for the different susceptibility of early *versus* late pregnant uteri are not well understood.

Different strains of EHV-1 display different levels of virulence in terms of their ability to induce abortion in pregnant mares. Mumford *et al.* (1994) demonstrated that experimental inoculation of mares in late gestation with EHV-1 strain Ab4/8 resulted in abortion in 3 out of 5 mares (60 %). In contrast, EHV-1 strain V592 induced abortion in 1 out of 10 infected mares only (10 %). Later studies showed that the level of virulence was associated with the degree of endotheliotropism of the respective EHV-1 strain (Smith *et al.*, 2000).

EHV-1-induced abortion typically occurs in the last trimester of pregnancy (7-11 months). Abortion may be preceded by mild respiratory disorders, but in most cases primary replication passes unnoticed. The incubation period varies from as less as 6 days (Gleeson and Coggins, 1980) to as much as 4 months (Mumford *et al.*, 1987). EHV-1-induced abortions mostly occur as single cases. Only occasionally, clusters of abortions or “abortion storms” are seen. Expulsion of the foetus usually occurs suddenly and mares that abort seldom show premonitory signs. Foetus and placenta are expelled in a fresh

state, except for the rare abortions that occur before 6 months of gestation. In those early abortions, foetuses show severe autolysis.

When a foetus becomes infected late in gestation, a living foal may be delivered. Such infected foals are usually weak, depressed and die within 24 hours post partum. Some foals appear normal at birth, but develop severe respiratory distress within 18 to 24 hours and succumb within 3 days.

Nervous system disorders

When EHV-1-infected PBMC reach the nervous system, neurological disorders may be induced. Initially, it was postulated that immune complexes played a key role in the pathogenesis of EHV-1-induced neurological disorders. This postulation was based on the observations that recovery of virus from the central nervous system frequently failed (Jackson *et al.*, 1977; Platt *et al.*, 1980) and that the occurrence of the neurological disorders coincided with high levels of circulating virus-specific antibodies (Edington *et al.*, 1986). However, Edington *et al.* (1986) provided clear evidence that EHV-1 itself can infect endothelial cells of the blood vessels of the nervous system and that this forms the initial step in the induction of vascular lesions. The vasculair lesions, mainly comprising of vasculitis and thrombosis, result in secondary hypoxic degeneration of adjacent neural tissue and, subsequently, in nervous system disorders. It is assumed that infection of endothelial cells in the nervous system occurs via transfer of virus from infected PBMC, as in the pregnant uterus. All parts of the central nervous system may be affected by EHV-1.

As observed for abortion, the different ability of EHV-1 strains to induce nervous system disorders (Mumford *et al.*, 1994) appears to be associated with their degree of endotheliotropism (Smith *et al.*, 2000). Whether EHV-1 isolates exist with a tropism for endothelial cells specific for either the pregnant uterus or the nervous system remains unclear.

The incubation period of EHV-1-induced neurological disorders is 6 to 8 days (Jackson and Kendrick, 1971; Jackson *et al.*, 1977). The disorders may be preceded by fever, general malaise and/or respiratory disorders 1 to 2 weeks earlier. Horses of all ages and breeds may be affected. Pregnancy or recent foaling appears to be predisposing

(Greenwood and Simson, 1980; McCartan *et al.*, 1995; Goehring and Sloet van Oldruitenborgh-Oosterbaan, 2001). EHV-1-induced neurological disorders may be seen in individual horses or in multiple horses during outbreaks. The onset of neurological disease is usually acute. Clinical signs vary in severity from mild hind limb incoordination to severe paralysis and quadriplegia. Additionally, distal limb oedema, head tilt, oedema of the testes, paralysis of the tail, penis prolaps as well as faecal and/or urinary incontinence may be observed. Horses that do not develop quadriplegia have a good chance of recovery, although this may take several weeks. Horses that have been unable to stand for 24 hours have a poor prognosis (van Maanen *et al.*, 2001).

Affected foals may develop gastro-intestinal disorders, uveitis, hypopyon as well as pneumonia and sudden death may occur (Patel *et al.*, 1982; McCartan *et al.*, 1995).

Latency

Similar to the other members of the family *Herpesviridae*, EHV-1 establishes a latent infection and persists in the host following a primary infection. During latency, the entire viral genome is present in infected cells, but only a limited part undergoes transcription, giving rise to latency-associated transcripts. No infectious virus is produced (Roizmann *et al.*, 1992). Latent EHV-1 has been detected in the trigeminal ganglion (Edington *et al.*, 1994; Slater *et al.*, 1994; Baxi *et al.*, 1995), in lymphoid tissue draining the respiratory tract (Welch *et al.*, 1992; Edington *et al.*, 1994; Slater *et al.*, 1994) and in peripheral blood leukocytes (Welch *et al.*, 1992; Chesters *et al.*, 1997; Smith *et al.*, 1998; Banbura *et al.*, 2000). EHV-1 latency in peripheral blood leukocytes was predominantly established in $CD5^+/CD8^+$ T-lymphocytes (Smith *et al.*, 1998).

A latent EHV-1 infection has been demonstrated in approximately 60 to 80 % of a randomly selected population of abatoir horses, indicating that it is a common feature in horses (Edington *et al.*, 1994; Smith *et al.*, 1998).

Immune response to primary EHV-1 infection

In general, a herpesvirus infection results in a local immune response at the primary site of replication as well as in a systemic immune response. Each comprises a humoral and a cell-mediated component. Breathnach *et al.* (2001) characterized the *local humoral*

immune response to primary EHV-1 infection. Six naive weanling foals were experimentally infected and EHV-1-specific antibody responses were measured in nasal wash samples using immunoglobulin (Ig) A, IgGa, IgGb, IgG(T) and IgM isotype-specific monoclonal antibodies. All these antibody isotypes were demonstrable in the nasal mucus following EHV-1 infection, with virus-specific IgA being the predominant antibody isotype detected throughout the sampling period. EHV-1-specific IgGa and IgGb were occasionally present in excess of IgA at 1 or 2 weeks post inoculation. These high levels of IgG in respiratory secretions were most likely caused by the exudation of serum antibody through the inflamed respiratory epithelium.

Up till now, the *local cell-mediated immune response* to primary EHV-1 infection has not been examined.

A *systemic humoral immune response* to primary EHV-1 infection is detectable as early as 5 or 6 days after inoculation. By then, IgM and IgG antibody titres start to increase (Stokes *et al*., 1991; Chong and Duffus, 1992). IgM antibodies rise to peak titres between day 9 and 18 and slowly decline afterwards. IgG antibody titres peak later (18-28 days) and remain high for at least several months (Stokes *et al*., 1991; Gibson *et al*., 1992b). When looking at the functionality of the evoked antibodies, it is shown that virus-neutralizing (VN) antibodies are first detectable between 10 and 21 days after primary infection of horses (Edington and Bridges, 1990; Stokes *et al*., 1991; Chong and Duffus, 1992; Gibson *et al*., 1992b; Tewari *et al*., 1993). They reach a maximum between 33 and 60 days and remain high for at least several months (Chong and Duffus, 1992; Gibson *et al*., 1992b; Tewari *et al*., 1993). Complement fixation (CF) antibodies start to rise between 7 and 15 days post inoculation (Edington and Bridges, 1990; Gibson *et al*., 1992b; Tewari *et al*., 1993), peak between 26 and 60 days post inoculation and decline thereafter (Gibson *et al*., 1992b; Tewari *et al*., 1993).

Studies on the *systemic cell-mediated immune response* to primary EHV-1 infection are rather restricted and only address the proliferative response of lymphocytes. This response has been examined by incubating lymphocytes from primary infected horses with EHV-1 antigens (virus-specific lymphocyte response) (Wilks and Coggins, 1976; Thomson and Mumford, 1977; Dutta *et al*., 1980; Hannant *et al*., 1991; Chong and Duffus, 1992) or with phytohemagglutinin (non-specific lymphocyte response) (Dutta *et*

al., 1980; Bumgardner *et al.*, 1982; Hannant *et al.*, 1991) and by subsequent determination of the uptake of ^{3}H-thymidine. The studies provided evidence that lymphocytes of primary infected horses display reactivity towards EHV-1 antigens. Most authors also reported an increase in the non-specific lymphocyte proliferation, indicative of a generalized activation of the lymphocyte responsiveness, except for Hannant *et al.* (1991), who described a suppression of the non-specific lymphocyte proliferation following EHV-1 infection. So far, no studies are available on the response of cytotoxic T-lymphocyte (CTL) precursors (memory CTL) or on the response of CTL effectors following a primary EHV-1 infection.

1.3.2 EHV-1 infection in the infection-immune horse

Horses may become re-infected as early as 2 months after primary infection (Gibson *et al.*, 1992a). Apparently, the protective potential of the local immunity resulting from an EHV-1 infection is of short duration. Virus replication in the respiratory tract of infection-immune horses is, however, restricted when compared to that in naive horses. Gibson *et al.* (1992a) found a 10- to 100-fold reduction in nasal virus titres upon re-infection of foals 2 months after primary infection. The duration of nasal viral shedding as well as the severity of respiratory disease are also reduced upon re-infection and depend on the time interval after which re-infection occurs. Re-infection within 103 days after primary infection resulted in nasal virus shedding for a maximum of 2 days and no clinical signs were apparent (Gibson *et al.*, 1992a; Slater *et al.*, 1993; Tewari *et al.*, 1993). When re-infection was performed at 5 (Edington *et al.*, 1990) or 6 (van der Meulen, unpublished results) months after primary infection, nasal viral shedding was also observed for a maximum of 2 days and horses showed fever. Other studies on the pathogenesis of an EHV-1 infection report nasal virus shedding lasting up to 5 - 9 days after inoculation of infection-immune horses and describe the occurrence of mild respiratory disease, comprising fever, anorexia and serous nasal discharge, between 1 to 5 days following inoculation (Gleeson and Coggins, 1980; Edington *et al.*, 1991; Kydd *et al.*, 1994a; Mumford *et al.*, 1994; Allen *et al.*, 1995). However, these studies lack information on the exact time point at which a prior EHV-1 infection had occurred.

An important feature in the pathogenesis of an EHV-1 infection in immune horses is that viremia may occur despite the presence of high levels of systemic EHV-1-specific antibodies (Doll and Bryans, 1963; Gleeson and Coggins, 1980; Mumford *et al.*, 1987) and EHV-1-specific CTL precursors (O'Neill *et al.*, 1999). Consequently, EHV-1 can spread to internal organs and cause abortion or nervous system disorders even though horses possess an active immunity. Re-infection with EHV-1 may result in viremia as early as 6 months after prior exposure to EHV-1 (van der Meulen, unpublished results). Infected PBMC may be present up to 9 - 13 days upon re-infection with EHV-1, which is almost similar as observed for naive horses (Gleeson and Coggins, 1980; Edington *et al.*, 1991; Kydd *et al.*, 1994a; Mumford *et al.*, 1994; van der Meulen, unpublished results). Up till now, it is unclear how EHV-1-infected PBMC are able to circumvent efficient elimination by the host's immune response. The studies presented in Chapter 4 of the present thesis will treat some aspects of the inefficient elimination of EHV-1-infected PBMC.

Nasal virus replication and viremia in infection-immune horses may also occur following reactivation of latent EHV-1 (Edington *et al.*, 1985; van Maanen *et al.*, 2000). Reactivation of latent virus may be induced by stress situations under field conditions (Burrows and Goodridge, 1984; van Maanen *et al.*, 2001) or by immunosuppression after treatment with corticosteroids (Edington *et al.*, 1985; Gibson *et al.*, 1992a; Slater *et al.*, 1994). Reactivation of EHV-1 with corticosteroids resulted in nasal virus shedding for a period of 7 to 10 days in 40 (Edington *et al.*, 1985) to 100 % (Gibson *et al.*, 1992a; Slater *et al.*, 1994) of the treated horses. Edington *et al.* (1985) and Gibson *et al.* (1992a) also detected viremia in 63 and 100 % of the animals, respectively. Viremia lasted for 1 to 4 days. Even though latent EHV-1 could be demonstrated in a large number of randomly selected horses (Edington *et al.*, 1994; Smith *et al.*, 1998), the incidence of reactivation of latent EHV-1 and the importance of reactivation in the induction of clinical disease is unclear.

Various studies addressed the immune response to EHV-1 in infection-immune horses. Breathnach (2001) examined the *local humoral immune response*. Nasal wash samples were collected at weekly intervals post inoculation and EHV-1-specific antibody responses were measured. As observed for naive foals, EHV-1 infection of infection-

immune horses mainly elicited IgA, which remained detectable till at least 26 weeks after inoculation. High levels of IgG in respiratory secretions of some horses, present in excess of IgA at 1 or 2 weeks post inoculation, were associated with the occurrence of respiratory disease. The authors postulated that these high levels of IgG resulted from an exudation of serum antibodies through the inflamed respiratory epithelium, as mentioned for naive weanling foals. Horses that remained clinically normal upon inoculation showed no excessive amounts of IgG in their respiratory secretions.

To study the *local cell-mediated immune response*, Breathnach (2001) collected nasal-associated lymphoid tissue and local draining lymph nodes at 1 week post inoculation and analyzed the response of CTL precursors, the response of CTL effectors and the EHV-1-specific lymphoproliferative response indicative for T-helper cell activity. CTL precursors and CTL effectors were elicited in all horses following EHV-1 inoculation, suggesting that prior EHV-1 infection had resulted in the establishment of virus-specific memory CTL, which were now rapidly recalled. Furthermore, a lymphoproliferative response was observed in the retropharyngeal lymph nodes. The duration of the local cell-mediated immune response and its role in the protection against challenge infection were not examined.

With regard to the *systemic humoral immune response* in infection-immune horses, re-exposure to EHV-1 induces an anamnestic antibody response for both CF and VN antibodies, which may be detected within 1 week post inoculation (Edington and Bridges, 1990; van der Meulen, personal observations).

With regard to the *systemic cell-mediated immune response* evoked upon re-infection of immune horses, studies by Charan *et al.* (1997) and Hannant *et al.* (1999) demonstrated a long-term suppression of the virus-specific as well as the non-specific lymphocyte proliferation response. Immunosuppression correlated with a rise in circulating activated transforming growth factor-β and could be reversed by an antiserum to transforming growth factor-β (Charan *et al.*, 1997). Alveolar macrophages, bronchial epithelium, lymphoid tissue and platelets were indicated as the potential sources of activated transforming growth factor-β during EHV-1 infection (Chesters *et al.*, 2000). Allen *et al.* (1995) studied the presence and activity of EHV-1-specific CTL precursors in PBMC from infection-immune horses. As mentioned for the local immune response, CTL

precursors or memory CTL have been established as a result of a prior exposure to EHV-1 and can rapidly differentiate and mature into CTL effectors which, in turn, will eliminate infected target cells. For their study, Allen *et al.* (1995) experimentally infected immune horses, collected their PBMC and restimulated the CTL precursors *in vitro* by culture with live, cell-free EHV-1. Subsequently, cytotoxic activity of the restimulated CTL precursors against EHV-1-infected target cells was assessed. EHV-1-specific CTL precursors were detected as early as 1 week post infection, reached maximum levels after 2 to 3 weeks and remained detectable for at least 1 year after infection. The cytotoxic activity was predominantly displayed by $CD8^+$ T-lymphocytes and was restricted by major histocompatibility complex (MHC) class I molecules. Siedek *et al.* (1999) modified the assay of Allen *et al.* and restimulated PBMC *in vitro* using EHV-1-infected dendritic cells instead of cell-free virus. In this manner, they showed that equine dendritic cells, after being exposed to infectious virus, were able to restimulate cytotoxic activity of EHV-1-specific CTL precursors, suggesting that they act as potent antigen-presenting cells *in vivo*. O'Neill *et al.* (1999) demonstrated that ponies with higher frequencies of MHC class I-restricted, EHV-1-specific CTL precursors were better protected against re-infection with EHV-1, as evidenced by reduced or absent clinical signs, virus shedding and viremia. Studies on the activity of EHV-1-specific CTL effectors in the blood of infection-immune horses following re-exposure to EHV-1 are very limited. Breathnach (2001) demonstrated the presence of circulating CTL effectors at 1 week after experimental inoculation. The CTL effector response was, however, not examined at any other time point post inoculation. Consequently, the course of the CTL effector response during an EHV-1 infection is still unknown.

1.3.3 EHV-1 infection in the vaccination-immune horse

The purpose of vaccination against EHV-1 is 2-fold. First, vaccination has to minimize virus replication in the respiratory tract upon subsequent exposure to virulent EHV-1. In this manner, nasal shedding of virus will be reduced and respiratory disorders may be prevented. Second, vaccination should prevent or at least minimize the occurrence of viremia upon challenge infection, in view of protecting pregnant mares

against subsequent abortion. The first purpose of vaccination seems to be fulfilled by the available vaccines, as demonstrated by reduced nasal virus titres and absence of respiratory disease upon challenge infection of vaccinated horses. In contrast, it seems difficult to develop a vaccine that guarantees a full protection against viremia and, consequently, against subsequent abortion upon challenge. In view of this, we will briefly review several vaccination/challenge studies and field studies performed with vaccines that have been developed for reducing the incidence of EHV-1-induced abortion.

Heldens *et al.* (2001) performed a vaccination/challenge study with Duvaxyn EHV$_{1,4}$, a whole virus inactivated and carbomer-adjuvanted vaccine containing EHV-1 and EHV-4. Infection-immune pregnant mares were vaccinated three times and challenged 4 weeks later. A non-vaccinated group was included as a control. All mares, irrespective of their vaccination status, became viremic upon challenge. Virus was isolated from PBMC up to 14 days after challenge in the vaccinated mares, despite the presence of virus-specific antibodies. In the non-vaccinated controls, infected PBMC were present up to 21 days post challenge. Interestingly, the incidence of EHV-1-induced abortion was clearly reduced in the vaccinates. Only 1 out of 5 vaccinated mares aborted, whereas all 6 mares aborted in the control group. This suggests that even though vaccination does not reduce the number of viremic mares, it may reduce the number of infected PBMC per mare and, subsequently, reduce the chance of their interaction with endothelial cells of the pregnant uterus. Additionally, Heldens *et al.* (2001) examined the occurrence of viremia in vaccinated and challenged naive foals. Only 3 out of 10 vaccinated foals became viremic upon challenge, compared to 4 out of 5 non-vaccinated control foals. This significant effect of Duvaxyn EHV$_{1,4}$ in a group of naive foals seems little surprising, knowing that the same vaccine was unable to reduce the number of viremic animals in a group of infection-immune mares.

Studies on the ability of Pneumabort-K, a whole virus inactivated and oil-adjuvanted vaccine containing EHV-1, to protect against viremia and abortion are not consistent. Upon introduction of vaccination in Kentucky in 1977, Bryans and Allen (1982) demonstrated a reduction in the incidence of EHV-1-induced abortion from 6.8/1000 in 1977 to 1.8/1000 in 1980. However, in an experimental vaccination/challenge study performed by Burrows *et al.* (1984), the incidence of EHV-1-induced abortion was

similar for vaccinated mares (41 %) and non-vaccinated control mares (33 %), as was the number of mares showing viremia (88 % and 77 %, respectively). Also the duration of viremia was unaffected by vaccination. Virus was isolated from PBMC as long as 8 to 14 days after challenge, both in the vaccinated and in the non-vaccinated mares. When yearlings and two-year-old ponies were vaccinated with Pneumabort K® and subsequently challenged, viremia was apparent in all animals upon challenge and lasted for 4 to 10 days. In an experimental vaccination/challenge experiment, performed by Bürki *et al*. (1990), 89% of the Pneumabort-K®-vaccinated animals became viremic upon challenge and 50 % of vaccinated pregnant mares aborted. No comparison was made with a non-vaccinated control group in this study.

Prevaccinol® is a live-attenuated Rac-H virus vaccine developed by attenuating the RacL11 strain of EHV-1 via serial passages on heterologous cells (Woyciechowska, 1960; Woyciechowska *et al*., 1980). The protective effects of Prevaccinol® against EHV-1-induced viremia and/or abortion have been tested in several field trials with variable outcome. Von Benten and Petzoldt (1977) performed a six-year survey in German thoroughbreds and reported that EHV-1 abortions occurred as frequently in vaccinated as in non-vaccinated mares. On the other hand, introduction of the vaccine on 6 farms in Poland resulted in a slight, but significant decrease in the number of foetal and neonatal foal losses from 11.8 % to 8.9 % (Frymus *et al*., 1986). In a field trial performed over a period of 6 years by Becker (1988), no cases of virus abortion were detected when mares were vaccinated according to the manufacturer's instructions. Bürki *et al*. (1990) carried out an experimental vaccination/challenge study with Prevaccinol®. Vaccination followed by subsequent challenge infection resulted in viremia in 8 out of 9 included horses. However, in 4 of the 8 viremic animals, infected PBMC were only detected at 4 days post challenge, suggesting that vaccination reduced the duration of viremia upon challenge. Two out of 4 vaccinated pregnant mares aborted upon challenge. It could not be concluded whether vaccination had reduced the abortion rate, since the study lacked a control group of non-vaccinated pregnant mares.

Apart from the above-mentioned, commercially available vaccines, several experimental vaccines have been designed for reducing the incidence of EHV-1-induced viremia and/or abortion in horses. Dolby *et al*. (1995) immunized 5 yearling ponies with

a vaccine containing the inactivated EHV-1 strain V592 in Freund's complete adjuvant, then revaccinated with inactivated V592 in Freund's incomplete adjuvant and, finally, challenged with virulent virus. Using this vaccination regime, cell-associated viremia was prevented in all 5 animals upon challenge.

Another inactivated experimental vaccine was described by Cook *et al.* (1990). This vaccine consisted of purified major EHV-1 envelope glycoproteins, instead of whole virus, and was presented using immune stimulating complexes or ISCOMs. Hannant *et al.* (1993) demonstrated that, although foals that were immunized with this vaccine all developed viremia upon challenge infection, the duration and amount of viremia were significantly reduced compared to non-vaccinated foals. On average, viremia lasted 11.5 days in the non-vaccinated foals and only 6 days in the vaccinated foals. The highest amount of virus was detected at 8 days post challenge in both groups, but the mean tissue culture infectious dose $_{50}$ ($TCID_{50}$) recovered from 1 ml whole blood was 20 times lower in the vaccinated animals than in the control animals.

Matsumura *et al.* (1996) examined the protective potential of an experimental vaccine containing the live-attenuated KyA strain of EHV-1. This attenuated strain was obtained by serial passages on heterologous cells (Randall and Lawson, 1962; Perdue *et al.*, 1974). Matsumura *et al.* (1996) immunized 6 weanling horses with attenuated KyA and challenged them 4 weeks later. All 6 horses developed viremia upon challenge, but the maximum duration of viremia was reduced when compared to challenge control horses. Infected PBMC were isolated for only 3 to 6 days from the blood samples of the vaccinated animals, while they were present for 16 to 19 days in the blood samples of the challenge control animals.

An experimental vaccine based on a thymidine kinase-negative deletion mutant of EHV-1 was described by Slater *et al.* (1993) and Tewari *et al.* (1993), whereas Matsumura *et al.* (1998) described an experimental vaccine containing an EHV-1 deletion mutant lacking the genes encoding gE and gI. It was found that immunization of foals with either the thymidine kinase-negative mutant (Slater *et al.*, 1993; Tewari *et al.*, 1993) or the gE^-/gI^- mutant (Matsumura *et al.*, 1998) did not prevent viremia upon subsequent challenge. The effect of vaccination on the amount of viremia was examined for the thymidine kinase-negative mutant only and it appeared that immunization with

this mutant induced a 10-fold reduction in the number of infected PBMC ($1/10^6$) when compared to non-immunized controls ($1/10^5$) (Slater *et al.*, 1993). The effect of vaccination on the duration of viremia was only addressed by Matsumura *et al.* (1998). They found that immunization of foals with the gE^-/gI^- mutant had very little effect in reducing the duration of viremia, with a mean duration of 8 to 10 days being observed in the non-vaccinated controls and of 7 to 8 days in the vaccinates.

Many other alternative approaches for potential vaccination against EHV-1-induced viremia and/or abortion have been explored, such as subunit vaccines comprising the envelope glycoproteins gB, gC, gD, gH and/or gL, deletion mutants lacking genes encoding gB, gC, gD, gM and/or gp2 and, finally, DNA vaccines based on the coding region for gB, gC or gD. Since none of these "alternative" vaccines have actually been tested for their ability to protect against EHV-1-induced viremia and abortion in horses, they will not be further discussed in the present thesis.

Taken all the data on vaccination together, it is clear that elimination of EHV-infected PBMC by the vaccination-induced immunity is inefficient, as demonstrated by the high incidence of viremia upon challenge infection. This is not unexpected knowing that even the immune response induced by natural infection does not efficiently eradicates EHV-1-infected PBMC, as previously discussed in Chapter 1.3.2.

In the next Chapter, we will take a closer look at the immune evasive strategies that are exploited by herpesvirus-infected PBMC in order to avoid their elimination by the different components of the host's immune system.

1.4 IMMUNE EVASION STRATEGIES OF HERPESVIRUS-INFECTED PBMC

When reviewing the literature on EHV-1 infection in horses, it attracts the attention that EHV-1-infected PBMC are able to circulate in the host despite the presence of an active immune response, either induced by natural infection (Chapter 1.3.2) or by vaccination (Chapter 1.3.3). Apparently, elimination of circulating EHV-1-infected

PBMC by the immune system is inefficient, allowing them to spread in the immune host and, consequently, to reach the internal organs.

In general, recognition and subsequent destruction of herpesvirus-infected cells involves three major immune effector mechanisms: the antibody-dependent lysis, the MHC class I-dependent, CTL-mediated lysis and the natural killer (NK) cell-mediated lysis. However, during evolution, herpesviruses have developed an impressive array of strategies to minimize recognition and/or destruction by these immune effector mechanisms. By doing so, they gain time to replicate in the host and spread to uninfected hosts, thereby securing their own survival. This chapter will focus on immune evasion strategies by which herpesvirus-infected PBMC can avoid elimination by the different components of the immune system. Even though many other viral immune evasion strategies may have been described, their relevance for herpesvirus-infected PBMC has not (yet) been addressed.

1.4.1 Antibody-dependent lysis and evasion strategies of herpesvirus-infected PBMC

Following infection of a cell with an enveloped virus, such as a herpesvirus, viral proteins are synthesized and, subsequently, incorporated into cellular membranes while being transported to the plasma membrane. When virus-specific antibodies bind to viral glycoproteins present on the plasma membrane, the infected cell becomes recognizable for the classical pathway of complement, for phagocytic cells and for NK cells, which are the three major arms of the antibody-dependent immunity. Destruction via the classical pathway of complement is then initiated by binding of complement component C1q to the Fragment crystalizable (Fc) domain of the virus-specific antibodies at the plasma membrane. This binding leads to the activation of the complement cascade, which finally results in the assembly of the Membrane Attack Complex and in subsequent lysis of the infected cell. Also NK cells and phagocytes bind to the Fc domain of the virus-specific antibodies at the plasma membrane. This binding is mediated via Fc receptors present on NK cells and phagocytes and results in lysis or phagocytosis of the infected cell, respectively. An overview of the antibody-dependent cell lysis is shown in Figure 5.

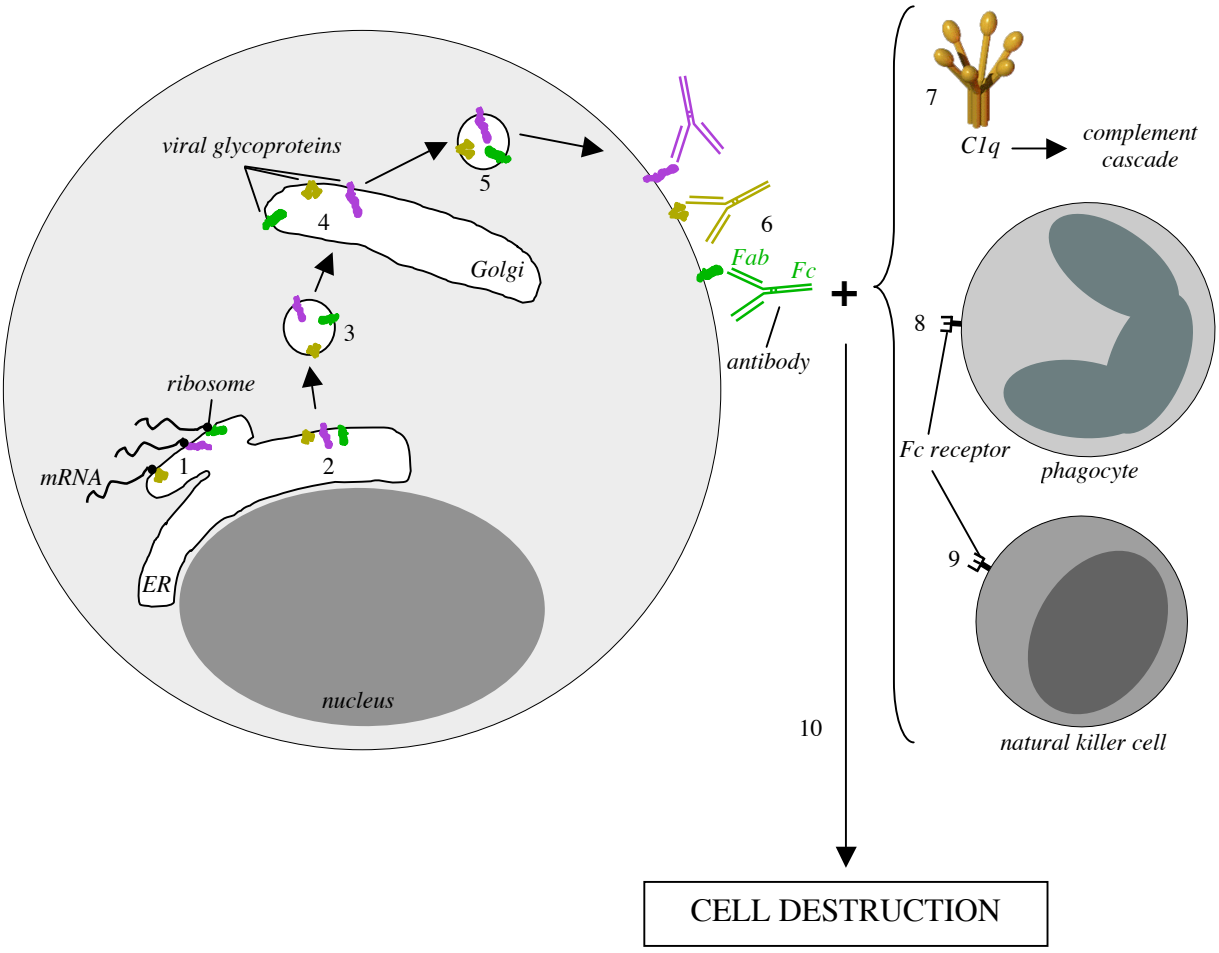

Figure 5. Mechanism of antibody-dependent cell lysis of a virus-infected cell

Viral proteins are co-translationally transported into the ER lumen (1) and incorporated into transport vesicles that bud off from the ER (2-3). Upon reaching the Golgi compartment, viral proteins undergo their final glycosylation (4) and the resulting envelope glycoproteins are transported to the cell surface via vesicle-budding from the Golgi (5). Here, the Fragment antigen binding (Fab) domain of virus-specific antibodies binds to the viral glycoproteins (6). The Fc domain of the antibodies may then be recognized by the C1q component of the classical complement pathway (7) or by the Fc receptors of phagocytes (8) and NK cells (9), which leads to the destruction of the infected cell (10).

Several herpesviruses have developed strategies to avoid elimination of infected PBMC by the antibody-dependent components of the immune system. First, SuHV-1-infected monocytes are able to clear their cell surface from viral glycoproteins, a process described as *antibody-induced clearance of viral glycoproteins from the plasma membrane* (Favoreel *et al.*, 1999a). In brief, addition of SuHV-1-specific antibodies to SuHV-1-infected blood monocytes *in vitro* results in an aggregation of viral glycoproteins on the plasma membrane and subsequent internalization of glycoprotein -

antibody complexes, leaving the SuHV-1-infected monocytes with only few viral glycoproteins on their surface. The viral proteins gB and gD are crucial for this internalization process to occur (Favoreel *et al.*, 1999a; Van de Walle *et al.*, 2001; Favoreel *et al.*, 2002). Antibody-induced clearance of viral glycoproteins from the plasma membrane renders SuHV-1-infected monocytes significantly less susceptible towards antibody-dependent, complement-mediated lysis (Van de Walle *et al.*, in press).

Activation of the three components of the antibody-dependent immune system relies on their interaction with the Fc domain of virus-specific antibodies. Some members of the herpesvirus family induce the *surface expression of viral proteins that display Fc receptor activity*. By doing so, they capture the Fc domain of the virus-specific antibodies, making it no longer available for interaction with C1q or with the Fc receptor of NK cells and phagocytes. This disarming of virus-specific antibodies occurs via a process called antibody bipolar bridging. Hereby, the antigen-specific Fab domain of an antibody is bound to the respective viral protein, whereas the Fc domain of the same antibody is bound to the viral Fc receptor (Frank and Friedman, 1989). The glycoprotein complex gE/gI of SuHV-1 has been shown to possess Fc receptor activity (Favoreel *et al.*, 1999b) and its expression on the surface of infected blood monocytes helps to avoid efficient antibody-dependent, complement-mediated lysis (Van de Walle *et al.*, in press). For VZV, the glycoprotein gE/gI complex also displays Fc receptor activity (Litwin *et al.*, 1992) and since Mainka *et al.* (1998) showed a clear surface expression of gE on VZV-infected PBMC during viremia, it seems likely that gE/gI Fc receptor activity can help VZV-infected PBMC to circumvent antibody-dependent lysis.

1.4.2 MHC class I-dependent, CTL-mediated lysis and evasion strategies of herpesvirus-infected PBMC

Beside the antibody-dependent lysis, destruction of infected cells also involves MHC class I-dependent, CTL-mediated lysis. When a cell becomes infected with a virus, proteasomes present in the cytoplasm of the infected cell will process short peptides from newly produced viral proteins. These viral peptides are translocated to the ER via transporters associated with antigen presentation, where they are loaded onto MHC class

I/2-microglobulin heterodimers. The resulting complex then enters the secretory pathway of the cell and is finally presented at the cell surface. CTL which specifically recognize MHC class I associated with a foreign peptide, but not with self-antigens, then induce the lysis of the infected cell. Figure 6 shows a schematic overview of the MHC class I-dependent, CTL-mediated lysis.

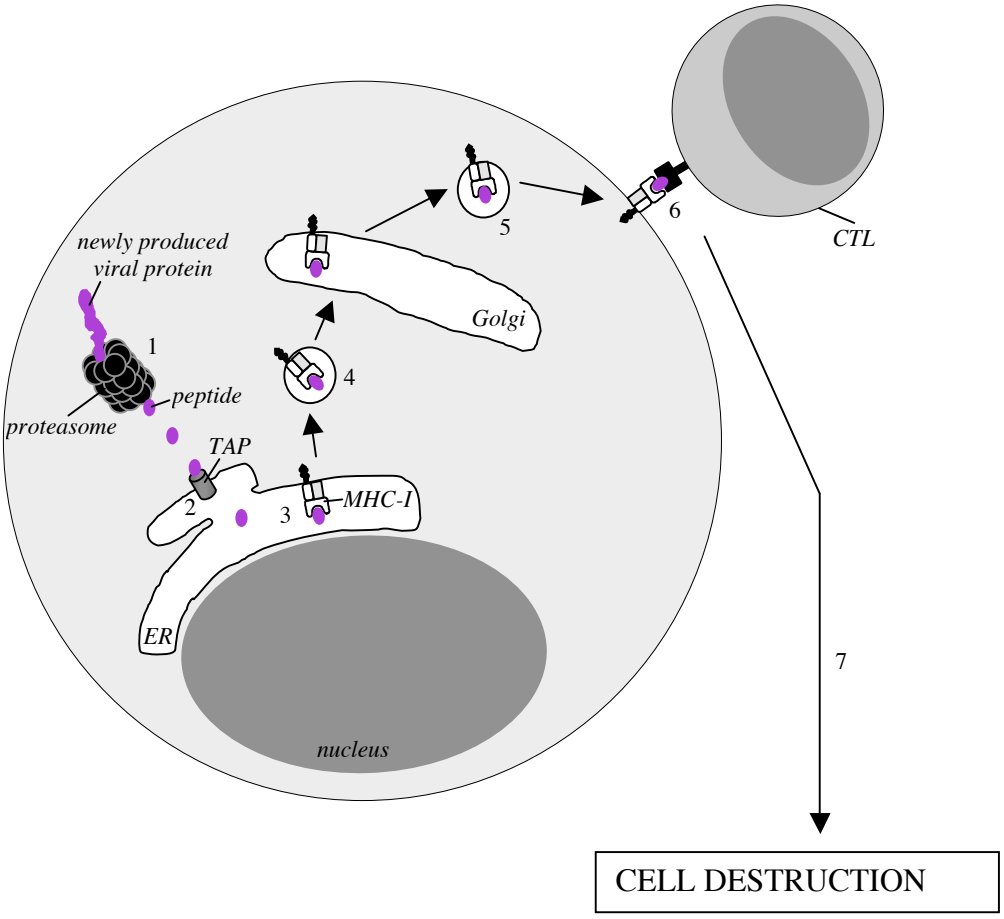

Figure 6. Mechanism of MHC class I-dependent, CTL-mediated lysis of a virus-infected cell

Viral proteins are proteolytically degraded into peptides inside the proteasome (1). The peptides are then transported into the lumen of the ER via transporters associated with antigen presentation (TAP) (2). In the ER, peptides are loaded onto MHC class I molecules (3). The resulting complex is transported to the cell surface via the secretory pathway (4-5). Once expressed at the cell surface, the infected cell becomes recognizable for CTL (6), leading to its destruction (7).

Numerous mechanisms have been described by which herpesviruses can inhibit expression of MHC class I - viral peptide complexes at the cell surface and, subsequently,

inhibit recognition and destruction of the infected cell by CTL. They include inhibition of peptide production and loading, inhibition of peptide transport to the ER, interference with MHC class I processing and inhibition of MHC class I - viral peptide trafficking through the cell. Up till now, only 2 of these mechanisms have been demonstrated in herpesvirus-infected PBMC. Firstly, Epstein-Barr virus (EBV) is able to *interfere with peptide production and loading* in PBMC. B-lymphocytes from individuals latently infected with EBV express Epstein-Barr nuclear antigen 1 (EBNA-1) (Levitskaya *et al.*, 1995). It was found that EBNA-1 is resistant to degradation by proteasomes and, consequently, its loading onto MHC class I molecules and its presentation to CTL are inhibited. Since EBNA-1 is the only protein in B-lymphocytes detected during latent infection, it is suggested that its resistance to degradation allows the latently infected B-lymphocytes to persist without being noticed by CTL (Levitskaya *et al.*, 1995). During the lytic phase of an EBV infection in B-lymphocytes, the peptide loading is compromised. EBV transcribes the BCRF-1 gene, which encodes a viral homologue of human interleukin-10 (Zeidler *et al.*, 1997). The viral interleukin-10 indirectly hampers the transport of peptides into the ER by downregulating the expression of the transporter associated with antigen presentation, TAP-1. The resulting reduction of MHC class I molecules on the cell surface involves all B-lymphocytes and may, therefore, affect the recognition of EBV-infected B-lymphocytes by CTL. Human cytomegalovirus (HCMV) can also interfere with peptide loading (Gilbert *et al.*, 1996). The virus encodes a viral kinase, designated pp65, which phosphorylates the immediate-early antigen IE-1 of HCMV. By doing so, the loading of IE-1 onto MHC class I molecules is blocked and, consequently, the presentation of IE-1 to CTL is inhibited. Pp65 is frequently monitored in the mononuclear cells from the blood of HCMV-infected humans (Greijer *et al.*, 2001; Lautenschlager *et al.*, 2002), indicating that it may contribute to an inefficient recognition of these cells by CTL.

Secondly, VZV *interferes with MHC class I - viral peptide trafficking* through the cell. Abendroth and Arvin (2001) found a significant decrease in MHC class I expression on the surface of VZV-infected T-lymphocytes during viremia in experimentally inoculated mice, when compared to non-infected T-lymphocytes. Co-localization studies demonstrated that MHC class I molecules maintained in the Golgi apparatus of the

infected cells, instead of continuing their way up to the plasma membrane. The identity of the viral gene(s) responsible for the retention of MHC class I molecules in the Golgi apparatus remains to be determined.

Alternatively, SuHV-1-infected blood monocytes undergo a *non-specific clearance of MHC class I molecules from their cell surface* (Favoreel *et al.*, 1999a). As mentioned in the previous chapter, SuHV-1-specific antibodies induce the internalization of viral glycoproteins from the plasma membrane of SuHV-1-infected blood monocytes. It was found that the internalization of viral glycoproteins was accompanied by a co-internalization of MHC class I molecules (Favoreel *et al.*, 1999a). Disappearance of MHC class I molecules from the cell surface reduced the susceptibility of the SuHV-1-infected monocytes to CTL activity, as demonstrated by comparing the percentage of lysed monocytes infected with wild type SuHV-1 with the percentage of lysed monocytes infected with a mutant virus unable to cause antibody-induced internalization (Favoreel, 1999).

1.4.3 NK cell-mediated lysis and evasion strategies of herpesvirus-infected PBMC

The viral strategies used to reduce the expression of MHC class I molecules on the surface of infected cells may be protective against CTL recognition and destruction; however, they render the infected cells highly susceptible to another arm of the host's immune system: the NK cells. NK cells are constitutively present in the host and they destroy any cell that lacks MHC class I expression on its surface. To secure their existence, viruses thus developed strategies to avoid recognition by NK cells. One of the best-studied strategies is the *surface expression of a viral MHC class I homologue which can bind to inhibitory receptors on NK cells* and, consequently, prevent NK cell-mediated lysis of the infected cell. Gene *UL18* of HCMV encodes such an MHC class I homologue, gpUL18, and transcripts of this gene have been clearly demonstrated in PBMC of patients with HCMV viremia (Hassan-Walker *et al.*, 1998, 2001). Glycoprotein UL18 binds to the Leukocyte Immunoglobulin-like Receptor 1 on NK cells, resulting in a reduced NK cell-mediated cytotoxicity (Reyburn *et al.*, 1997; Cosman *et al.*, 1999).

Until now, it has not been elucidated how EHV-1-infected PBMC are able to avoid elimination by the major immune effector mechanisms. The research presented in the present thesis addresses some aspects of the inefficient elimination of EHV-1-infected PBMC *in vitro* and *in vivo*.

References

Abendroth, A., Arvin, A. M. (2001). Immune evasion as a pathogenic mechanism of varicella zoster virus. *Seminars in Immunology* **13**, 27-39.

Allen, G. P., Bryans, J. T. (1986). Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. *Progress in Veterinary Microbiology and Immunology* **2**, 78-144.

Allen, G., Yeargan, M., Costa, L. R., Cross, R. (1995). Major histocompatibility complex class I-restricted cytotoxic T-lymphocyte responses in horses infected with equine herpesvirus 1. *Journal of Virology* **69**, 606-612.

Banbura, M., Chmielewska, A., Tucholska, A., Malicki, K. (2000). Occurrence of equine herpes virus type-1 (EHV-1)-specific DNA sequences in peripheral blood leukocytes of horses. *Medycyna Weterynaryjna* **56**, 521-523.

Baxi, M. K., Efstathiou, S., Lawrence, G., Whalley, J. M., Slater, J. D., Field, H. J. (1995). The detection of latency-associated transcripts of equine herpesvirus 1 in ganglionic neurons. *Journal of General Virology* **76**, 3113-3118.

Becker, W. (1988). Preventative vaccination against EHV (equine herpesvirus) abortion. *Tierärztliche Praxis* **16**, 61-63.

Breathnach, C. C. (2001). Mucosal humoral and cellular defence mechanism of the horse's upper respiratory tract against equine herpesvirus 1-infection. PhD thesis, University of Kentucky, Lexington, Kentucky.

Breathnach, C. C., Yeargan, M. R., Sheoran, A. S., Allen, G. P. (2001). The mucosal humoral immune response of the horse to infective challenge and vaccination with equine herpesvirus-1 antigens. *Equine Veterinary Journal* **33**, 651-657.

Browning, G. F., Ficorilli, N., Studdert, M. J. (1988). Asinine herpesvirus genomes: comparison with those of the equine herpesviruses. *Archives of Virology* **101**, 183-190.

Bryans, J. T., Allen, G. P. (1982). Application of a chemically inactivated, adjuvanted vaccine to control abortigenic infection of mares by equine herpesvirus I. *Developments in Biological Standardization* **52**, 493-498.

Bumgardner, M. K., Dutta, S. K., Campbell, D. L., Myrup, A. C. (1982). Lymphocytes from ponies experimentally infected with equine herpesvirus 1: subpopulation dynamics and their response to mitogens. *American Journal of Veterinary Research* **43**, 1308-1310.

Bürki, F., Rossmanith, W., Nowotny, N., Pallan, C., Möstl, K., Lussy, H. (1990). Viraemia and abortions are not prevented by two commercial equine herpesvirus-1 vaccines after experimental challenge of horses. *Veterinary Quarterly* **12**, 80-86.

Burrows, R., Goodridge, D. (1984). Studies of persistent and latent equid herpesvirus-1 and herpesvirus-3 infections in the Pirbright pony herd. *In* Wittmann, G., Gaskell, R. M., Rziha, H.-J. (eds.). Latent herpesvirus infections in veterinary medicine. Martinus Nijhoff, The Hague, pp 307–319.

Burrows, R., Goodridge, D., Denyer, M. S. (1984). Trials of an inactivated equid herpesvirus 1 vaccine: challenge with a subtype 1 virus. *Veterinary Record* **114**, 369-374.

Caughman, G. B., Staczek, J., O'Callaghan, D. J. (1985). Equine herpesvirus type 1-infected cell polypeptides: evidence for immediate early / early / late regulation of viral gene expression. *Virology* **145**, 49-61.

Caughman, G. B., Robertson, A. T., Gray, W. L., Sullivan, D. C., O'Callaghan, D. J. (1988). Characterization of equine herpesvirus type 1 immediate early proteins. *Virology* **163**, 563-571.

Charan, S., Palmer, K., Chester, P., Mire-Sluis, A. R., Meager, A., Edington, N. (1997). Transforming growth factor-beta induced by live or ultraviolet-inactivated equid herpes virus type-1 mediates immunosuppression in the horse. *Immunology* **90**, 586-591.

Chesters, P. M., Allsop, R., Purewal, A., Edington, N. (1997). Detection of latency-associated transcripts of equid herpesvirus 1 in equine leukocytes but not in trigeminal ganglia. *Journal of Virology* **71**, 3437-3443.

Chesters, P. M., Hughes, A., Edington, N. (2000). Equid herpesvirus 1: platelets and alveolar macrophages are potential sources of activated TGF-B1 in the horse. *Veterinary Immunology and Immunopathology* **75**, 71-79.

Chong, Y. C., Duffus, W. P. (1992). Immune responses of specific pathogen free foals to EHV-1 infection. *Veterinary Microbiology* **32**, 215-228.

Chowdhury, S. I., Buhk, H. J., Ludwig, H., Hammerschmidt, W. (1990). Genomic termini of equine herpesvirus 1. *Journal of Virology* **64**, 873-880.

Colle, C. F. 3rd, Flowers, C. C., O'Callaghan, D. J. (1992). Open reading frames encoding a protein kinase, homolog of glycoprotein gX of pseudorabies virus, and a novel glycoprotein map within the unique short segment of equine herpesvirus type 1. *Virology* **188**, 545-557.

Cook, R. F., O'Neill, T., Strachan, E., Sundquist, B., Mumford, J. A. (1990). Protection against lethal equine herpes virus type 1 (subtype 1) infection in hamsters by immune stimulating complexes (ISCOMs) containing the major viral glycoproteins. *Vaccine* **8**, 491-496.

Cosman, D., Fanger, N., Borges, L. (1999). Human cytomegalovirus, MHC class I and inhibitory signalling receptors: more questions than answers. *Immunological Reviews* **168**, 177-185.

Csellner, H., Walker, C., Wellington, J. E., McLure, L. E., Love, D. N., Whalley, J. M. (2000). EHV-1 glycoprotein D (EHV-1 gD) is required for virus entry and cell-cell fusion, and an EHV-1 gD deletion mutant induces a protective immune response in mice. *Archives of Virology* **145**, 2371-2385.

Darlington, R. W., Randall, C. C. (1963). The nucleic acid content of equine abortion virus. *Virology* **19**, 322-327.

Dimock, W. W., Edwards, P. R. (1933). Is there a filterable virus of abortion in mares? *The Kentucky Agricultural Experiment Station Bulletin* **333**, 291-301.

Dolby, C. A., Hannant, D., Mumford, J. A. (1995). Response of ponies to adjuvanted EHV-1 whole virus vaccine and challenge with virus of the homologous strain. *British Veterinary Journal* **151**, 27-37.

Doll, E. R. (1952). Seasonal incidence and fetal age in equine virus abortion. *Cornell Veterinarian* **42**, 505-509.

Doll, E. R., Bryans, J. T. (1963). Immunisation of young horses against viral rhinopneumonitis. *Cornell Veterinarian* **53**, 24-41.

Dutta, S. K., Myrup, A., Bumgardner, M. K. (1980). Lymphocyte responses to virus and mitogen in ponies during experimental infection with equine herpesvirus 1. *American Journal of Veterinary Research* **41**, 2066-2068.

Dutta, S. K., Myrup, A. C. (1983). Infectious center assay of intracellular virus and infective virus titer for equine mononuclear cells infected *in vivo* and *in vitro* with equine herpesviruses. *Canadian Journal of Comparative Medicine* **47**, 64-69.

Edington, N., Bridges, C. G., Huckle, A. (1985). Experimental reactivation of equid herpesvirus 1 (EHV 1) following the administration of corticosteroids. *Equine Veterinary Journal* **17**, 369-372.

Edington, N., Bridges, C. G., Patel, J. R. (1986). Endothelial cell infection and thrombosis in paralysis caused by equid herpesvirus-1: equine stroke. *Archives of Virology* **90**, 111-124.

Edington, N., Bridges, C. G. (1990). One way protection between equid herpesvirus 1 and 4 *in vivo*. *Research in Veterinary Science* **48**, 235-239.

Edington, N., Smyth, B., Griffiths, L. (1991). The role of endothelial cell infection in the endometrium, placenta and foetus of equid herpesvirus 1 (EHV-1) abortions. *Journal of Comparative Pathology* **104**, 379-387.

Edington, N., Welch, H. M., Griffiths, L. (1994). The prevalence of latent Equid herpesviruses in the tissues of 40 abattoir horses. *Equine Veterinary Journal* **26**, 140-142.

Favoreel, H. W. (1999). Antibody-induced clearance of viral and cellular plasma membrane proteins from pseudorabies virus-infected cells and its possible role in viral immune evasion. PhD thesis, Ghent University, Merelbeke, Belgium.

Favoreel, H. W., Nauwynck, H. J., Halewyck, H. M., Van Oostveldt, P., Mettenleiter, T. C., Pensaert, M. B. (1999a). Antibody-induced endocytosis of viral glycoproteins and major histocompatibility complex class I on pseudorabies virus-infected monocytes. *Journal of General Virology* **80**, 1283-1291.

Favoreel, H. W., Nauwynck, H. J., Pensaert, M. B. (1999b). Role of the cytoplasmic tail of gE in antibody-induced redistribution of viral glycoproteins expressed on pseudorabies virus-infected cells. *Virology* **259**, 141-147.

Favoreel, H. W., Van Minnebruggen, G., Nauwynck, H. J., Enquist, L. W., Pensaert, M. B. (2002). A tyrosine-based motif in the cytoplasmic tail of pseudorabies virus glycoprotein B is important for both antibody-induced internalization of viral glycoproteins and efficient cell-to-cell spread. *Journal of Virology* **76**, 6845-6851.

Flowers, C. C., O'Callaghan, D. J. (1992). The equine herpesvirus type 1 (EHV-1) homolog of herpes simplex virus type 1 US9 and the nature of a major deletion within the unique short segment of the EHV-1 KyA strain genome. *Virology* **190**, 307-315.

Frank, I., Friedman, H. M. (1989). A novel function of the herpes simplex virus type 1 Fc receptor: participation in bipolar bridging of antiviral immunoglobulin G. *Journal of Virology* **63**, 4479-4488.

Frymus, T., Kita, J., Woyciechowska, S., Ganowicz, M. (1986). Foetal and neonatal foal losses on equine herpesvirus type 1 (EHV-1) infected farms before and after EHV-1 vaccination was introduced. *Polskie Archiwum Weterynaryjne* **26**, 7-14.

Fukushi, H., Tomita, T., Taniguchi, A., Ochiai, Y., Kirisawa, R., Matsumura, T., Yanai, T., Masegi, T., Yamaguchi, T., Hirai, K. (1997). Gazelle herpesvirus 1: a new neurotropic herpesvirus immunologically related to equine herpesvirus 1. *Virology* **227**, 34-44.

Gibson, J. S., Slater, J. D., Awan, A. R., Field, H. J. (1992a). Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. *Archives of Virology* **123**, 351-366.

Gibson, J. S., O'Neill, T., Thackray, A., Hannant, D., Field, H. J. (1992b). Serological responses of specific pathogen-free foals to equine herpesvirus-1: primary and secondary infection, and reactivation. *Veterinary Microbiology* **32**, 199-214.

Gilbert, M. J., Riddell, S. R., Plachter, B., Greenberg, P. D. (1996). Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature* **383**, 720-722.

Gleeson, L. J., Coggins, L. (1980). Response of pregnant mares to equine herpesvirus 1 (EHV1). *Cornell Veterinarian* **70**, 391-400.

Goehring, L. S., Sloet van Oldruitenborgh-Oosterbaan, M. M. (2001). The mystery of equine herpes myeloencephalopathy. *Equine Veterinary Education* **13**, 36-42.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N., Mettenleiter, T. C. (2001). Egress of alphaherpesviruses: comparative ultrastructural study. *Journal of Virology* **75**, 3675-3684.

Gray, W. L., Baumann, R. P., Robertson, A. T., Caughman, G. B., O'Callaghan, D. J., Staczek, J. (1987a). Regulation of equine herpesvirus type 1 gene expression: characterization of immediate early, early, and late transcription. *Virology* **158**, 79-87.

Gray, W. L., Baumann, R. P., Robertson, A. T., O'Calaghan, D. J., Staczek, J. (1987b). Characterization and mapping of equine herpesvirus type 1 immediate early, early, and late transcripts. *Virus Research* **8**, 233-244.

Greenwood, R. E., Simson, A. R. (1980). Clinical report of a paralytic syndrome affecting stallions, mares and foals on a thoroughbred studfarm. *Equine Veterinary Journal* **12**, 113-117.

Greijer, A. E., Verschuuren, E. A., Harmsen, M. C., Dekkers, C. A., Adriaanse, H. M., The, T. H., Middeldorp, J. M. (2001). Direct quantification of human cytomegalovirus immediate-early and late mRNA levels in blood of lung transplant recipients by competitive nucleic acid sequence-based amplification. *Journal of Clinical Microbiology* **39**, 251-259.

Hannant, D., O'Neill, T., Jessett, D. M., Mumford, J. A. (1991). Evidence for non-specific immunosuppression during the development of immune responses to equid herpesvirus-1. *Equine Immunology Supplement* **12**, 41-45.

Hannant, D., Jessett, D. M., O'Neill, T., Dolby, C. A., Cook, R. F., Mumford, J. A. (1993). Responses of ponies to equid herpesvirus-1 ISCOM vaccination and challenge with virus of the homologous strain. *Research in Veterinary Science* **54**, 299-305.

Hannant, D., O'Neill, T., Ostlund, E. N., Kydd, J. H., Hopkin, P. J., Mumford, J. A. (1999). Equid herpesvirus-induced immunosuppression is associated with lymphoid cells and not soluble circulating factors. *Viral Immunology* **12**, 313-321.

Harty, R. N., Colle, C. F., Grundy, F. J., O'Callaghan, D. J. (1989). Mapping the termini and intron of the spliced immediate-early transcript of equine herpesvirus 1. *Journal of Virology* **63**, 5101-5110.

Harty, R. N., O'Callaghan, D. J. (1991). An early gene maps within and is 3' coterminal with the immediate-early gene of equine herpesvirus 1. *Journal of Virology* **65**, 3829-3838.

Hassan-Walker, A. F., Cope, A. V., Griffiths, P. D., Emery, V. C. (1998). Transcription of the human cytomegalovirus natural killer decoy gene, UL18, *in vitro* and *in vivo*. *Journal of General Virology* **79**, 2113-2116.

Hassan-Walker, A. F., Mattes, F. M., Griffiths, P. D., Emery, V. C. (2001). Quantity of cytomegalovirus DNA in different leukocyte populations during active infection *in vivo* and the presence of gB and UL18 transcripts. *Journal of Medical Virology* **64**, 283-289.

Heldens, J. G. M., Hannant, D., Cullinane, A. A., Prendergast, M. J., Mumford, J. A., Nelly, M., Kydd, J. H., Weststrate, M. W., van den Hoven, R. (2001). Clinical and virological evaluation of the efficacy of an inactivated EHV1 and EHV4 whole virus vaccine (Duvaxyn $EHV_{1,4}$). Vaccination/challenge experiments in foals and pregnant mares. *Vaccine* **19**, 4307-4317.

Henry, B. E., Robinson, R. A., Dauenhauer, S. A., Atherton, S. S., Hayward, G. S., O'Callaghan, D. J. (1981). Structure of the genome of equine herpesvirus type 1. *Virology* **115**, 97-114.

Jackson, T., Kendrick, J. W. (1971). Paralysis of horses associated with equine herpesvirus 1 infection. *Journal of the American Veterinary Medical Association* **158**, 1351-1357.

Jackson, T. A., Osburn, B. I., Cordy, D. R., Kendrick, J. W. (1977). Equine herpesvirus 1 infection of horses: studies on the experimentally induced neurologic disease. *American Journal of Veterinary Research* **38**, 709-719.

Karger, A., Mettenleiter, T. C. (1993). Glycoproteins gIII and gp50 play dominant roles in the biphasic attachment of pseudorabies virus. *Virology* **194**, 654-664.

Klupp, B. G., Granzow, H., Mettenleiter, T. C. (2000). Primary envelopment of pseudorabies virus at the nuclear membrane requires the UL34 gene product. *Journal of Virology* **74**, 10063-10073.

Kydd, J. H., Smith, K. C., Hannant, D., Livesay, G. J., Mumford, J. A. (1994a). Distribution of equid herpesvirus-1 (EHV-1) in respiratory tract of ponies: implications for vaccination strategies. *Equine Veterinary Journal* **26,** 466-469.

Kydd, J. H., Smith, K. C., Hannant, D., Livesay, G. J., Mumford, J. A. (1994b). Distribution of equid herpesvirus-1 (EHV-1) in respiratory tract associated lymphoid tissue: implications for cellular immunity. *Equine Veterinary Journal* **26**, 470-473.

Lautenschlager, I., Lappalainen, M., Linnavuori, K., Suni, J., Hockerstedt, K. (2002). CMV infection is usually associated with concurrent HHV-6 and HHV-7 antigenemia in liver transplant patients. *Journal of Clinical Virology* **25** *Supplement 2*, 57-61.

Levitskaya, J., Coram, M., Levitsky, V., Imreh, S., Steigerwald-Mullen, P. M., Klein, G., Kurilla, M. G., Masucci, M. G. (1995). Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* **375**, 685-688.

Litwin, V., Jackson, W., Grose, C. (1992). Receptor properties of two varicella-zoster virus glycoproteins, gpI and gpIV, homologous to herpes simplex virus gE and gI. *Journal of Virology* **66**, 3643-3651.

Mainka, C., Fuss, B., Geiger, H., Hofelmayr, H., Wolff, M. H. (1998). Characterization of viremia at different stages of varicella-zoster virus infection. *Journal of Medical Virology* **56**, 91-98.

Matsumura, T., O'Callaghan, D. J., Kondo, T., Kamada, M. (1996). Lack of virulence of the murine fibroblast adapted strain, Kentucky A (KyA), of equine herpesvirus type 1 (EHV-1) in young horses. *Veterinary Microbiology* **48**, 353-365.

Matsumura, T., Kondo, T., Sugita, S., Damiani, A. M., O'Callaghan, D. J., Imagawa, H. (1998). An equine herpesvirus type 1 recombinant with a deletion in the gE and gI genes is avirulent in young horses. *Virology* **242**, 68-79.

McGeoch, D. J., Cook, S. (1994). Molecular phylogeny of the alphaherpesvirinae subfamily and a proposed evolutionary timescale. *Journal of Molecular Biology* **238**, 9-22.

McCartan, C. G., Russell, M. M., Wood, J. L. N., Mumford, J. A. (1995). Clinical, serological and virological characteristics of an outbreak of paresis and neonatal foal disease due to equine herpesvirus-1 on a stud farm. *Veterinary Record* **136**, 7-12.

Mettenleiter, T. C., Zsak, L., Zuckermann, F., Sugg, N., Kern, H., Ben-Porat, T. (1990). Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. *Journal of Virology* **64**, 278-286.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *Journal of Virology* **76**, 1537-1547.

Mumford, J. A., Rossdale, P. D., Jessett, D. M., Gann, S., Ousey, J., Cook, R. F. (1987). Serological and virological investigations of an equid herpesvirus 1 (EHV_1) abortion storm on a stud farm in 1985. *Journal of Reproduction and Fertility Supplement* **35**, 509-518.

Mumford, J. A., Hannant, D., Jessett, D. M., O'Neill, T., Smith, K. C., Ostlund, E. N. (1994). Abortigenic and neurological disease caused by experimental infection with equid herpesvirus-1. Equine Infectious Diseases VII, Proceedings of the Seventh International Conference of Equine Infectious Diseases. Tokyo, pp 261-275.

Neubauer, A., Braun, B., Brandmuller, C., Kaaden, O. R., Osterrieder, N. (1997). Analysis of the contributions of the equine herpesvirus 1 glycoprotein gB homolog to virus entry and direct cell-to-cell spread. *Virology* **227**, 281-294.

O'Neill, T., Kydd, J. H., Allen, G. P., Wattrang, E., Mumford, J. A., Hannant, D. (1999). Determination of equid herpesvirus 1-specific, $CD8^+$, cytotoxic T lymphocyte precursor frequencies in ponies. *Veterinary Immunology and Immunopathology* **70**, 43-54.

Osterrieder, N., Neubauer, A., Brandmuller, C., Braun, B., Kaaden, O. R., Baines, J. D. (1996). The equine herpesvirus 1 glycoprotein gp21/22a, the herpes simplex virus type 1 gM homolog, is involved in virus penetration and cell-to-cell spread of virions. *Journal of Virology* **70**, 4110-4115.

Osterrieder, N. (1999). Construction and characterization of an equine herpesvirus 1 glycoprotein C negative mutant. *Virus Research* **59**, 165-177.

Patel, J. R., Edington, N., Mumford, J. A. (1982). Variation in cellular tropism between isolates of equine herpesvirus-1 in foals. *Archives of Virology* **74**, 41-54.

Perdue, M. L., Kemp, M. C., Randall, C. C., O'Callaghan, D. J. (1974). Studies of the molecular anatomy of the L-M cell strain of equine herpes virus type 1: proteins of the nucleocapsid and intact virion. *Virology* **59**, 201-216.

Platt, H., Singh, H., Whitwell, K. E. (1980). Pathological observations on an outbreak of paralysis in broodmares. *Equine Veterinary Journal* **12**, 118-126.

Randall, C. C., Lawson, L. A. (1962). Adaptation of equine abortion virus to Earle's L-cells in serum-free medium with plaque formation. *Proceedings of the Society for Experimental Biology and Medicine* **110**, 487-489.

Reyburn, H. T., Mandelboim, O., Vales-Gomez, M., Davis, D. M., Pazmany, L., Strominger, J. L. (1997). The class I MHC homologue of human cytomegalovirus inhibits attack by natural killer cells. *Nature* **386**, 514-517.

Robertson, A. T., Caughman, G. B., Gray, W. L., Baumann, R. P., Staczek, J., O'Callaghan, D. J. (1988). Analysis of the *in vitro* translation products of the equine herpesvirus type 1 immediate early mRNA. *Virology* **166**, 451-462.

Robertson, G. R., Scott, N. A., Miller, J. M., Sabine, M., Zheng, M., Bell, C.W., Whalley, J. M. (1991). Sequence characteristics of a gene in equine herpesvirus 1 homologous to glycoprotein H of herpes simplex virus. *DNA Sequence* **1**, 241-249.

Roizman, B. (1991). Herpesviridae: a brief introduction. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, B. R. Roizman (eds.). Fundamental Virology, 2nd edition. Raven Press, Ltd., New York, pp 841- 848.

Roizmann, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C., Studdert, M. J. (1992). The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Archives of Virology* **123**, 425-449.

Roller, R. J., Zhou, Y., Schnetzer, R., Ferguson, J., DeSalvo, D. (2000). Herpes simplex virus type 1 U(L)34 gene product is required for viral envelopment. *Journal of Virology* **74**, 117-129.

Rudolph, J., Osterrieder, N. (2002). Equine herpesvirus type 1 devoid of gM and gp2 is severely impaired in virus egress but not direct cell-to-cell spread. *Virology* **293**, 356-367.

Rudolph, J., Seyboldt, C., Granzow, H., Osterrieder, N. (2002). The gene 10 (UL49.5) product of equine herpesvirus 1 is necessary and sufficient for functional processing of glycoprotein M. *Journal of Virology* **76**, 2952-2963.

Ruyechan, W. T., Dauenhauer, S. A., O'Callaghan, D. J. (1982). Electron microscopic study of equine herpesvirus type 1 DNA. *Journal of Virology* **42**, 297-300.

Sabine, M., Robertson, G. R., Whalley, J. M. (1981). Differentiation of sub-types of equine herpesvirus I by restriction endonuclease analysis. *Australian Veterinary Journal* **57**, 148-149.

Scott, J. C., Dutta, S. K., Myrup, A. C. (1983). *In vivo* harboring of equine herpesvirus-1 in leukocyte populations and subpopulations and their quantitation from experimentally infected ponies. *American Journal of Veterinary Research* **44**, 1344-1348.

Siedek, E. M., Whelan, M., Edington, N., Hamblin, A. (1999). Equine herpesvirus type 1 infects dendritic cells *in vitro*: stimulation of T lymphocyte proliferation and cytotoxicity by infected dendritic cells. *Veterinary Immunology and Immunopathology* **67**, 17-32.

Slater, J. D., Gibson, J. S., Field, H. J. (1993). Pathogenicity of a thymidine kinase-deficient mutant of equine herpesvirus 1 in mice and specific pathogen-free foals. *Journal of General Virology* **74**, 819-828.

Slater, J. D., Borchers, K., Thackray, A. M., Field, H. J. (1994). The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. *Journal of General Virology* **75**, 2007-2016.

Smith, K. C., Whitwell, K. E., Binns, M. M., Dolby, C. A., Hannant, D., Mumford, J. A. (1992). Abortion of virologically negative foetuses following experimental challenge of pregnant pony mares with equid herpesvirus 1. *Equine Veterinary Journal* **24**, 256-259.

Smith, K. C., Whitwell, K. E., Mumford, J. A., Gower, S. M., Hannant, D., Tearle, J. P. (1993). An immunohistological study of the uterus of mares following experimental infection by equid herpesvirus 1. *Equine Veterinary Journal* **25**, 36-40.

Smith, K. C., Mumford, J. A., Lakhani, K. (1996). A comparison of equid herpesvirus-1 (EHV-1) vascular lesions in the early versus late pregnant equine uterus. *Journal of Comparative Pathology* **114**, 231-247.

Smith, D. J., Iqbal, J., Purewal, A., Hamblin, A. S., Edington, N. (1998). *In vitro* reactivation of latent equid herpesvirus-1 from CD5+/CD8+ leukocytes indirectly by IL-2 or chorionic gonadotrophin. *Journal of General Virology* **79**, 2997-3004.

Smith, K. C., Whitwell, K. E., Mumford, J. A., Hannant, D., Blunden, A. S., Tearle, J. P. (2000). Virulence of the V592 isolate of equid herpesvirus-1 in ponies. *Journal of Comparative Pathology* **122**, 288-297.

Smith, D. J., Hamblin, A. S., Edington, N. (2001). Infection of endothelial cells with equine herpesvirus-1 (EHV-1) occurs where there is activation of putative adhesion molecules: a mechanism for transfer of virus. *Equine Veterinary Journal* **33**, 138-142.

Smith, K. C., Borchers, K. (2001). A study of the pathogenesis of equid herpesvirus-1 (EHV-1) abortion by DNA in-situ hybridization. *Journal of Comparative Pathology* **125**, 304-310.

Smith, D., Hamblin, A., Edington, N. (2002). Equid herpesvirus 1 infection of endothelial cells requires activation of putative adhesion molecules: an *in vitro* model. *Clinical and Experimental Immunology* **129**, 281-287.

Soehner, R. L., Gentry, G. A., Randall, C. C. (1965). Some physicochemical properties of equine abortion virus nucleic acid. *Virology* **26**, 394-405.

Spear, P. G. (1993). Entry of alphaherpesviruses into cells. *Seminars in Virology* **4**, 167-180.

Stokes, A., Corteyn, A. H., Murray, P. K. (1991). Clinical signs and humoral immune response in horses following equine herpesvirus type-1 infection and their susceptibility to equine herpesvirus type-4 challenge. *Research in Veterinary Science* **51**, 141-148.

Stokes, A., Alber, D. G., Greensill, J., Amellal, B., Carvalho, R., Taylor, L. A., Doel, T. R., Killington, R. A., Halliburton, I. W., Meredith, D. M. (1996). The expression of the proteins of equine herpesvirus 1 which share homology with herpes simplex virus 1 glycoproteins H and L. *Virus Research* **40**, 91-107.

Studdert, M. J., Simpson, T., Roizman, B. (1981). Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. *Science* **214**, 562-564.

Sun, Y., MacLean, A. R., Dargan, D., Brown, S. M. (1994). Identification and characterization of the protein product of gene 71 in equine herpesvirus 1. *Journal of General Virology* **75**, 3117-3126.

Sun, Y., MacLean, A. R., Aitken, J. D., Brown, S. M. (1996). The role of the gene 71 product in the life cycle of equine herpesvirus 1. *Journal of General Virology* **77**, 493-500.

Taniguchi, A., Fukushi, H., Matsumura, T., Yanai, T., Masegi, T., Hirai, K. (2000). Pathogenicity of a new neurotropic equine herpesvirus 9 (gazelle herpesvirus 1) in horses. *Journal of Veterinary Medical Science* **62**, 215-218.

Telford, E. A., Watson, M. S., McBride, K., Davison, A. J. (1992). The DNA sequence of equine herpesvirus-1. *Virology* **189**, 304-316.

Tewari, D., Gibson, J. S., Slater, J. D., O'Neill, T., Hannant, D., Allen, G. P., Field, H. J. (1993). Modulation of the serological response of specific pathogen-free (EHV-free) foals to EHV-1 by previous infection with EHV-4 or a TK-deletion mutant of EHV-1. *Archives of Virology* **132**, 101-120.

Thein, P., Brown, K. (1988). Infection mit equinen Herpesviren und Manifestation am Zentralnervensystem beim Pferd. *Tierärztliche Praxis* **16**, 295-302.

Thomson, G. R., Mumford, J. A. (1977). *In vitro* stimulation of foal lymphocytes with equid herpesvirus 1. *Research in Veterinary Science* **22**, 347-352.

Turtinen, L. W., Allen, G. P., Darlington, R. W., Bryans, J. T. (1981). Serologic and molecular comparisons of several equine herpesvirus type 1 strains. *American Journal of Veterinary Research* **42**, 2099-2104.

Van de Walle, G. R., Favoreel, H. W., Nauwynck, H. J., Van Oostveldt, P., Pensaert, M. B. (2001). Involvement of cellular cytoskeleton components in antibody-induced internalization of viral glycoproteins in pseudorabies virus-infected monocytes. *Virology* **288**, 129-138.

van de Walle, G. R., Favoreel, H. W., Nauwynck, H. J., Pensaert, M. B. (2003). Antibody-induced internalization of viral glycoproteins and gE-gI receptor activity protect pseudorabies virus-infected monocytes from efficient complement-mediated lysis. *Journal of General Virology* **84** (in press).

van Maanen, C., Willink, D. L., Smeenk, L. A., Brinkhof, J., Terpstra, C. (2000). An equine herpesvirus 1 (EHV1) abortion storm at a riding school. *Veterinary Quarterly* **22**, 83-87.

van Maanen, C., Sloet van Oldruitenborgh-Oosterbaan, M. M., Damen, E. A., Derksen, A. G. (2001). Neurological disease associated with EHV-1-infection in a riding school: clinical and virological characteristics. *Equine Veterinary Journal* **33**, 191-196.

von Benten, C., Petzoldt, K. (1977). Several years of diagnostic studies on the EHV 1 abortion in thoroughbred studs following the introduction of vaccination. *Berliner und Münchener Tierärztliche Wochenschrift* **90**, 176-180.

Welch, H. M., Bridges, C. G., Lyon, A. M., Griffiths, L., Edington, N. (1992). Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. *Journal of General Virology* **73**, 261-268.

Wellington, J. E., Love, D. N., Whalley, J. M. (1996a). Evidence for involvement of equine herpesvirus 1 glycoprotein B in cell-cell fusion. *Archives of Virology* **141**, 167-175

Wellington, J. E., Lawrence, G. L., Love, D. N., Whalley, J. M. (1996b). Expression and characterization of equine herpesvirus 1 glycoprotein D in mammalian cell lines. *Archives of Virology* **141**, 1785-1793.

Whalley, J. M., Robertson, G. R., Davison, A. J. (1981). Analysis of the genome of equine herpesvirus type 1: arrangement of cleavage sites for restriction endonucleases EcoRI, BglII and BamHI. *Journal of General Virology* **57**, 307-323.

Wilks, C. R., Coggins, L. (1976). Immunity to equine herpesvirus type 1 (rhinopneumonitis): *in vitro* lymphocyte response. *American Journal of Veterinary Research* **37**, 486-492.

Woyciechowska, S. (1960). Adaptation of equine abortion virus (isolated in Poland) strain RAC-Heraldia to Syrian hamster. *Medycyna Doswiadczalna i Mikrobiologia* **12**, 255-263.

Woyciechowska, S., Kita, J., Frymus, T., Górski, J., Michalak, T., Chmielewska, A. (1980). A vaccine against rhinopneumonitis equorum – studies of safety and immunogenicity in pregnant mares. *Medycyna Weterynaryjne* **36**, 525-529.

WuDunn, D., Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *Journal of Virology* **63**, 52-58.

Yalamanchili, R. R., O'Callaghan, D. J. (1990). Sequence and organization of the genomic termini of equine herpesvirus type 1. *Virus Research* **15**, 149-161.

Yanai, T., Sakai, T., Fukushi, H., Hirai, K., Narita, M., Sakai, H., Masegi, T. (1998). Neuropathological study of gazelle herpesvirus 1 (equine herpesvirus 9) infection in Thomson's gazelles (Gazella thomsoni). *Journal of Comparative Pathology* **119**, 159-168.

Zeidler, R., Eissner, G., Meissner, P., Uebel, S., Tampe, R., Lazis, S., Hammerschmidt, W. (1997). Downregulation of TAP1 in B lymphocytes by cellular and Epstein-Barr virus-encoded interleukin-10. *Blood* **90**, 2390-2397.

Zhao, Y., Holden, V. R., Harty, R. N., O'Callaghan, D. J. (1992). Identification and transcriptional analyses of the UL3 and UL4 genes of equine herpesvirus 1, homologs of the ICP27 and glycoprotein K genes of herpes simplex virus. *Journal of Virology* **66**, 5363-5372.

2. AIMS OF THE STUDY

AIMS OF THE STUDY

Equine herpesvirus 1 (EHV-1) is recognized worldwide as a major pathogen of horses, causing serious economic losses due to abortion, neonatal foal death and, less frequently, nervous system disorders. Peripheral blood mononuclear cells (PBMC) play a crucial role in the pathogenesis of an EHV-1 infection. During the viremic phase of the infection, infected PBMC transport EHV-1 from the primary site of replication to the target organs, such as the pregnant uterus and the nervous system. As already mentioned in Chapter 1.2.3 of the present thesis, basic knowledge on the interaction of EHV-1 with equine PBMC is rather scarce. The first aim of this thesis was to obtain more detailed information on the kinetics of EHV-1 replication in freshly isolated PBMC and on the identity of the infected PBMC *in vitro*. Since earlier studies indicated that mitogens may promote viral replication in PBMC, we also wanted to investigate the replication kinetics in mitogen-stimulated PBMC and, subsequently, to obtain better insights in the mechanism(s) by which mitogen stimulation affects the susceptibility of PBMC to EHV-1.

An important feature in the pathogenesis of an EHV-1 infection is the ability of EHV-1 to cause viremia in the horse in the presence of an active immunity. Consequently, EHV-1-induced abortion or nervous system disorders may occur in infection-immune and vaccination-immune horses. Apparently, elimination of circulating EHV-1-infected PBMC by the immune system is inefficient. For other herpesviruses, several immune evasion strategies have been described that enable herpesvirus-infected PBMC to circumvent recognition and/or destruction by the major immune effector mechanisms. Up till now, no such strategies have been described for EHV-1. The second aim of this thesis was to obtain some insights in how EHV-1-infected PBMC are able to avoid efficient elimination by humoral and cellular components of the host's immune system. Studies were performed using *in vitro* infected PBMC as well as infected PBMC isolated from experimentally inoculated ponies.

3. REPLICATION OF EQUINE HERPESVIRUS 1 IN FRESHLY ISOLATED AND MITOGEN-STIMULATED PERIPHERAL BLOOD MONONUCLEAR CELLS OF THE HORSE

3.1 REPLICATION OF EQUINE HERPESVIRUS 1 IN FRESHLY ISOLATED EQUINE PERIPHERAL BLOOD MONONUCLEAR CELLS AND CHANGES IN SUSCEPTIBILITY FOLLOWING MITOGEN STIMULATION

3.2 MITOGEN STIMULATION FAVOURS REPLICATION OF EQUINE HERPESVIRUS 1 IN EQUINE BLOOD MONONUCLEAR CELLS BY INDUCING CELL PROLIFERATION AND FORMATION OF CLOSE INTERCELLULAR CONTACTS

3.1 REPLICATION OF EQUINE HERPESVIRUS 1 IN FRESHLY ISOLATED EQUINE PERIPHERAL BLOOD MONONUCLEAR CELLS AND CHANGES IN SUSCEPTIBILITY FOLLOWING MITOGEN STIMULATION

Journal of General Virology (2000), 81, 21-25

van der Meulen, K., Nauwynck, H., Buddaert, W. and Pensaert, M.

3.1.1 Summary

In the present study, the outcome of an inoculation of equine peripheral blood mononuclear cells (PBMC) with equine herpesvirus 1 (EHV-1) was studied *in vitro*. Cytoplasmic and plasma membrane expression of viral antigens, intra- and extracellular virus titres and plaque formation in co-culture were determined. EHV-1 replicated in monocytes, although in a highly restricted way. Viral antigens were found in a maximum number of 8.7 % of the monocytes at 12 hours post inoculation (h pi). The infection was productive in 0.16 % of the monocytes. The virus yield was $10^{0.7}$ tissue culture infectious dose $_{50}$ ($TCID_{50}$) per productive cell. In a population of resting lymphocytes, 0.9 % of the cells were infected and less than 0.05 % produced infectious virus. The virus yield was $10^{0.1}$ $TCID_{50}$ per productive cell. After prestimulation with different mitogens, the number of infected lymphocytes increased 4 to 12 times. The susceptible lymphocytes were T-lymphocytes. None of the infected PBMC were B-lymphocytes. In mitogen-stimulated lymphocytes, a clear expression of viral antigens was found on the plasma membrane. In conclusion, it can be stated that in freshly isolated PBMC, monocytes are the most important cell fraction in which EHV-1 replicates. After mitogen stimulation of the PBMC, T-lymphocytes become more susceptible.

3.1.2 Introduction

Equine herpesvirus 1 (EHV-1), a member of the *Alphaherpesvirinae*, is a major pathogen of horses, responsible for respiratory disorders, abortion, neonatal foal disease and neurological disorders. Starting from 4-6 days after EHV-1 infection, an extensive cell-associated viremia is detected which lasts until 9-14 days after infection (Gibson *et al.*, 1992). T-lymphocytes seem to be the most susceptible of the peripheral blood mononuclear cells (PBMC) (Scott *et al.*, 1983). Viremia is associated with e.g. T-cell lymphopaenia and appearance of blastic cells (McCulloch *et al.*, 1993) and may occur in the presence of virus-neutralizing antibodies (Doll and Bryans, 1963; Mumford *et al.*, 1987). Carried by infected PBMC, EHV-1 spreads to internal organs.

Information on the interaction between EHV-1 and PBMC is rather scarce. Scott *et al.* (1983) demonstrated EHV-1 infection of leukocytes by co-cultivation from 2 to 14 days after experimental inoculation of ponies. Virus was not detected by co-cultivation of disrupted leukocytes. After mitogen stimulation of the leukocytes in culture, infectious virus was detected in a higher number of cells and was also found by co-cultivation of disrupted leukocytes. Dutta and Myrup (1983) performed a similar study, but extended it with *in vitro* infection of leukocytes. For *in vivo*-infected leukocytes they obtained similar results as Scott *et al.* (1983). *In vitro* infected, non-stimulated leukocytes gave a higher number of plaques than mitogen-stimulated leukocytes, in contrast to the results obtained *in vivo*.

The main purpose of this study is to obtain more detailed information about the replication of EHV-1 in freshly isolated, equine PBMC and to investigate the effect of mitogen stimulation on the replication kinetics of EHV-1 in lymphocytes.

3.1.3 Materials and Methods

Separation of PBMC in monocytes, T-lymphocytes and B-lymphocytes

PBMC were isolated by density centrifugation of heparinized blood from adult, infection-immune horses on Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) and afterwards separated in 2 subpopulations by plasma-mediated adhesion as described by

Nauwynck and Pensaert (1994). In brief, polystyrene tissue culture dishes (Corning, New York, U.S.) were coated with 20 % autologous plasma in RPMI during 1 hour at 37 °C. Afterwards, the dishes were washed and PBMC were layered on the dishes for 1 hour at 37 °C. Adherent cells consisted predominantly of monocytes and non-adherent cells consisted predominantly of lymphocytes. Remaining monocytes in the lymphocyte-enriched population were removed by plastic adhesion during 1 hour at 37 °C.

Mitogen stimulation

PBMC and lymphocytes were stimulated by adding pokeweed mitogen (PWM), concanavalin A (ConA), phytohemagglutinin (PHA) or ionomycin and phorbol dibutyrate (IONO/PDB) (Sigma, Bornem, Belgium) to the medium. Medium was further supplemented with 10 U/ml of heparin (Leo, Zaventem, Belgium). Cellular DNA analysis was performed at different time points as described by Darzynkiewicz *et al.* (1984). Briefly, cells were fixed in ice-cold ethanol (70 %) for 30 minutes, washed and stained for 30 minutes at room temperature with a solution containing 10 □g/ml propidium iodide, 0.1 % Triton X-100, 2 mM $MgCl_2$, 0.1 M NaCl, 10 mM PIPES buffer and 20 U/ml Rnase A in distilled water. Cells were analyzed by flow cytometry. Optimal stimulation was obtained after 48 hours of treatment with concentrations of 4 μg/ml for PWM, ConA or PHA and 0.5 μM and 10 nM for IONO and PDB, respectively.

Virus and infection

The Belgian EHV-1-strain 97P70 was used throughout the study. The strain was isolated from lungs of an aborted fetus in 1997 and was plaque-purified twice in equine embryonic lung cells. The strain was identified as EHV-1 using the monoclonal antibody 13B2 (Yeargan *et al.*, 1985). Virus stocks used for inoculation were at the 6^{th} passage.

PBMC and subpopulations were inoculated at a multiplicity of infection of 10, either directly following isolation and separation or after 48 hours of mitogen stimulation. Mock-inoculated cells were included as a control. After 1 hour of incubation at 37 °C, extracellular virus was removed by treating the cells with a citrate buffer of pH 3 (Mettenleiter, 1989). After 2 washing steps, cells were resuspended in leukocyte medium

(RPMI 1640, 10 % fetal bovine serum, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 0.3 mg/ml glutamin, 1 % non-essential amino acids 100x (GIBCO BRL, Life Technologies Inc., Gaithersburg, Maryland, USA), 1 mM sodium pyruvate) and incubated at 37 °C and 5 % CO_2.

Cell viability was determined by flow cytometry using propidium iodide (1 μg/ml), at 3 and 24 hours post inoculation (h pi).

Immunofluorescence stainings

To determine the composition of PBMC and subpopulations immediately after isolation and separation, an immunofluorescence staining was performed using monoclonal antibodies HB88A and DH59B (VMRD Inc., Pullman, U.S.) to stain T-lymphocytes and monocytes, respectively (Tumas *et al.*, 1994). A hyperimmune goat serum against horse IgM (Kirkegaard and Perry Laboratories Inc., Gaithersburg, U.S.) was used to stain B-lymphocytes. Cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, U.S.).

To determine the percentages of freshly isolated as well as mitogen-stimulated cells expressing viral antigens in the cytoplasm, cells were collected at various time points pi, as indicated in Figure 1 and Table 2. Subsequently, cells were smeared on microscopic slides using a Cytospin 3 (Shandon), fixed with aceton during 20 minutes at -20 °C and stained using polyclonal, protein A-purified and biotinylated rabbit anti-EHV-1 antibodies followed by streptavidine labeled with fluoresceine isothyocyanate (FITC). Samples were analyzed by fluorescence microscopy (Leica DM RBE, Wetzlar, Germany).

Expression of viral antigens on the plasma membrane was determined in a total population of IONO/PDB-stimulated PBMC only. PBMC were collected at 48 h pi and an indirect immunofluorescence staining was performed, using polyclonal, protein G-purified and biotinylated horse anti-EHV-1 antibodies followed by streptavidine-FITC. A non-inoculated sample was included as control. Cells were analyzed by flow cytometry.

The identity of IONO/PDB-stimulated and infected PBMC was determined after 1, 24, 48 and 72 h pi. First, PBMC were stained in suspension at 4 °C with the specific cell

markers as described above. Then, PBMC were smeared on microscopic slides, fixed with paraformaldehyde (4 %) and permeabilized with Triton X-100. Finally, intracellular viral antigens were stained using polyclonal, protein G-purified and biotinylated horse anti-EHV-1 antibodies followed by streptavidine-FITC.

Virus titration

To quantitate extracellular virus titres, medium was collected at various time points pi, as indicated in Figure 1 and Table 2. Medium was then centrifuged at 390 x g and titrated on rabbit kidney (RK13) cells. To quantitate intracellular virus titres, cells were collected and freeze-thawed twice. The cell lysate was centrifuged and the supernatant was titrated.

Co-cultivation

For co-cultivation, cells were collected at 1 h pi and seeded on RK13 monolayers. The monolayers were overlaid with 0.94 % carboxymethylcellulose (Sigma, Bornem, Belgium) in leukocyte medium and centrifuged at 750 x g for 30 minutes to sediment the cells onto the monolayer. Duplicate preparations were made after disruption of PBMC by ultrasonication. Monolayers were placed at 37 °C and 5 % CO_2. After 7 days of incubation, the number of plaques was counted. Additionally, the percentage of PBMC expressing viral antigens in their cytoplasm was determined at 12 h pi by indirect immunofluorescence staining as described above.

3.1.4 Results

Composition of freshly isolated, non-inoculated PBMC and subpopulations

In the total population of PBMC, the percentages of T-lymphocytes, B-lymphocytes and monocytes were 54.3 ± 0.1 %, 24.4 ± 1.7 % and 9.0 ± 0.3 %, respectively. In the non-adherent population, the percentages were 66.4 ± 5.7 %, 24.9 ± 8.7 % and 1.0 ± 0.6 %, respectively. In the adherent population, 79.1 ± 5.2 % of the cells were identified as monocytes.

Replication of EHV-1 in freshly isolated PBMC, monocytes and lymphocytes

The percentages of PBMC, monocytes and lymphocytes expressing viral antigens in the cytoplasm are presented in Table 1.

Table 1. Viral antigen expression in freshly isolated, EHV-1-inoculated PBMC

Selected PBMC fraction	% EHV-1 antigen-positive cells at … h pi					
	3	5	7	9	12	24
Total PBMC	0.5 ± 0.2	1.5 ± 0.8	2.1 ± 0.4	2.3 ± 0.1	2.3 ± 2.1	2.5 ± 2.4
Monocytes	0.3 ± 0.3	3.9 ± 2.1	6.6 ± 2.6	8.1 ± 3.8	8.7 ± 4.2	7.5 ± 4.4
T- and B-lymphocytes	0	0.5 ± 0.2	0.6 ± 0.3	0.7 ± 0. 4	0.5 ± 0.3	0.9 ± 0.6

All data are expressed as the mean value of three experiments ± standard deviation.
h pi: hours post inoculation

Viral antigens appeared from 5 h pi. The number of infected monocytes and PBMC peaked at 12 (8.7 ± 4.2 %) and 24 h pi (2.5 ± 2.4 %), respectively, whereas the percentage of infected lymphocytes remained below 1 % (Table 1). Mock-infected cells yielded negative data in each case.

Figure 1 shows the kinetics of intra- and extracellular virus titres in freshly isolated PBMC, monocytes and lymphocytes following EHV-1 inoculation. In PBMC and lymphocytes, intracellular virus titres started to increase at 5 and 7 h pi, respectively and reached a maximum at 12 h p.i (Figure 1). In the enriched monocytes, intracellular titres increased from 3 h pi and reached a maximum at 7 h pi (Figure 1). Extracellular titres increased only in the PBMC from 9 h pi (Figure 1).

The total number of plaques per 10^4 cells as determined by co-cultivation was 11.2 ± 0.8 for PBMC, 15.7 ± 7.5 for monocytes and 3.8 ± 1.4 for lymphocytes. The percentage of infected cells at 12 h pi as determined by immunofluorescence was 1.3 ± 0.6 % for PBMC, 1.2 ± 0.3 % for monocytes and 0.7 ± 0.3 % for lymphocytes. It was calculated that plaque formation was induced by 13.7 ± 8.3 % of the infected monocytes, 9.2 ± 2.8

% of the infected PBMC and 5.3 ± 0.9 % of the infected lymphocytes. No plaques were observed in the duplicate preparations.

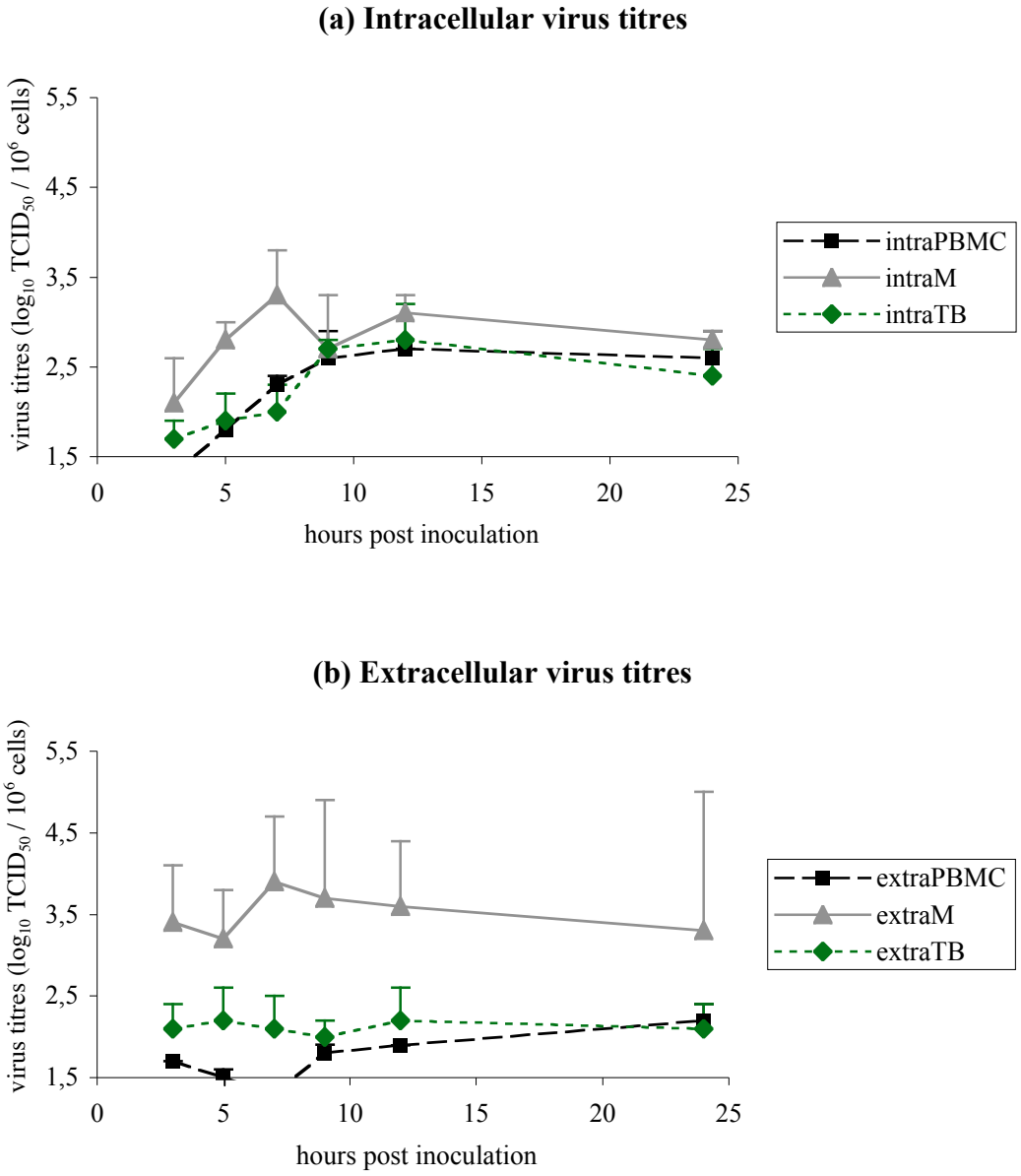

Figure 1. Replication kinetics of EHV-1 in freshly isolated PBMC and subpopulations

(a) Intracellular virus titres for the total population of PBMC and for the enriched subpopulations of monocytes and T- and B-lymphocytes. (b) Extracellular virus titres. The detection limit for these assays was 1.5 $\log_{10}$ $TCID_{50}$. All data are expressed as the mean value of three experiments ± standard deviation.

Viability was always higher than 65 % and no differences were observed between EHV-1-infected and mock-infected cells.

Replication of EHV-1 in mitogen-stimulated PBMC and lymphocytes

The percentages of viral antigen-positive cells in mitogen-stimulated, EHV-1-inoculated lymphocytes as well as intra- and extracellular virus titres are shown in Table 2.

Table 2. EHV-1 infection in a subpopulation of T- and B-lymphocytes stimulated with different mitogens

Mitogen*	EHV-1 antigen-positive cells at … h pi (%)			Intracellular virus titres at … h pi (log_{10} $TCID_{50}$ / 10^6 cells)			Extracellular virus titres at … h pi (log_{10} $TCID_{50}$ / 10^6 cells)		
	1	12	24	1	12	24	1	12	24
None	0	0.5 ± 0.8	0.5 ± 0.3	≤1.5 ± 0	1.8 ± 0.2	1.9 ± 0.3	≤1.5 ± 0	1.6 ± 0.1	1.8 ± 0.2
ConA	0	1.0 ± 0.3	2.9 ± 1.5	≤1.5 ± 0	1.9 ± 0.3	3.2 ± 0.4	1.6 ± 0.1	1.7 ± 0.2	2.4 ± 0.3
PHA	0	1.2 ± 0.2	2.2 ± 1.2	≤1.5 ± 0	1.7 ± 0	2.3 ± 0.3	≤1.5 ± 0	1.8 ± 0.4	1.9 ± 0.4
PWM	0	2.4 ± 1.0	4.5 ± 1.3	≤1.5 ± 0	2.1 ± 0.2	3.1 ± 0.9	≤1.5 ± 0	1.6 ± 0.1	2.6 ± 0.1
IONO/ PDB	0	2.9 ± 1.3	6.0 ± 1.9	≤1.5 ± 0	2.1 ± 0.4	3.2 ± 0.5	1.6 ± 0.1	2.2 ± 0.4	3.3 ± 0.3

* stimulation for 48 hours
Abbreviations: ConA, concanavalin A; PHA, phytohaemagglutinin; PWM, pokeweed mitogen; IONO/PDB, ionomycin and phorbol dibutyrate; h pi, hours post inoculation
All data are expressed as mean values of three experiments ± standard deviation.

For all mitogens used, the percentage of infected lymphocytes increased 4 to 12 times. PWM and IONO/PDB showed the largest effect. ConA-, PWM- and IONO/PDB-stimulated lymphocytes showed an increase in intra- and extracellular virus titres, when compared to non-stimulated lymphocytes (Table 2). PHA only induced a slight increase in intracellular titres (Table 2).

When identification was performed in a total population of mitogen-stimulated, EHV-1-inoculated PBMC, it was found that most of the infected cells were T-

lymphocytes (0.9, 7.3 and 3.3 % of the PBMC at 24, 48 and 72 h pi, respectively for experiment 1 and 0.5, 2.6 and 2.0 % of the PBMC respectively for experiment 2). Infected monocytes were only detected at 48 h pi (0.5 % of the PBMC). None of the infected cells were B-lymphocytes.

Study of the expression of viral antigens on the plasma membrane at 48 h pi, revealed that approximately 8 % of the mitogen-stimulated, EHV-1-inoculated PBMC showed plasma membrane expression of viral antigens.

3.1.5 Discussion

Our results show that in fresh, unstimulated equine PBMC, monocytes are the most important cell fraction in which EHV-1 replicates. Other *Alphaherpesviruses* such as bovine herpesvirus 1 (BoHV-1) (Rouse and Babiuk, 1975; Nyaga and McKercher, 1980), suid herpesvirus 1 (SuHV-1) (Wang *et al.*, 1988) and herpes simplex virus (HSV) (Plaeger-Marshall and Smith, 1978; Mintz *et al.*, 1980) also mainly replicate in monocytes / macrophages. However, for EHV-1, replication in monocytes is clearly restricted. Less than 10 % of the monocytes express viral antigens and only 0.16 % induces plaques in co-culture. Lack of viral antigen expression in more than 90 % of the monocytes indicates that an early block exists in the replication cycle of EHV-1. Moreover, most of the infected monocytes do not produce detectable amounts of EHV-1, which may be explained by a block at another level in the replication cycle. This finding that several blocks exist, is in agreement with results obtained with other *Alphaherpesviruses* in monocytes (Albers *et al.*, 1989; Nauwynck and Pensaert, 1994). Based upon the highest extracellular virus progeny titer ($10^{3.9}$ $TCID_{50}$ per 10^6 inoculated cells) and the percentage of virus producing monocytes (0.16 %), it is estimated that $10^{0.7}$ $TCID_{50}$ virus was formed per virus-producing cell, which demonstrates that even virus-producing monocytes are not fully productive. This is in contrast with SuHV-1 (Nauwynck and Pensaert, 1994).

Our results with regard to the susceptibility of lymphocytes to EHV-1 infection are somewhat different from those obtained *in vivo* by Scott *et al.* (1983). They suggested that EHV-1 was mainly T-lymphotropic in unstimulated PBMC. We found that most of

the unstimulated lymphocytes were refractory to infection. Very low percentages of infected lymphocytes were detected and less than 0.05 % produced infectious virus on co-culture.

EHV-1 showed an increased replication in mitogen-stimulated lymphocytes, which is consistent with the results of Scott *et al.* (1983). Mitogens mimic the initial signals, required to initiate cell proliferation or to induce a state of competence (Terada *et al.*, 1991). The ability of T-lymphocytes to support viral replication following mitogen stimulation has been recognized for other herpesviruses as well (Nyaga and McKercher, 1980; Teute *et al.*, 1983; Wang *et al.*, 1988). HSV has long been known to replicate more efficiently in actively dividing than in growth-arrested cells. Recently, Schang *et al.* (1998) found that olomoucine and roscovitine inhibit HSV replication. Both substances exert an influence on the cell cycle by inhibiting certain cyclin-dependent kinases. The authors suggested that one or more of these kinases, which are active during the cell cycle from the late G1-phase onward, are required for HSV replication. For EHV-1 similar specific cell cycle events may play a role in virus replication.

Activation of T-lymphocytes may be an important pathogenetic feature during an EHV-1 infection. McCulloch *et al.* (1993) demonstrated an increase in the percentage of blastic cells up to 40 % in the blood circulation of horses from 4-8 days and on day 10 after experimental inoculation with EHV-1. This coincides with the viremic phase of an EHV-1 infection. Blastic transformation of lymphocytes, *in vitro* induced by mitogens or *in vivo* induced during an EHV-1 infection, most likely provides a signal for the virus to start its replication. The factor(s) inducing the proliferation of lymphocytes *in vivo* during an EHV-1 infection are not yet examined.

Scott *et al.* (1983) experimentally infected ponies and collected blood samples at different time intervals after infection. Blastic transformation of lymphocytes may have taken place during infection before culturing the cells *in vitro*, which may explain why EHV-1 replication was found in T-lymphocytes even without mitogen stimulation. In our experiments, PBMC were obtained from healthy horses and were infected *in vitro*. It is plausible that almost no blastic transformation occurred before mitogens were added *in vitro*. The very low percentage of infected cells that we detected in the unstimulated

lymphocytes may represent a small fraction of lymphoblasts, present in the blood of healthy horses.

We demonstrated a clear plasma membrane expression of EHV-1 antigens on mitogen-stimulated PBMC. Such an expression makes infected cells recognizable for antibodies. After binding of the antibodies to their respective antigens, anchored in the plasma membrane, cell lysis occurs by the activation of complement or phagocytes. However, EHV-1-induced viremia occurs in the presence of virus-neutralizing antibodies (Doll and Bryans, 1963; Mumford *et al.*, 1987). How EHV-1-infected PBMC escape from elimination by the host's immunity is unknown and will be examined in the future.

Acknowledgements

We thank Chantal Vanmaercke and Chris Bracke for their excellent technical assistance and Prof. Dr. M.T. Ysebaert for her help in the statistical analysis of the results. We also thank Dr. M. Yeargan of the Gluck Equine Research Centre for supplying the specific monoclonal antibody 13B2 to identify the Belgian EHV-strain.

References

Albers, I., Kirchner, H., Domke-Opitz, I. (1989). Resistance of human blood monocytes to infection with herpes simplex virus. *Virology* **169**, 466-469.

Darzynkiewicz, Z., Williamson, B., Carswell, E. A., Old, L. J. (1984). Cell cycle-specific effects of tumor necrosis factor. *Cancer Research* **44**, 83-90.

Doll, E. R., Bryans, J. T. (1963). Immunisation of young horses against viral rhinopneumonitis. *Cornell Veterinarian* **53**, 24-41.

Dutta, S. K., Myrup, A. C. (1983). Infectious center assay of intracellular virus and infective virus titer for equine mononuclear cells infected *in vivo* and *in vitro* with equine herpesvirus. *Canadian Journal of Comparative Medicine* **47**, 64-69.

Gibson, J. S., Slater, J. D., Awan, A. R., Field, H. J. (1992). Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. *Archives of Virology* **123**, 351-366.

McCulloch, J., Williamson, S. A., Powis, S. J., Edington, N. (1993). The effect of EHV-1 infection upon circulating leucocyte populations in the natural equine host. *Veterinary microbiology* **37**, 147-161.

Mettenleiter, T. C. (1989). Glycoprotein gIII deletion mutants of pseudorabies virus are impaired in virus entry. *Virology* **171**, 623-625.

Mintz, L., Drew, W. L., Hoo, R., Finley, T. N. (1980). Age-dependent resistance of human alveolar macrophages to herpes simplex virus. *Infection and Immunity* **28**, 417-420.

Mumford, J. A., Rossdale, P. D., Jesset, D. M., Gann, S., Ousey, J., Cook, R. F. (1987). Serological and virological investigations of an equid herpesvirus 1 (EHV_1) abortion storm on a stud farm in 1985. *Journal of Reproduction and Fertility Supplement* **35**, 509-518.

Nauwynck, H. J., Pensaert, M. B. (1994). Virus production and viral antigen expression in porcine blood monocytes inoculated with pseudorabies virus. *Archives of Virology* **137**, 69-79.

Nyaga, P. N., McKercher, D. G. (1980). Pathogenesis of bovine herpesvirus-1 (BHV_1) infections: interactions of the virus with peripheral bovine blood cellular components. *Comparative Immunobiology, Microbiology and Infectious Diseases* **2**, 587-602.

Plaeger-Marshall, S., Smith, J. W. (1978). Inhibition of mitogen- and antigen-induced lymphocyte blastogenesis by herpes simplex virus. *Journal of Infectious Diseases* **138**, 506-511.

Rouse, B. T., Babiuk, L. A. (1975). Host defence mechanisms against infectious bovine rhinotracheitis virus II. Inhibition of viral plaque formation by immune peripheral blood lymphocytes. *Cellular Immunology* **17**, 43-56.

Schang, L. M., Phillips, J., Schaffer, P. A. (1998). Requirement for cellular cyclin-dependent kinases in herpes simplex virus replication and transcription. *Journal of Virology* **72**, 5626-5637.

Scott, J. C., Dutta, S. K., Myrup, A. C. (1983). *In vivo* harbouring of equine herpesvirus-1 in leukocyte populations and subpopulations and their quantitation from experimentally infected ponies. *American Journal of Veterinary Research* **44**, 1344-1348.

Terada, N., Lucas, J. J., Gelfand, E. W. (1991). Differential regulation of the tumor suppressor molecules, retinoblastoma susceptibility gene product (Rb) and p53, during cell cycle progression of normal human T-cells. *Journal of Immunology* **147**, 698-704.

Teute, H., Braun, R., Kirchner, H., Becker, H., Munk, K. (1983). Replication of herpes simplex virus in human T lymphocytes. *Intervirology* **20**, 32-41.

Tumas, D. B., Brassfield, A. L., Travenor, A. S., Hines, M. T., Davis, W. C., McGuire, T. C. (1994). Monoclonal antibodies to the equine CD2 T lymphocyte marker, to a pan-granulocyte/monocyte marker and to a unique pan-B lymphocyte marker. *Immunobiology* **192**, 48-64.

Wang, F., Pang, V. F., Hahn, E. C. (1988). Flow cytometric analysis of porcine peripheral blood leukocytes infected with Aujeszky's disease virus. *Journal of Leukocyte Biology* **43**, 256-264.

Yeargan, M. R., Allen, G., Bryans, J. T. (1985). Rapid subtyping of equine herpesvirus 1 with monoclonal antibodies. *Journal of Clinical Microbiology* **21**, 694-697.

3.2 MITOGEN STIMULATION FAVOURS REPLICATION OF EQUINE HERPESVIRUS 1 IN EQUINE BLOOD MONONUCLEAR CELLS BY INDUCING CELL PROLIFERATION AND FORMATION OF CLOSE INTERCELLULAR CONTACTS

Journal of General Virology (2001), 82, 1951-1957

van der Meulen, K., Nauwynck, H. and Pensaert, M.

3.2.1 Summary

In the present study, equine herpesvirus 1 (EHV-1)-infected cells were identified in ionomycin/phorbol dibutyrate (IONO/PDB)-stimulated peripheral blood mononuclear cells (PBMC) and the mechanism by which stimulation increases the percentage of infected cells was examined. In the population of viral antigen-positive PBMC, 38.4 ± 4.5 % were $CD5^+$ T-lymphocytes (18.1 ± 3.2 % $CD4^+$; 13.6 ± 1.8 % $CD8^+$), 18.1 ± 5.4 % were B-lymphocytes, 8.5 ± 3.9 % were monocytes and 35 % remained unidentified. The role of the cell cycle in the increased susceptibility to EHV-1 upon stimulation was examined by stimulating PBMC during 0, 12, 24 and 36 hours prior to inoculation. A high correlation was found between the increase of cells in the S- ($r = 0.974$) and G2/M-phase ($r = 0.927$) at the moment of inoculation and the increase of infected cells at 12 hours post inoculation (h pi). This suggests that a specific stage of the S-phase or S- and G2/M-phase facilitates viral replication. At 24 h pi, lower correlations were found, suggesting that other effects are involved. From 12 hours after addition of IONO/PDB, formation of clusters of PBMC became manifest. It was examined if close intercellular contacts in these clusters facilitated cell-to-cell transmission of EHV-1. Between 8 and 17 h pi, the percentage of clusters containing adjacent infected cells increased from 1.6 to 13.4 % and the maximal number of adjacent infected cells increased from 2 to 4. Confocal microscopy visualized close intercellular contacts between adjacent infected cells. It can be concluded that mitogen stimulation favours EHV-1 infection of PBMC (i) by initiating specific cell cycle events and (ii) by inducing formation of clusters, thereby facilitating transmission of virus between cells.

3.2.2 Introduction

Equine herpesvirus 1 (EHV-1), a member of the *Alphaherpesvirinae*, is an important pathogen of horses causing abortion, neonatal foal death, nervous system disorders and, less frequently, respiratory disorders. An extensive cell-associated viremia during EHV-1 infection enables the virus to reach internal organs, even in the presence of virus-neutralizing antibodies (Doll and Bryans, 1963; Mumford *et al.*, 1987). During viremia, the virus is carried by peripheral blood mononuclear cells (PBMC), mainly by lymphocytes and to a lesser extent by monocytes, as described by Scott *et al.* (1983).

Somewhat different from these results obtained *in vivo* by Scott *et al.* (1983), we found during *in vitro* studies that the majority of the EHV-1-infected equine PBMC are monocytes, whereas most of the lymphocytes are refractory to infection (van der Meulen *et al.*, 2000). However, mitogen stimulation of PBMC prior to EHV-1 infection increases the percentage of infected T-lymphocytes (van der Meulen *et al.*, 2000). The identity of the EHV-1-infected subtypes of mitogen-stimulated T-lymphocytes has not been determined so far.

The finding that mitogen stimulation increases the number of infected T-lymphocytes led to the hypothesis that EHV-1 replication in T-lymphocytes might benefit from a specific phase of the cell cycle, induced by mitogen stimulation. Cell cycle-dependent replication has already been demonstrated for several herpesviruses. For example, herpes simplex virus (HSV) needs one or more cyclin-dependent kinases active from late G1 onward and required for cellular progression into the S-phase, for the accumulation of HSV transcripts, viral DNA replication and production of infectious virus (Schang *et al.*, 1998; Schang *et al.*, 1999; Schang *et al.*, 2000). Cyclin-dependent kinases are also required for the replication of human cytomegalovirus (HCMV) (Bresnahan *et al.*, 1997). An effect of the cell cycle on the replication of EHV has already been described by Lawrence (1971) in a human carcinoma cell line. He showed that synthesis of EHV DNA takes place, only when the cells are in or just entering the S-phase of the cell cycle. McCulloch *et al.* (1993) demonstrated an increase in the percentage of blastic cells of up to 40 % in the blood circulation of experimentally inoculated horses during a time period coinciding with the viremic phase of an EHV-1

infection. Cell cycle-dependent replication of EHV-1 may be an important pathogenic feature during an infection *in vivo*.

The main purpose of this study is to identify the EHV-1-infected subtype(s) of mitogen-stimulated PBMC *in vitro* and to obtain better insights in the mechanism(s) by which mitogen stimulation affects the course of an EHV-1 infection.

3.2.3 Materials and methods

PBMC

Blood samples were obtained from infection-immune horses by jugular venipuncture and collected on heparin (Leo, Zaventem, Belgium). PBMC were then isolated by density centrifugation on Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). Cells were washed three times with phosphate-buffered saline and resuspended in medium (RPMI 1640, 10 % fetal bovine serum, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 0.3 mg/ml glutamin, 1 % non-essential amino-acids 100x (GibcoBRL), 1 mM sodium pyruvate).

Virus and in vitro infection

The Belgian EHV-1 strain 97P70 was used throughout all experiments (van der Meulen *et al.*, 2000). Virus used for inoculation was at the 5th passage on equine embryonic lung cells and 1 subsequent passage on rabbit kidney (RK13) cells.

PBMC were infected at a multiplicity of infection of 10. After 1 hour of incubation at 37 °C, the cells were washed thoroughly with RPMI and cultured in medium supplemented with 10 U/ml heparin (Leo, Zaventem, Belgium) and with or without addition of mitogens.

Mitogen stimulation

In a previous study, we have demonstrated that the highest number of EHV-1 antigen-positive PBMC was obtained after stimulation with a combination of the phorbol ester, phorbol 12,13-dibutyrate (PDB), and the calcium ionophore, ionomycin (IONO) (van der Meulen *et al.*, 2000). Therefore, IONO and PDB were used throughout the

experiments in the current study. PBMC were stimulated with 0.5 µM of IONO and 10 nM of PDB (Sigma, Bornem, Belgium) in medium as decribed earlier. Mitogen stimulation was performed before or during EHV-1 infection, dependent on the experiment.

Detection of viral antigen-positive PBMC

The percentage of EHV-1 antigen-positive cells was determined by indirect immunofluorescence staining on acetone-fixed cell smears. Cells were incubated during 1 hour at 37 °C, first with mouse polyclonal antibodies against EHV-1 and, after 3 washing steps in PBS, with goat anti-mouse antibodies labeled with fluoresceine isothyocyanate (FITC). Samples were analyzed with a Leica DM BRE fluorescence microscope (Leica Microsystems Holdings GmbH, Wetzlar, Germany). At least 200 cells were counted for each sample.

Cell cycle analysis

Cells were fixed in ice-cold ethanol (70 %) during 30 minutes, washed with phosphate-buffered saline and stained during 30 minutes at room temperature with a solution containing 10 µg/ml propidium iodide, 0.1 % Triton X-100, 2 mM $MgCl_2$, 0.1 M NaCl, 10 mM Pipes buffer and 20 units/ml RNAse A in distilled water. The cells were analyzed by flow cytometry using a Becton-Dickinson FACSCalibur and BD Cellquest software (Becton-Dickinson, San Jose, CA, USA). The following parameters were stored for further analysis: FSC, SSC, FL2-W and FL2-A. Gates were placed, based on FSC and SSC profiles, to include viable cells only. At least 5000 cells were analyzed for each sample.

Identification of EHV-1-infected, mitogen-stimulated PBMC

A double immunofluorescence staining was performed in 2 steps. First, the cells were fixed in paraformaldehyde (3 %), washed with phosphate-buffered saline and incubated during 1 hour with one of the monoclonal antibodies HT23A (anti-equine CD5), HB61A (anti-equine CD4), 73/6.9.1. (anti-equine CD8) or DH59B (equine monocyte marker) (VMRD, Pullman, WA, USA). Then, an FITC-conjugated goat anti-

mouse antibody was added for 1 hour. To stain B-lymphocytes, an FITC-labeled goat anti-horse IgM antibody (Kirkegaard and Perry Laboratories Inc., Gaithersburg, U.S.) was used. After the first step, cells were washed in phosphate-buffered saline and permeabilized with saponin (0.1 %). Then, the cells were incubated during 1 hour with protein G-purified and biotinylated horse antibodies raised against EHV-1 and finally with phycoerythrin-labeled streptavidin (Molecular Probes, Eugene, Oregon, USA). An irrelevant monoclonal antibody 41D3 against a putative receptor for a porcine arterivirus (Duan *et al.*, 1998) as well as a non-infected sample were included as controls.

Staining was performed either in suspension or within the recipient of cultivation. For cells in suspension, all manipulations were performed at 4 °C. Samples were analyzed by flow cytometry. Four parameters were stored for further analysis: FSC, SSC, FL1 and FL2. Gates were placed, based on FSC and SSC profiles, to include viable cells only. At least 10000 cells were analyzed for each sample. Within the recipient of cultivation, all manipulations during the staining were performed at room temperature. Cells were analyzed within their recipient with a Leica DM IL inverted fluorescence microscope (Leica Microsystems Holdings GmbH, Wetzlar, Germany). At least 100 cells or cell groups were counted for each sample. Additionally, cells were analyzed with a Bio-Rad Radiance 2000 confocal laser scanning system (Bio-Rad House, Hertfordshire, UK) linked to a Nikon Diaphot 300 microscope (Nikon Corporation, Tokyo, Japan).

3.2.4 Results

Identification of EHV-1-infected, IONO/PDB-stimulated PBMC by flow cytometry

PBMC were simultaneously stimulated and infected. Cells were collected at 48 hours post inoculation (h pi) and a double immunofluorescence staining was performed. The percentage of the different cell types within the total population of PBMC and within the population of EHV-1 antigen-positive PBMC are shown in Table 1.

The total population of PBMC consisted mainly of $CD5^+$ T-lymphocytes (including $CD4^+$ and $CD8^+$ T-lymphocytes), followed by B-lymphocytes. Monocytes formed the smallest fraction of the PBMC. With the markers used in this experiment, 36.9 % of the PBMC could not be identified.

Most of the EHV-1 antigen-positive cells were $CD5^+$ T-lymphocytes, followed by B-lymphocytes. The minority of the EHV-1 antigen-positive cells were monocytes. Thirty-five percent of the infected cells could not be identified. The composition of EHV-1-infected PBMC reflected well the composition of the total population of PBMC.

Table 1. Identification of the EHV-1-infected cells in IONO/PDB-stimulated PBMC*

	Percentage of cells identified as …					
	$CD5^+$ T-lymphocytes			B-lymphocytes	Monocytes	Unidentified†
Cell population	total	$CD4^+$	$CD8^+$			
Total population of PBMC	46.2 ± 5.8	27.6 ± 3.6	10.6 ± 2.1	12.8 ± 2.2	4.1 ± 2.0	36.9
EHV-1-infected PBMC	38.4 ± 4.5	18.1 ± 3.2	13.6 ± 1.8	18.1 ± 5.4	8.5 ± 3.9	35

*: PBMC were simultaneously stimulated and infected, cells were collected at 48 h pi
†: 100 - (% $CD5^+$ T-lymphocytes + % B-lymphocytes + % monocytes)
All data are expressed as the mean value of at least three experiments ± s.d.

Replication of EHV-1 in PBMC stimulated with IONO/PDB during different time periods prior to inoculation

PBMC were stimulated for 0, 12, 24 or 36 hours and subsequently infected. Stimulation was continued after inoculation. Non-stimulated PBMC were included as a control. Cells were collected at 0, 12 and 24 h pi. Table 2 shows the distribution of cells in the different stages of the cell cycle (G0/G1, S, G2/M) at the time point of inoculation with EHV-1 and the percentage of EHV-1 antigen-positive cells at different time points after inoculation for PBMC of three different infection-immune horses.

Based on the results of the 5 experiments, the mean percentage of PBMC in the G0/G1-phase of the cell cycle decreased from 98.2 ± 1.0 % before stimulation to 93.3 ± 1.3 % after 36 hours of stimulation. The mean percentage of PBMC in the S- and G2/M-phase increased from 1.5 ± 1.2 % and 0.4 ± 0.1 %, respectively before stimulation to 5.7 ± 0.8 % and 1.1 ± 0.5 %, respectively after 36 hours of stimulation.

Table 2. Replication of EHV-1 in PBMC stimulated with IONO/PDB during different time periods prior to inoculation

Horse number	Stimulation with IONO/PDB		Cell cycle distribution at EHV-1 inoculation (%)			EHV-1 antigen-positive cells at … h pi (%)		
	prior to EHV-1 inoculation (hours)	after EHV-1 inoculation	G0/G1	S	G2/M	0	12	24
1*	No (0)	No	98.2 ± 1.4	1.6 ± 1.6	0.3 ± 0	0	2.5 ± 2.8	1.4 ± 1.5
	No (0)	Yes	98.2 ± 1.4	1.6 ± 1.6	0.3 ± 0	0	3.8 ± 3.1	3.5 ± 3.2
	Yes (12)	Yes	96.9 ± 1.0	2.5 ± 0.5	0.7 ± 0.4	0	5.4 ± 3.9	10.9 ± 7.6
	Yes (24)	Yes	96.5 ± 0.4	2.9 ± 0.2	0.7 ± 0.2	0	5.3 ± 3.9	10.9 ± 5.9
	Yes (36)	Yes	93.1 ± 1.6	5.9 ± 1.1	1.0 ± 0.6	0	6.5 ± 3.3	9.7 ± 4.6
2†	No (0)	No	99.0	0.5	0.5	0	2.0	2.1
	No (0)	Yes	99.0	0.5	0.5	0	1.3	3.2
	Yes (12)	Yes	98.2	1.6	0.3	0	4.8	6.0
	Yes (24)	Yes	96.4	3.1	0.5	0	3.0	9.5
	Yes (36)	Yes	94.2	5.0	0.9	0	2.8	9.2
3†	No (0)	No	97.4	2.3	0.4	0	0.6	0.5
	No (0)	Yes	97.4	2.3	0.4	0	0.6	0.7
	Yes (12)	Yes	95.3	3.0	1.7	0	0.6	1.8
	Yes (24)	Yes	95.7	3.0	1.3	0	1.8	4.0
	Yes (36)	Yes	92.7	5.8	1.5	0	1.4	3.9

*: mean value of three experiments ± s.d.
†: value of a single experiment

Due to a large biological variation observed between the individual experiments, significant differences were not found between the absolute mean percentages of EHV-1 antigen-positive cells in the 0, 12, 24 or 36 hours-prestimulated samples and the non-stimulated control at 12 h pi. However, when relative values were calculated based on the data of the individual experiments and with the non-stimulated control as a reference value of 1, a significant difference was observed between the 36 hours-prestimulated PBMC and the control (relative values were 0.9 ± 0.2, 1.9 ± 1.0, 2.3 ± 0.7 and 3.1 ± 2.3 for the 0, 12, 24 and 36 hours-prestimulated samples, respectively). At 24 h pi, a significant difference was observed between the absolute percentages of viral antigen-positive cells in the 12, 24 and 36 hours-prestimulated samples and the non-stimulated control. For the relative values, a significant difference was found between the 24 and 36 hours-prestimulated samples on the one hand and the non-stimulated control on the other hand (relative values were 1.6 ± 0.5, 5.7 ± 2.9, 9.1 ± 5.3 and 9.7 ± 7.8 for the 0, 12, 24 and 36 hours-prestimulated samples, respectively). Statistical analysis was based on the *least significant difference (LSD, rejection level 0.050).*

A correlation coefficient r *(Pearson's correlation)* between the increase in the percentage of cells in the S- or G2/M-phase at the moment of inoculation with EHV-1 and the increase in the percentage of EHV-1 antigen-positive cells was calculated for 0, 12, 24 and 36 hours-prestimulated PBMC. The relative values were used for both the percentage of viral antigen-positive cells and the percentage of cells in the S- and G2/M-phase. The highest correlation was observed between the relative value of cells in the S-phase at the moment of inoculation and the relative value of EHV-1 antigen-positive cells at 12 h pi (r = 0.974). A lower correlation was found between the relative value of cells in the G2/M-phase and the EHV-1 antigen-positive cells at 12 h pi (r = 0.927), between the relative value of cells in the S-phase and the EHV-1 antigen-positive cells at 24 h pi (r = 0.909) and, finally, between the relative value of cells in the G2/M-phase and the EHV-1 antigen-positive cells at 24 h pi (r = 0.865). However, all calculated correlations were significant *(t-test, rejection level 0.050)*.

Formation of clusters of PBMC by stimulation with IONO/PDB

When PBMC were stimulated with IONO/PDB, intercellular contacts were initiated which resulted in the formation of aggregates or clusters of PBMC, consisting of at least 2 to more than 50 cells (Figure 3.2.1a, page 110). Clusters became manifest between 12 and 24 hours after addition of IONO/PDB and remained clearly visible till at least 60 hours after IONO/PDB addition (end of the experiment).

EHV-1 replication in clusters of IONO/PDB-stimulated PBMC

PBMC were stimulated during 24 hours and infected afterwards. At 8, 17 and 24 h pi, a double immunofluorescence staining was carried out. The results are shown in Table 3. At 8 h pi, most of the infected clusters contained 1 viral antigen-positive cell (Table 3; Figure 3.2.1b, page 110). Between 8 and 17 h pi, a 2.2-fold increase in the percentage of clusters with infected cells was seen and the number of adjacent infected cells per cluster increased from a maximum of 2 to a maximum of 4 (Table 3; Figure 3.2.1c and d, page 110). The percentages obtained at 24 h pi were similar to those obtained at 17 h pi.

Table 3. EHV-1 replication within clusters of IONO/PDB-stimulated PBMC*

Hours post inoculation	Percentage of clusters of PBMC with … adjacent infected cells				
	0	1	2	3	4
8	83.3 ± 2.7	15.1 ± 3.5	1.6 ± 1.1	0	0
17	62.6 ± 9.5	24.1 ± 4.5	7.9 ± 1.8	3.0 ± 2.7	2.5 ± 1.3
24	60.6 ± 5.2	25.3 ± 4.3	9.9 ± 1.9	3.6 ± 0.6	0.6 ± 0.9

*: PBMC were stimulated during 24 hours prior to EHV-1 inoculation
All data are expressed as the mean value of 5 experiments ± s.d.

By using confocal laser scanning microscopy, the presence of close intercellular contacts between adjacent infected cells was visualized in more detail, as shown in Figure 3.2.2a and b (page 110).

Between 8 and 17 h pi, an increase was seen in the percentage of clusters containing 1 infected cell (Table 3). Identification with DH59B, an equine monocyte marker, revealed that this increase was caused by an increase in the percentage of clusters containing 1 infected cell, not belonging to the monocytes (from 10.5 ± 4.1 % at 8 h pi to 19.3 ± 4.0 % at 17 h pi). The percentage of clusters containing 1 infected monocyte, on the other hand, remained at a constant level (4.6 ± 1.3 % at 8 h pi and 4.9 ± 1.0 % at 17 h pi). The percentages obtained at 24 h pi were similar to those obtained at 17 h pi.

A similar pattern was seen in the population of cells that remained individually in the recipient without any visual contact to other cells. In the population of cells, not belonging to the monocytes, an increase was seen in the percentage of viral antigen-positive cells between 8 (2.2 ± 1.1 %) and 24 h pi (6.9 ± 6.0 %), whereas a constant number of monocytes was infected during the entire course of the EHV-1 infection (10.0 ± 4.0, 11.0 ± 2.1 and 11.1 ± 3.4 % at 8, 17 and 24 h pi, respectively).

In clusters with 2 or more infected cells in contact, the infected cell groups consisted of either monocytes and non-monocytes or non-monocytes only (maximum 6.8 ± 4.0 and

7.6 ± 3.6 % of all the clusters, respectively). Clusters consisting of 2 or more infected monocytes formed 1.3 ± 1.0 % of all the clusters.

3.2.5 Discussion

The present study shows that stimulation of equine PBMC with IONO/PDB affects an EHV-1 infection in 2 ways. First, stimulation initiates proliferation events in lymphocytes, which enable EHV-1 to replicate in a limited number of cells. Second, stimulation induces the formation of aggregates or clusters of PBMC. The close interactions between the PBMC within these clusters facilitate the transmission of EHV-1 from an infected cell to an uninfected adjacent cell.

EHV-1 infection was studied in a population of PBMC stimulated with IONO/PDB during different time periods prior to virus inoculation. PBMC were isolated from infection-immune, seropositive horses which may be assumed to be latently infected. It has been described that latent EHV-1 can be reactivated *in vitro* from leukocytes by the mitogens phytohaemagglutinin and pokeweed mitogen, as detected by co-cultivation and immunofluorescence (Smith *et al.*, 1998). Since IONO/PDB also acts as a mitogen, it is reasonable to suggest, that these products induce reactivation of EHV-1 in the leukocytes of the three infection-immune horses, thereby confounding the results of the present study. However, to exclude an influence of reactivation of latenly infected leukocytes on the percentage of viral antigen-positive cells, we always determined the percentage of EHV-1 antigen-positive cells in uninoculated PBMC after 0, 12, 24 and 36 of stimulation (0 h pi). No viral antigen-positive cells were detected (Table 2). Therefore, we may conclude that stimulation with IONO/PDB did not cause a reactivation of EHV-1 and did not affect the percentage of EHV-1 antigen-positive cells.

Based on the relative values of viral antigen-positive cells at 12 h pi, a significant difference was observed between the 36 hours-prestimulated PBMC and the non-stimulated control. This suggests that stimulation with IONO/PDB induces one or more specific events that enable EHV-1 to replicate in a limited number of cells. Viral transmission from cell-to-cell, as will be discussed later on, cannot account for the increase, since groups consisting of 2 or more adjacent viral antigen-positive cells

occurred mainly from 14 h pi onwards (data not shown). Moreover, when EHV-1 replication was studied at 8 and 17 h pi in prestimulated PBMC, a 2-fold increase in the percentage of clusters containing 1 infected lymphocyte (from 10.5 % at 8 h pi to 19.3 % at 17 h pi) as well as in the percentage of individually located, infected lymphocytes (from 2.2 % at 8 h pi to 4.4 % at 17 h pi) was observed. Since there is no direct interaction with other infected cells, these observations support the idea that the increase in the percentage of infected cells is not caused by cell-to-cell transmission of the virus, but by another effect induced by the mitogens, for example one or more cell cycle-dependent events.

To determine whether the effect, induced by IONO/PDB, was related to the S-phase or the G2/M-phase of the cell cycle, the correlation coefficient (r) was calculated between the relative value of cells in the S- or G2/M-phase of the cell cycle at the moment of inoculation and the relative value of EHV-1 antigen-positive cells. A very high correlation ($r = 0.974$) was observed between the relative value of cells in the S-phase and the relative value of EHV-1 antigen-positive cells at 12 h pi. This means that an increase in the percentage of cells in the S-phase after a certain time interval of prestimulation is accompanied by a similar increase in the percentage of EHV-1 antigen-positive cells at 12 h pi. Therefore, we assume that viral replication is facilitated during a specific stage of the S-phase of the cell cycle. This is consistent with a previous report by Lawrence (1971), who found evidence for a relationship between EHV DNA-synthesis and the S-phase of the cell cycle in KB cells, a human carcinoma cell line. Since most cells entering the S-phase will continue into the G2/M-phase, a significant, but lower, correlation was found between the relative value of cells in the G2/M-phase and the relative value of EHV-1 antigen-positive cells at 12 h pi. At 24 h pi, there was still a significant correlation between the different phases of the cell cycle and the relative percentage of EHV-1 antigen-positive cells. However, the correlation coefficients were clearly lower than at 12 h pi, suggesting that also other effects are involved at this time point after inoculation.

We hypothesized that the close intercellular interactions formed within the clusters of IONO/PDB-stimulated equine PBMC favoured the transmission of EHV-1 from an infected to an uninfected adjacent cell. Our results show that EHV-1 was indeed

transferred from infected cells to adjacent cells. At 8 h pi, only 1.6 % of the clusters contained a group of adjacent infected cells, whereas this percentage significantly increased to 13.4 % at 17 h pi. Moreover, the number of adjacent infected cells per cluster increased from a maximum of 2 at 8 h pi to a maximum of 4 at 17 and 24 h pi. With confocal laser scanning microscopy the existence of close intercellular contacts between adjacent infected cells was confirmed. The exact mechanism by which EHV-1 is transmitted from one cell to another within the clusters of PBMC remains to be determined. Since addition of virus-neutralizing antibodies in the medium during infection did not alter the percentage of infected clusters or the number of adjacent infected cells per cluster (data not shown), we suggest that a direct cell-associated spread of the virus is involved. Since cluster formation seems to be required for transmission and since cluster formation depends on discrete interactions between cell adhesion molecules (reviewed by Hogg and Landis), it seems likely that EHV-1 transmission relies on the close contacts initiated by cell adhesion molecules, as described for human immunodeficiency virus (Fais *et al.*, 1995; Tsunetsugu-Yokota *et al.*, 1997).

The results of the present study concerning the subfractions of lymphocytes that were scored as infected differed from those obtained by Scott *et al.* (1983). However, it is important to mention that the study of Scott *et al.* was performed *in vivo*. They experimentally infected ponies to detect and quantitate the *in vivo* presence of the virus in leukocytes. Cultivation and identification were performed on cells obtained from the infected ponies. In the present study, the interaction between leukocytes and EHV-1 was studied *in vitro*. Leukocytes were isolated from healthy horses and, afterwards, stimulated and infected *in vitro*. Furthermore, Scott *et al.* used infectious centre assays and plaque assays to detect the infected cells and, therefore, their results represent the “productively infected” leukocytes. In our experiments, an immunofluorescence staining was used and, therefore, the results represent the “viral antigen-positive” leukocytes. In our previous report, we demonstrated that only 13.7 % of the viral antigen-positive monocytes and 5.3 % of the viral antigen-positive lymphocytes were productively infected. This means that the number of viral antigen-positive cells will be quite different from the number of productively infected cells. The above-mentioned differences may be the cause of the disparity between the study of Scott *et al.* and the present study.

Remarkably, one-third of the PBMC could not be identified by the markers used in our experiments. This finding cannot be due to an effect of infection, since the same observation was made in non-infected cells (30.1 ± 5.7 % unidentified). Modulation of the expression of cell surface molecules in response to phorbol esters has been described in horses by Zhang *et al.* (1994). Since they found that phorbol-12-myristate-13-acetate (PMA)-mediated down-regulation of CD4 expression was reversible and the percentage of $CD4^+$ molecules was returned to control levels after 48 hours (Zhang *et al.*, 1994), it seems unlikely that the PMA stimulation is responsible for the loss of expression of leukocyte markers on PBMC in this study. Whether a synergistic effect of PDB and IONO, as described in mice by Anderson and Coleclough (1993), causes a more extensive and long-lasting down-regulation of surface molecules in equine T-lymphocytes than PDB alone has not been determined as it remained outside the scope of this study.

We can conclude from this study that mitogen stimulation positively influences an EHV-1 infection in 2 ways: (i) by initiating specific cell cycle events and (ii) by inducing the formation of clusters of PBMC, thereby facilitating transmission of EHV-1 from cell to cell.

Acknowledgements

We thank Chantal Vanmaercke and Chris Bracke for their excellent technical assistance, Professor Dr. M. T. Ysebaert for helping with the statistical analysis of the results and Dr. H. Favoreel, Dr. K. Van Reeth and Dr. N. Vanderheijden for critically reading the manuscript. We also thank Prof. Dr. P. Van Oostveldt for the confocal microscopy.

References

Anderson, S. J., Coleclough, C. (1993). Regulation of CD4 and CD8 expression on mouse T cells: active removal from the cell surface by 2 mechanisms. *Journal of Immunology* **151**, 5123-5134.

Bresnahan, W. A., Boldogh, I., Chi, P., Thompson, E. A., Albrecht, T. (1997). Inhibition of cellular Cdk2 activity blocks human cytomegalovirus replication. *Virology* **231**, 239-247.

Doll, E. R., Bryans, J. T. (1963). Immunisation of young horses against viral rhinopneumonitis. *Cornell Veterinarian* **53**, 24-41.

Duan, X., Nauwynck, H. J., Favoreel, H. W., Pensaert, M. B. (1998). Porcine reproductive and respiratory syndrome virus infection of alveolar macrophages can be blocked by monoclonal antibodies against cell surface antigens. *Advances in Experimental Medicine and Biology* **440**, 461-467.

Fais, S., Capobianchi, M. R., Abbate, I., Castilletti, C., Gentile, M., Cordiali Fei, P., Ameglio, F., Dianzani, F. (1995). Unidirectional budding of HIV-1 at the site of cell-to-cell contact is associated with co-polarization of intercellular adhesion molecules and HIV-1 viral matrix protein. *AIDS* **9**, 329-335.

Hogg, N., Landis, R. C. (1993). Adhesion molecules in cell interactions. *Current Opinions in Immunology* **5**, 383-390.

Lawrence, W. C. (1971). Evidence for a relationship between equine abortion (herpes) virus deoxyribonucleic acid synthesis and the S-phase of the KB cell mitotic cycle. *Journal of Virology* **7**, 736-748.

McCulloch, J., Williamson, B., Powis, S. J., Edington, N. (1993). The effect of EHV_1 infection upon circulating leucocyte populations in the natural equine host. *Veterinary Microbiology* **37**, 147-161.

Mumford, J. A., Rossdale, P. D., Jesset, D. M., Gann, S., Ousey, J., Cook, R. F. (1987). Serological and virological investigations of an equid herpesvirus 1 (EHV_1) abortion storm on a stud farm in 1985. *Journal of Reproduction and Fertility Supplement* **35**, 5091-518.

Schang, L. M., Phillips, J., Schaffer, P. A. (1998). Requirement for cellular cyclin-dependent kinases in herpes simplex virus replication and transcription. *Journal of Virology* **72**, 5626-5637.

Schang, L. M., Rosenberg, A., Schaffer, P. A. (1999). Transcription of herpes simplex virus immediate-early and early genes is inhibited by roscovitine, an inhibitor specific for cellular cyclin-dependent kinases. *Journal of Virology* **73**, 2161-2172.

Schang, L. M., Rosenberg, A., Schaffer, P. A. (2000). Roscovitine, a specific inhibitor of cellular cyclin-dependent kinases, inhibits herpes simplex virus DNA synthesis in the presence of viral early proteins. *Journal of Virology* **74**, 2107-2120.

Scott, J. C., Dutta, S. K., Myrup, A. C. (1983). *In vivo* harboring of equine herpesvirus-1 in leukocyte populations and subpopulations and their quantitation from experimentally infected ponies. *American Journal of Veterinary Research* **44**, 1344-1348.

Smith, D. J., Iqbal, J., Purewal, A., Hamblin, A. S., Edington, N. (1998). *In vitro* reactivation of latent equid herpesvirus-1 from CD5 / CD8 leukocytes indirectly by IL-2 or chorionic gonadotrophin. *Journal of General Virology* **79**, 2997-3004.

Tsunetsugu-Yokota, Y., Yasuda, S., Sugimoto, A., Yagi, T., Azuma, M., Yagita, H., Akagawa, K., Takemori, T. (1997). Efficient virus transmission from dendritic cells to CD4+ T cells in response to antigen depends on close contact through adhesion molecules. *Virology* **239**, 259-268.

van der Meulen, K. M., Nauwynck, H. J., Buddaert, W., Pensaert, M. B. (2000). Replication of equine herpesvirus type 1 in freshly isolated equine peripheral blood mononuclear cells and changes in susceptibility following mitogen stimulation. *Journal of General Virology* **81**, 21-25.

Zhang, C. H., Grünig, G., Davis, W., Antczak, D. F. (1994). Down-regulation followed by re-expression of equine CD4 molecules in response to phorbol myristate acetate. *Veterinary Immunology and Immunopathology* **42**, 71-82.

4. ABSENCE OF VIRAL ANTIGENS ON THE SURFACE OF EQUINE HERPESVIRUS 1-INFECTED BLOOD MONONUCLEAR CELLS *IN VITRO* AND *IN VIVO* AS A STRATEGY OF IMMUNE ESCAPE

4.1 ABSENCE OF VIRAL ANTIGENS ON THE SURFACE OF EQUINE HERPESVIRUS 1-INFECTED BLOOD MONONUCLEAR CELLS: A STRATEGY TO AVOID COMPLEMENT-MEDIATED LYSIS

4.2 EXPRESSION OF VIRAL ANTIGENS AND MHC CLASS I ON THE SURFACE OF EQUINE HERPESVIRUS 1-INFECTED BLOOD MONONUCLEAR CELLS DURING VIREMIA IN IMMUNE PONIES

4.1 ABSENCE OF VIRAL ANTIGENS ON THE SURFACE OF EQUINE HERPESVIRUS 1-INFECTED BLOOD MONONUCLEAR CELLS: A STRATEGY TO AVOID COMPLEMENT-MEDIATED LYSIS

Journal of General Virology (2003), 84, 93-97

van der Meulen, K., Nauwynck, H., and Pensaert, M.

4.1.1 Summary

Equine herpesvirus 1 (EHV-1) may cause abortion in vaccination- and infection-immune horses. EHV-1-infected peripheral blood mononuclear cells (PBMC) play an important role in the escape of the virus from immunity. We examined how infected PBMC can avoid destruction by EHV-1-specific antibody and equine complement. The majority of EHV-1-infected PBMC (68.6 %) lacked surface expression of viral antigens and these cells were not susceptible to complement-mediated lysis. In infected PBMC with surface expression of viral antigens, 63 % showed focal and 37 % showed general surface expression. General surface expression rendered infected PBMC susceptible to lysis by antibody and complement (from 5.4 to 31.2 % lysed cells depending on antibody and complement concentration). Infected PBMC with focal surface expression showed significant lysis only in the presence of high antibody and complement concentrations. Thus, absence of surface expression protects infected PBMC against complement-mediated lysis.

4.1.2 Introduction

Equine herpesvirus 1 (EHV-1), an *Alphaherpesvirus*, is an important pathogen of horses. After exposure, EHV-1 replicates in the respiratory tract. Replication is followed by a leukocyte-associated viremia, enabling EHV-1 to reach internal organs where its replication can result in abortion, neonatal death or nervous system disorders (Allen and Bryans, 1986). Viremia may occur in the presence of virus-neutralizing antibodies in infection-immune (Doll and Bryans, 1963; Gleeson and Coggins, 1980; Mumford *et al.*, 1987) and vaccination-immune horses (Bürki *et al.*, 1990; Heldens *et al.*, 2001). Apparently, recognition of circulating EHV-1-infected peripheral blood mononuclear cells (PBMC) by the antibody-mediated immune system is inefficient.

Following infection of cells with enveloped viruses, viral glycoproteins are incorporated in cellular membranes. Binding of virus-specific antibodies to glycoproteins present in the plasma membrane makes infected cells recognizable for the classical complement pathway, phagocytes and natural killer (NK) cells, leading to lysis of the cell (Harper, 1994). Several herpesviruses have developed strategies to avoid antibody-dependent cell lysis. For suid herpesvirus 1 (SuHV-1)-infected monocytes, addition of SuHV-1-specific antibodies results in clearance of viral glycoproteins from the plasma membrane by antibody-induced internalization (Favoreel *et al.*, 1999). Clearance of the plasma membrane renders infected monocytes significantly less susceptible towards antibody-dependent, complement-mediated lysis (Van de Walle *et al.*, in press). In human cytomegalovirus (HCMV)-infected monocyte-derived macrophages, transport of viral glycoproteins to the plasma membrane is prevented, due to destruction of the microtubule network (Fish *et al.*, 1996). Absence of HCMV glycoproteins on the cell surface may be another strategy to avoid recognition by the antibody-dependent immune responses. Finally, several herpesviruses are known to encode proteins that interfere with activation of the complement cascade (reviewed by Favoreel *et al.*, 2000).

The main purpose of the present study was to investigate how EHV-1-infected PBMC are able to avoid recognition and destruction by the antibody-dependent immune responses.

4.1.3 Materials and methods

Cells

PBMC were isolated from adult, infection-immune horses by density centrifugation on Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). After isolation, PBMC were incubated during 24 hours in medium, supplemented with ionomycin (IONO) (0.5 µM) and phorbol dibutyrate (PDB) (10 nM) (Sigma, Bornem, Belgium) to favour EHV-1 replication (van der Meulen *et al.*, 2001). Rabbit kidney (RK13) and equine embryonic lung (EEL) cells were maintained in MEM with 5 % fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin and 0.3 mg/ml glutamin, until trypsinisation and inoculation.

Virus and in vitro infection

All cells were inoculated with the Belgian EHV-1 strain 97P70 at a multiplicity of infection of 10 (van der Meulen *et al.*, 2000). After 1 hour incubation, cells were washed and cultured at 37 °C. To ensure that the percentages of infected PBMC obtained in the present study, were not confounded by a possible reactivation in or re-infection of the horses during the sampling period, we always included stimulated but non-inoculated PBMC. Viral antigen expression was never detected in these control samples.

Immunofluorescence stainings

Cell surface and intracellular expression of EHV-1 antigens in PBMC were examined by means of a double immunofluorescence staining at 0, 6, 9, 12 and 24 hours post inoculation (h pi). PBMC were fixed with paraformaldehyde (3 %) and viral proteins on the surface were stained with polyclonal, fluoresceine isothyocyanate (FITC)-labeled horse anti-EHV-1 immunoglobulin (Ig) G. Intracellular viral proteins were stained upon permeabilisation with saponin (0.1 %) using polyclonal, biotinylated anti-EHV-1 IgG and streptavidin-Alexa Fluor® 350 (Molecular Probes, Eugene, OR, USA). Anti-EHV-1 IgG was raised by hyperimmunization of a horse. An infection-immune horse was inoculated intranasally with EHV-1 strain 97P70. After 2 weeks, a second immunization was performed intramuscularly using the same virus strain mixed with adjuvans (Suvaxyn®

Aujeszky im o/w, Fort Dodge Animal Health, Benelux). Two weeks later, serum was collected. IgG was purified on a protein G column and labeled with FITC (Becton Dickinson, San Jose, CA, USA) or biotinylated (Amersham International, Buckinghamshire, UK) following the manufacturer's instructions.

To determine if expression of viral antigens on the surface relied on the cell type, EEL and RK13 cells were examined at 12 h pi using the immunofluorescence staining as described before. For PBMC, an additional step was included prior to permeabilisation, using monoclonal antibodies HT23A (anti-equine CD5), 1.9/3.2 (anti-equine B-lymphocytes) or DH59B (equine monocyte marker) (VMRD, Pullman, WA, USA) followed by goat anti-mouse-Texas Red® (Molecular Probes, Eugene, OR, USA), allowing identification of different PBMC subpopulations.

Antibody-dependent, complement-mediated cell lysis (ADCML) assay

To examine the effect of antibody and complement on the viability of EHV-1-infected PBMC with and without surface expression, PBMC were infected for 24 hours and, subsequently, incubated for 1 hour at 37 °C with the previously described FITC-labeled anti-EHV-1 antibodies. IgG concentrations ranged from 0 to 1.6 mg IgG per ml. Afterwards, different concentrations of unheated EHV-1-negative horse serum were added during 1 h at 37 °C as a source of complement. The maximum concentration of serum was 20 %, since higher concentrations were cytotoxic. Ethidium monoazide bromide (EMA) (Molecular Probes, Eugene, OR, USA) was then added during 30 min at 4 °C to stain the nucleus of lysed cells. No photocrosslinking of EMA was performed, since it reduced the fluorescence intensity of the FITC-labeled antibodies and did not affect the observed percentages of lysed cells when compared to non-photocrosslinked samples (data not shown). Finally, intracellular viral proteins were stained upon fixation and permeabilisation as described earlier. In order to visualize surface expression in PBMC incubated in the absence of FITC-labeled antibodies during the first step of the assay, an additional labeling step was included in between fixation and permeabilisation. For that, the polyclonal, FITC-labeled anti-EHV-1 IgG was used.

To determine whether lysis was complement-mediated, the assay was repeated in the presence of ethylenediamine tetra-acetic acid (EDTA). EDTA is a Ca^{2+}- and Mg^{2+}-

chelator that blocks activation of the complement system in different species, including the horse (Joseph *et al.*, 1975; Leid *et al.*, 1985; Friedman *et al.*, 2000). EDTA (10 mM) was added 30 minutes before and during complement incubation. Antibody and serum concentration used were 0.8 mg/ml and 20 %, respectively.

Fluorescence and confocal laser scanning microscopy

Cells were analyzed using an inverted fluorescence microscope (Leica DM IRBE, Leica Microsystems Holdings GmbH, Wetzlar, Germany). Confocal images were obtained using a Bio-rad Radiance 2000 MP confocal laser scanning system attached to an inverted microscope (Nikon, Eclipse TE300, Japan).

Statistics

Mean values were obtained from the data of at least three independent experiments. Statistical analysis was based on analysis of variance (ANOVA) with a rejection level of 0.050 using SPSS (SPSS Inc., Chicago, Illinois, USA)

4.1.4 Results

Cell surface expression of viral antigens in EHV-1-infected PBMC

Only if intracellular expression of viral antigens was present, cells were considered for further examination on a possible surface expression. Total percentages of infected cells were 2.0 ± 1.3 % at 6 h pi, 4.3 ± 1.9 % at 9 h pi, 6.6 ± 1.2 % at 12 h pi and 14.2 ± 4.3 % at 24 h pi. Of these infected PBMC, 4.0 ± 0.8 % showed cell surface expression at 6 h pi, 14.4 ± 3.7 % at 9 h pi, 26.8 ± 6.9 % at 12 h pi and 31.4 ± 4.5 % at 24 h pi. Thus, only a minority of EHV-1-infected PBMC showed expression of viral antigens on their surface. Among these, two-thirds expressed antigens on part of the cell surface only, a status further designated as ‘focal expression’, whereas the remaining expressed EHV-1 antigens on the entire surface, a status further designated as ‘general expression’. No differences were observed in the ratio of these expression patterns between the various time points post inoculation. Figure 4.1.1 (see page 111) represents confocal images of an

EHV-1-infected PBMC without surface expression (a), with focal expression (b), and with general expression (c).

Cell surface expression of viral antigens in different cell types upon EHV-1 infection

EHV-1 antigens were present on the surface of 12.6 ± 3.0 % of infected T-lymphocytes, 12.2 ± 1.8 % of infected B-lymphocytes, 25.7 ± 8.7 % of infected monocytes, 11.0 ± 5.6 % of infected EEL cells and 13.0 ± 1.0 % of infected RK13 cells. Thus, cell surface expression was absent on the majority of infected cells independently of the cell type.

ADCML of EHV-1-infected PBMC with and without cell surface expression

Table 1 presents the percentages of lysed PBMC when using varying antibody (a) and complement (b) concentrations in the ADCML assay. A minimal number of 100 cells were scored per data point.

In non-infected PBMC, between 1.2 and 2.2 % of the cells were lysed and addition of antibodies and complement had no effect on viability. For infected PBMC without cell surface expression, percentages of lysed cells were similar to that in non-infected PBMC. For infected PBMC showing focal surface expression, the percentage of lysed cells was only significantly higher compared to non-infected PBMC when an antibody concentration of 1.6 mg/ml and a serum concentration of 20 % were used (8.3 %). For infected PBMC showing general surface expression, between 19.4 and 31.2 % of the cells were lysed in the presence of antibodies and complement, which was significantly higher compared to non-infected cells. When complement was added in the absence of antibodies, 10.5 % of PBMC showing general surface expression were lysed, whereas addition of antibodies in the absence of complement resulted in 5.4 % lysed cells.

Table 1. Complement-mediated cell lysis in EHV-1-inoculated PBMC

a. The effect of antibodies

		Percentage of lysed cells in …			
		Non-infected PBMC	Infected PBMC		
Antibody (mg/ml)	Complement (% horse serum)		without surface expression	with focal surface expression	with general surface expression
0	0	1.2 ± 0.4	1.2 ± 0.5	2.0 ± 0.9	2.2 ± 1.2
0	20	2.2 ± 0.8	1.5 ± 0.8	2.2 ± 0.8	10.5 ± 1.6
0.4	20	1.7 ± 0.8	3.6 ± 0.7	4.3 ± 0.9	28.3 ± 3.3
0.8	20	2.0 ± 0.4	2.5 ± 0.4	3.8 ± 1.5	27.6 ± 3.6
1.6	20	1.9 ± 0.4	2.6 ± 0.3	8.3 ± 1.4	31.2 ± 5.0

b. The effect of complement

		Percentage of lysed cells in …			
		Non-infected PBMC	Infected PBMC		
Antibody (mg/ml)	Complement (% horse serum)		without surface expression	with focal surface expression	with general surface expression
0	0	1.2 ± 0.4	1.2 ± 0.5	2.0 ± 0.9	2.2 ± 1.2
0.8	0	1.3 ± 0.8	0.8 ± 0.1	2.0 ± 0.4	5.4 ± 1.7
0.8	5	1.2 ± 0.6	1.8 ± 1.1	5.1 ± 0.8	19.4 ± 2.5
0.8	10	1.7 ± 0.3	1.2 ± 0.6	3.7 ± 0.9	20.0 ± 1.6
0.8	20	2.0 ± 0.4	2.5 ± 0.4	3.8 ± 1.5	27.6 ± 3.6

All data are expressed as the mean value of at least three experiments ± SD.

In contrast to infected PBMC without and with focal surface expression, PBMC showing general surface expression are apparently destroyed by three different immune mechanisms. Binding of antibodies to the surface of these infected PBMC in the absence of complement triggered destruction most likely via antibody-dependent, cell-mediated cytotoxicity. Lysis by complement in the absence of antibodies most likely occurred via the antibody-independent, complement-mediated pathway, whereas addition of antibodies increased the percentage of lysed cells by activation of the antibody-dependent, complement-mediated pathway. Figure 4.1.2 (see page 111) shows confocal images of a lysed, infected PBMC expressing viral antigens on the entire cell surface (a) and a viable, infected PBMC without cell surface expression (b), respectively.

Addition of EDTA significantly reduced the percentage of lysed PBMC showing general surface expression from 27.6 ± 3.6 to 8.8 ± 2.8 %, thereby demonstrating involvement of complement in the process of lysis. The percentages of lysed cells in infected PBMC without and with focal surface expression showed a slight increase to 3.9 ± 1.3 and 5.6 ± 2.3 %, respectively. The increase was due to cytotoxic effects of EDTA, since it was also observed when EDTA was used alone, in the absence of antibodies and complement.

4.1.5 Discussion

The present study describes a potential strategy of immune evasion for EHV-1-infected PBMC. We demonstrate that the majority of EHV-1-infected PBMC lacks cell surface expression of viral antigens. Absence of surface expression protects infected PBMC from lysis by equine complement.

Infected PBMC showing focal cell surface expression could escape from complement-mediated lysis as well. Circumvention of antibody-dependent, complement-mediated lysis may be explained by the fact that efficiency of lysis depends on the amount of antibody bound per infected cell (Joseph *et al.*, 1975; Perrin *et al.*, 1976; Sissons *et al.*, 1979). In our study, the extent of antigen expression on the surface of infected PBMC showing focal expression varied from only a small area of expression to an almost complete surface expression. It is very well possible that only cells with more extensive antigen expression may bind enough IgG to induce cell lysis. How infected PBMC without surface expression and with focal surface expression escape from antibody-independent, complement-mediated lysis seems less obvious. Analogous to the antibody-dependent pathway, the extent of antigen expression on the surface of infected cells may be important in turning the scale for either prevention or activation of the antibody-independent, complement-mediated pathway.

The maximal percentage of lysed PBMC showing general surface expression was 31.2 %. This suggests that the efficiency of equine complement to lyse EHV-1-infected PBMC is rather low. A low efficiency of equine complement, both of the antibody-independent and the antibody-dependent pathway, compared to other mammalian species

has already been described by Ish *et al.* (1993). Low efficiency of complement in our study may also result from viral interference with the complement cascade. For herpes simplex virus (HSV), varicella-zoster virus (VZV) and SuHV-1, it has been demonstrated that the viral glycoprotein complex gE/gI displays Fc receptor activity, which interferes with efficient antibody-dependent complement activation (Johnson *et al.*, 1988; Frank and Friedman, 1989; Litwin *et al.*, 1992; Favoreel *et al.*, 1997; Nagashunmugam *et al.*, 1998; Van de Walle *et al.*, in press). Homologues of gE and gI are also expressed by EHV-1 (Audonnet *et al.*, 1990; Elton *et al.*, 1991; Telford *et al.*, 1992), but whether they exert Fc receptor activity is not yet known. Glycoprotein gC of HSV, VZV, EHV-1 and 4, SuHV-1 and bovine herpesvirus 1 (BoHV-1) is capable of binding with complement factor C3, a pivotal component of complement activation, which may result in inhibition of the antibody-independent pathway (Friedman *et al.*, 1984; Harris *et al.*, 1990; Huemer *et al.*, 1992, 1993, 1995).

In conclusion, at least two-thirds of EHV-1-infected PBMC lack cell surface expression of viral antigens. Absence of surface expression was observed independently of the subtype of PBMC and protects infected cells from antibody-dependent and antibody-independent lysis by equine complement.

Acknowledgements

We thank Carine Boone, Chantal Vanmaercke and Chris Bracke for their excellent technical assistance. We also thank Prof. Dr. P. Van Oostveldt from the Department of Molecular Biotechnology at Ghent University for his help with the confocal microscopy and Armand De Smet from the Veterinary and Agrochemical Research Centre for labeling the horse anti-EHV-1 antibodies with FITC.

References

Allen, G. P., Bryans, J. T. (1986). Molecular epizootiology, pathogenesis and prophylaxis of equine herpesvirus-1 infections. *Progress in Veterinary Microbiology and Immunology* **1**, 78-144.

Audonnet, J. C., Winslow, J., Allen, G., Paoletti, E. (1990). Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI and gE. *Journal of General Virology* **71**, 2969-2978.

Bürki, F., Rossmanith, W., Nowotny, N., Pallan, C., Möstl, K., Lussy, H. (1990). Viraemia and abortions are not prevented by two commercial equine herpesvirus-1 vaccines after experimental challenge of horses. *Veterinary Quarterly* **12**, 80-86.

Doll, E. R., Bryans, J. T. (1963). Immunisation of young horses against viral rhinopneumonitis. *Cornell Veterinarian* **53**, 24-41.

Elton, D. M., Bonass, W. A., Killington, R. A., Meredith, D. M., Halliburton, I. W. (1991). Location of open reading frames coding for equine herpesvirus type-1 glycoproteins with homology to gE and gI of herpes simplex virus. *American Journal of Veterinary Research* **52**, 1252-1257.

Favoreel, H. W., Nauwynck, H. J., Van Oostveldt, P., Mettenleiter, T. C., Pensaert, M. B. (1997). Antibody-induced and cytoskeleton-mediated redistribution and shedding of viral glycoproteins expressed on pseudorabies virus-infected cells. *Journal of Virology* **71**, 8254-8261.

Favoreel, H. W., Nauwynck, H. J., Pensaert, M. B. (1999). Antibody-induced endocytosis of viral glycoproteins and major histocompatibility complex class I on pseudorabies virus-infected monocytes. *Journal of General Virology* **80**, 1283-1291.

Favoreel, H. W., Nauwynck, H. J., Pensaert, M. B. (2000). Immunological hiding of herpesvirus-infected cells. *Archives of Virology* **145**, 1269-1290.

Fish, K. N., Britt, W., Nelson, J. A. (1996). A novel mechanism for persistence of human cytomegalovirus in macrophages. *Journal of Virology* **70**, 1855-1862.

Frank, I., Friedman, H. M. (1989). A novel function of the herpes simplex virus type I Fc receptor: participation in bipolar bridging of antiviral immunoglobulin G. *Journal of Virology* **63**, 4479-4488.

Friedman, H. M., Cohen, G. H., Eisenberg, R. J., Seidel, C. A., Cines, D. B. (1984). Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. *Nature* **309**, 633-635.

Friedman, H. M., Wang, L., Pangburn, M. K., Lambris, J. D., Lubinski, J. (2000). Novel mechanism of antibody-independent complement neutralization of herpes simplex virus type 1. *Journal of Immunology* **15**, 4528-4536.

Gleeson, L. J., Coggins, L. (1980). Response of pregnant mares to equine herpesvirus 1 (EHV1). *Cornell Veterinarian* **70,** 391-400.

Harper, D. R. (1994). Viral interactions with the immune system. *In* Molecular Virology, 1st edition. BIOS Scientific Publishers Ltd., Oxford, pp 51-73.

Harris, S. L., Frank, I., Yee, A., Cohen, G. H., Eisenberg, R. J., Friedman, H. M. (1990). Glycoprotein C of herpes simplex virus type 1 prevents complement-mediated cell lysis and virus neutralization. *Journal of Infectious Diseases* **162**, 331-337.

Heldens, J. G. M., Hannant, D., Cullinane, A. A., Prendergast, M. J., Mumford, J. A., Nelly, M., Kydd, J. H., Weststrate, M. W., van den Hoven, R. (2001). Clinical and virological evaluation of the efficacy of an inactivated EHV1 and EHV4 whole virus vaccine (Duvaxyn $EHV_{1,4}$). Vaccination/challenge experiments in foals and pregnant mares. *Vaccine* **19**, 4307-4317.

Huemer, H. P., Larcher, C., Coe, N. E. (1992). Pseudorabies virus glycoprotein III derived from virions and infected cells binds to the third component of complement. *Virus Research* **23**, 271-280.

Huemer, H. P., Larcher, C., van Drunen Littel-van den Hurk, S., Babiuk, L. A. (1993). Species selective interaction of Alphaherpesvirinae with the "unspecific" immune system of the host. *Archives of Virology* **130**, 353-364.

Huemer, H. P., Nowotny, N., Crabb, B. S., Meyer, H., Hübert, P. H. (1995). gp13 (EHV-gC): a complement receptor induced by equine herpesviruses. *Virus Research* **37**, 113-126.

Ish, C., Ong, G. L., Desai, N., Mattes, M. J. (1993). The specificity of alternative complement pathway-mediated lysis of erythrocytes: a survey of complement and target cells from 25 species. *Scandinavian Journal of Immunology* **38**, 113-122.

Johnson, D. C., Frame, M. C., Ligas, M. W., Cross, A. M., Stow, N. D. (1988). Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. *Journal of Virology* **62**, 1347-1354.

Joseph, B. S., Cooper, N. R., Oldstone, M. B. A. (1975). Immunologic injury of cultured cells, infected with measles virus. I. Role of IgG antibody and the alternative complement pathway. *Journal of Experimental Medicine* **141**, 761-774.

Leid, R. W., Coley, S. C., Blanchard, D. P., Perryman, L. E. (1985). Equine alternative pathway activation by unsensitized rabbit red blood cells. *Veterinary Immunology and Immunopathology* **9**, 71-85.

Litwin, V., Jackson, W., Grose, C. (1992). Receptor properties of two varicella-zoster virus glycoproteins, gpI and gpIV, homologous to herpes simplex virus gE and gI. *Journal of Virology* **66**, 3643-3651.

Mumford, J. A., Rossdale, P. D., Jesset, D. M., Gann, S., Ousey, J., Cook, R. F. (1987). Serological and virological investigations of an equid herpesvirus 1 (EHV_1) abortion storm on a stud farm in 1985. *Journal of Reproduction and Fertility Supplement* **35**, 509-518.

Nagashunmugam, T., Lubinski, J., Wang, L., Goldstein, L. T., Weeks, B. S., Sundaresan, P., Kang, E. H., Dubin, G., Friedman, H. M. (1998). *In vivo* immune evasion mediated by the herpes simplex virus type I immunoglobulin G Fc receptor. *Journal of Virology* **72**, 5351-5359.

Perrin, L. H., Joseph, B. S., Cooper, N. R., Oldstone, M. B. A. (1976). Mechanism of injury of virus-infected cells by antiviral antibody and complement: participation of IgG, F(ab')2, and the alternative complement pathway. *Journal of Experimental Medicine* **143**, 1027-1041.

Sissons, J. G. P., Schreiber, R. D., Perrin, L. H., Cooper, N. R., Müller-Eberhard, H. J., Oldstone, M. B. A. (1979). Lysis of measles virus-infected cells by the purified cytolytic alternative complement pathway and antibody. *Journal of Experimental Medicine* **150**, 445-454.

Telford, E. A., Watson, M. S., McBride, K., Davison, A. J. (1992). The DNA sequence of equine herpesvirus-1. *Virology* **189**, 304-316.

van der Meulen, K. M., Nauwynck, H. J., Buddaert, W., Pensaert, M. B. (2000). Replication of equine herpesvirus type 1 in freshly isolated equine peripheral blood mononuclear cells and changes in susceptibility following mitogen stimulation. *Journal of General Virology* **81**, 21-25.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2001). Mitogen stimulation favours replication of equine herpesvirus-1 in equine blood mononuclear cells by inducing cell proliferation and formation of close intercellular contacts. *Journal of General Virology* **82**, 1951-1957.

van de Walle, G. R., Favoreel, H. W., Nauwynck, H. J., Pensaert, M. B. (2003). Antibody-induced internalization of viral glycoproteins and gE-gI receptor activity protect pseudorabies virus-infected monocytes from efficient complement-mediated lysis. *Journal of General Virology* **84** (in press).

4.2 EXPRESSION OF VIRAL ANTIGENS AND MHC CLASS I ON THE SURFACE OF EQUINE HERPESVIRUS 1-INFECTED BLOOD MONONUCLEAR CELLS DURING VIREMIA IN IMMUNE PONIES

_____*Veterinary Microbiology, submitted*

van der Meulen, K., Nauwynck, H. and Pensaert, M.

4.2.1 Summary

Previous *in vitro* studies demonstrated that the majority of equine herpesvirus 1 (EHV-1)-infected peripheral blood mononuclear cells (PBMC) lack viral antigen expression on their cell surface. Absence of cell surface expression protects infected PBMC from complement-mediated lysis *in vitro* and represents a potential strategy of immune evasion. The purpose of the present study was to examine if infected PBMC lack surface expression of viral antigens during viremia upon experimental inoculation of immune ponies. Moreover, surface expression of major histocompatibility complex (MHC) class I molecules was examined in infected cells, since MHC class I molecules present viral peptides to cytotoxic T-lymphocytes (CTL) and thus also play a role in making infected cells recognizable for the immune system. Four infection-immune ponies and 1 EHV-1-negative control pony were inoculated with the Belgian EHV-1 strain 97P70. PBMC were collected at various time points between 0 and 28 days post inoculation (d pi). Surface expression of viral antigens and MHC class I molecules on EHV-1-infected PBMC was determined by immunofluorescence labeling. Cell surface expression of viral antigens was present in only 1 EHV-1-infected PBMC in the immune ponies (1/31) and in none of the EHV-1-infected PBMC in the non-immune pony (0/18). MHC class I expression was found on 80 to 100 % of EHV-1-infected PBMC in both the immune ponies and the non-immune pony, which was similar as observed in non-infected PBMC (83 - 100 %). In conclusion, lack of surface expression of viral antigens, but not of MHC class I molecules, may allow EHV-1-infected PBMC to avoid recognition by the immune system.

4.2.2 Introduction

Equine herpesvirus 1 (EHV-1), an *Alphaherpesvirus*, is an important pathogen of horses worldwide. After infection, EHV-1 replicates in epithelia of the respiratory tract and, subsequently, spreads to regional lymph nodes (Patel *et al.*, 1982; Kydd *et al.*, 1994a, 1994b). In young horses, virus replication in the respiratory tract may result in acute respiratory disease (Allen and Bryans, 1986). Primary replication is followed by an extensive cell-associated viremia which lasts until 9 to 27 days after infection (Thein and Brown, 1988; Gibson *et al.*, 1992; McCartan *et al.*, 1995). Carried by infected peripheral blood mononuclear cells (PBMC), mainly T-lymphocytes, EHV-1 spreads to internal organs, where its replication within endothelium of the vasculature may cause late term abortion or myeloencephalopathy (Scott *et al.*, 1983; Allen and Bryans, 1986).

Protective immunity resulting from EHV-1 infection is of short duration. Despite persistence of virus-neutralizing antibodies, the respiratory mucosa may be asymptomatically re-infected within 2 to 4 months (Doll, 1961; Gibson *et al.*, 1992). Notwithstanding the presence of high levels of systemic antibodies (Doll and Bryans, 1963; Gleeson and Coggins, 1980; Bryans and Allen, 1982; Burrows *et al.*, 1984; Frymus *et al.*, 1986; Mumford *et al.*, 1987; Bürki *et al.*, 1990; van der Meulen *et al.*, 2000a; Heldens *et al.*, 2001) and EHV-1-specific cytotoxic T-lymphocyte (CTL) precursors (O'Neill *et al.*, 1999), EHV-1-infected PBMC can be detected in the blood circulation upon challenge of both infection-immune and vaccination-immune horses. This means that infectious virus can still be delivered to permissive endothelial cells and, consequently, cause abortion or nervous system disorders even though horses possess an active immunity.

Following infection with enveloped viruses, viral glycoproteins are incorporated in the plasma membrane of infected cells. Subsequent binding of virus-specific antibodies to these glycoproteins leads to recognition and destruction of infected cells via the classical complement pathway, phagocytes and natural killer (NK) cells (Harper, 1994). Recent *in vitro* studies on EHV-1-infected PBMC demonstrated that 70 % of the infected cells lack visually detectable levels of viral glycoproteins on their surface and that this protects the infected cells from lysis by equine complement (van der Meulen *et al.*, 2003). The

hypothesis is put forward that absence of viral antigens on the surface of EHV-1-infected PBMC also occurs *in vivo*, thereby providing a potential strategy for infected PBMC to escape from antibody-mediated immune responses in the horse.

Major histocompatibility complex (MHC) class I molecules play an important role in the destruction of virus-infected cells in a cell-mediated fashion. Once a cell is infected, newly produced viral proteins become degraded into peptides. These peptides are then loaded onto MHC class I molecules and transported to the cell surface, where their presentation results in recognition and lysis of infected cells by CTL (Harper, 1994). *In vitro*, several herpesviruses are known to affect MHC class I processing and loading, leading to retention or untimely degradation of these molecules in the cell. Furthermore, some herpesviruses are known to directly inhibit MHC class I - viral peptide trafficking to the surface of infected cells (reviewed by Favoreel *et al.*, 2000). *In vivo*, such phenomenon may result in inefficient recognition of virus-infected cells by CTL, thereby enabling them to circumvent destruction by the cell-mediated immunity.

The main purpose of the present study was to examine whether infected PBMC, present in the circulation of immune horses upon EHV-1 exposure, lack expression of viral antigens and/or MHC class I molecules on their cell surface.

4.2.3 Materials and methods

Virus

A 5th passage of EHV-1 strain 97P70 was used for experimental inoculation (van der Meulen *et al.*, 2000b). Virus was propagated on equine embryonic lung cells.

Animals

Four adult ponies and 1 pony foal were used in the present study. The adult ponies (A-D) were aged between 5 and 15 years and had been previously infected with EHV-1, as evidenced by the presence of antibodies at the start of the experiment using a complement-dependent seroneutralization (SN)-test or the more sensitive immunoperoxidase monolayer assay (IPMA), as described later. Pony E, a 9-month old foal, had no detectable EHV-1-specific antibody titres in either the SN-test or the IPMA

and was included as a control. To ensure that the foal was not latently infected, an attempt was made to reactivate virus using dexamethasone treatment. Dexamethasone natriumphosphate (Certa International, Braine-l'Alleud, Belgium) was administered 1 month prior to EHV-1 inoculation by intravenous injection at a dose of 2 mg/kg daily for three consecutive days (Gibson *et al.*, 1992; Slater *et al.*, 1994). Three weeks after dexamethasone administration, a complement-dependent SN-test and an IPMA were performed. Absence of detectable antibody titres ruled out a possible reactivation, thereby confirming the EHV-1-negative status of pony E.

All ponies were housed inside isolated stables. They were fed daily with a commercial, complete feed. Drinking water and hay were supplied *ad libitum*.

Experimental inoculation

Each pony was inoculated oronasally with 20 ml of a virus suspension containing $10^{6.8}$ tissue culture infectious dose $_{50}$ ($TCID_{50}$). Ten ml were administrated intranasally (5 ml per nostril) and 10 ml were inoculated orally. The virus titre was confirmed by titration of the inoculum.

Clinical observation

Following virus inoculation, the ponies were monitored daily for clinical signs via physical examination and measurement of rectal temperatures. Rectal temperatures ≥ 38.5 °C were regarded as fever.

Sampling procedure

A nasopharyngeal mucus sample and 100 ml of heparinized blood were taken at 0, 1, 3, 5, 7, 9,14, 21 and 28 days post inoculation (d pi).

Nasopharyngeal samples were collected using a swab with a length of 40 cm that was inserted through the nostrils up to the nasopharynx. Immediately after collection, swabs were immersed in transport medium containing phosphate-buffered saline supplemented with 10 % fetal bovine serum, 1000 U/ml penicillin, 1 mg/ml streptomycin, 0.5 mg/ml kanamycin and 0.1 mg/ml gentamycin.

Heparinized blood was separated in PBMC and plasma immediately after collection by means of density gradient centrifugation on Ficoll Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Virological examination

EHV-1 was titrated in the nasopharyngeal secretions. Therefore, nasopharyngeal swabs were vortexed in the transport medium, the medium was then centrifuged and the supernatant was titrated on monolayers of rabbit kidney (RK13) cells.

Immediately after separation from whole blood, PBMC were co-cultivated to determine the number of infected cells. Briefly, 1 x 10^7 PBMC were placed on a monolayer of RK13 cells. The monolayers were overlayed with carboxymethylcellulose (0.94 %), centrifuged for 30 min at 750 x g and incubated at 37 °C and 5 % CO_2. Monolayers were checked daily for plaques.

An additional sample of freshly isolated PBMC was freeze-thawed twice and the cell lysate was titrated for the presence of intracellular infectious virus.

Plasma samples were examined for the presence of extracellular virus by means of isolation on RK13 cells.

Immunofluorescence

In order to determine whether viral antigens and/or MHC class I molecules were expressed on the cell surface of EHV-1-infected PBMC during viremia, a double immunofluorescence labeling was performed. First, PBMC were fixed with paraformaldehyde (3 %) immediately after isolation and incubated with either protein G-purified, fluoresceine isothyocyanate (FITC)-labeled polyclonal horse anti-EHV-1 antibodies (van der Meulen *et al.*, 2003) or with the mouse monoclonal antibody PT85A directed against equine MHC class I (VMRD, Pullman, WA, USA) followed by goat anti-mouse FITC. Second, PBMC were permeabilized with saponin (0.1 %) and incubated with protein G-purified, biotinylated polyclonal horse anti-EHV-1 antibodies, followed by streptavidin-Texas Red® (Molecular Probes, Eugene, OR, USA). After staining, cells were placed on cover slips and analyzed using fluorescence (Leica DM BRE, Leica Microsystems GmbH, Wetzlar, Germany) and confocal laser scanning

microscopy (Leica TCS SP2 laser scanning spectral confocal system, Leica Microsystems GmbH, Wetzlar, Germany). For each sample, approximately 0.4 x 10^7 cells were analyzed.

Serological examination

The SN titre was determined at 0, 7, 14, 21 and 28 d pi by means of a complement-dependent SN-test. Two-fold serum dilutions were prepared in duplicate rows of 96-well microtiter plates using MEM as diluent and mixed with 300 $TCID_{50}$ of the EHV-1 isolate 97P70. After 23 hours of incubation at 37 °C and 5 % CO_2, unheated guinea pig serum was added to each well as a source of complement. Plates were then incubated for an additional hour and the content of each well was subsequently transferred to a monolayer of RK13 cells. Monolayers were incubated at 37 °C and 5 % CO_2 and examined for cytopathic effect after 5 days. The SN titre was calculated as the reciprocal value of the serum dilution that neutralized cytopathic effect in 50 % of the wells.

In case no detectable SN titre was observed, serum samples were tested using the more sensitive IPMA. RK13 cells were seeded in 96-well microtiter plates, grown to confluency and inoculated with 10^3 $TCID_{50}$ per well of the EHV-1 isolate 97P70. After 28 hours, cells were washed, dried at 37 °C for 1 hour and stored at -20 °C until use. Plates were then thawed and subsequently fixed with 4 % paraformaldehyde and a solution containing 1 % hydrogen in methanol. Following extensive washing, serial 2-fold dilutions of the sera were added and cells were incubated for 1 hour at 37 °C. Then, cells were incubated with peroxidase-labeled goat anti-horse antibodies (Jackson ImmunoResearch Laboratories Inc., PA, USA). After 1 hour, a substrate solution of 3-amino-9-ethylcarbazole in 0.05 M acetate buffer with 0.05 % hydrogen was added to each well. Following 20 minutes of incubation at 37 °C, substrate solution was replaced with acetate buffer to block the enzymatic staining reaction. The IPMA titre was calculated as the reciprocal value of the highest serum dilution that induced visual staining of infected RK13 cells, as determined by light microscopy.

4.2.4 Results

Clinical observation

The clinical signs as observed in the EHV-1-inoculated ponies are summarized in Table 1. Clinical signs were mild and very similar in the immune ponies and in the non-immune pony, except for swelling of the lymph nodes, which was observed only in the non-immune pony. Additionally, the peak temperature observed in the non-immune pony (40.2 °C at 1 d pi) was higher than that observed in the infection-immune animals (39.3 °C in pony D at 8 d p.i). In the immune pony B, the observed tachypnee caused severe discomfort and did not improve during the course of time. Therefore, the animal was treated with antibiotics (Duplocillin® L.A., Mycofarm Belga, Mechelen, Belgium) at 16, 18 and 21 d pi, which resulted in a full recovery.

Table 1. Clinical signs in ponies following experimental inoculation with EHV-1

Pony	Status at inoculation	SN (IPMA) titre at inoculation	Duration of clinical signs (from … to … d pi)				
			pyrexia	nasal discharge		tachypnee	swelling lnn*
				serous	purulent		
A	immune	48	-	2 - 9	-	-	-
B	immune	32	6 - 11	2 - 12	-	6 - 17	-
C	immune	4	-	2 - 10	-	-	-
D	immune	< 2 (10)	7 - 10	2 - 10	7	8 - 9	-
E	non-immune	< 2 (< 10)	1 - 2 / 4 - 5	2 - 12	-	-	2 – 23

*: submandibular and retropharyngeal lymph nodes

Virus isolation from the upper respiratory tract

The results of virus isolation from the nasopharyngeal swabs are shown in Table 2. All ponies shed virus on day 1 pi. For the immune ponies, the duration of virus excretion was 1 day in pony A, 5 days in ponies B and C, and 3 days in pony D. The non-immune pony E shed virus for 14 days.

Table 2. Virus shedding and viremia in ponies following an experimental inoculation with EHV-1

Pony	Status at inoculation	SN (IPMA) titre at inoculation	1 Virus titre (log_{10} $TCID_{50}$/gr nasopharyngeal mucus) 2 No. of plaques/10^7 PBMC (co-cultivation) 3 No. of infected cells/0.8x10^7 PBMC (IF) at … d pi								
				0	1	3	5	7	9	14	21	28
A	immune	48	(1)	-	4.6	-	-	-	-	-	-	-
			(2)	0	0	0	1	5	2	0	0	0
			(3)	0	0	0	4	3	4	2	0	0
B	immune	32	(1)	-	4.3	4.8	4.5	-	-	-	-	-
			(2)	0	0	0	14	2	0	1	+	0
			(3)	0	0	1	2	3	3	1	1	0
C	immune	4	(1)	-	2.5	3.3	5.3	-	-	-	-	-
			(2)	0	0	0	8	2	0	0	0	0
			(3)	0	1	1	1	4	2	2	1	0
D	immune	< 2 (10)	(1)	-	4.5	3.8	-	-	-	-	-	-
			(2)	0	0	0	0	4	1	0	0	0
			(3)	0	2	3	2	3	1	2	0	0
E	non-immune	< 2 (< 10)	(1)	-	5.9	5.0	3.9	3.6	3.6	3.1	-	-
			(2)	0	0	2	20	18	6	1	0	0
			(3)	0	3	7	10	6	3	3	3	0

-: < 0.8 log_{10} $TCID_{50}$ per gram nasopharyngeal mucus
IF: immunofluorescence
+: plaques were present, but the number could not be determined

Viremia

The numbers of infected PBMC as determined by co-cultivation and immunofluorescence labeling are also presented in Table 2. In the immune ponies, infected PBMC were detected by co-cultivation between 5 and 9 d pi, 5 and 7 d pi and 7 and 9 d pi in pony A, C and D, respectively. For pony B, PBMC were positive at 5 and 7 d pi and at 14 and 21 d pi, but no virus was recovered at 9 d pi. In the non-immune pony

E, cell-associated viremia was detected between 3 and 14 d pi. In none of the 5 ponies, virus was recovered from the cell lysate of PBMC or from plasma.

Infected PBMC as determined by immunofluorescence labeling were observed from 5 till 14 d pi, from 3 till 21 d pi, from 1 till 21 d pi and from 1 till 14 d pi in the immune ponies A, B, C and D, respectively. In the non-immune pony E, infected PBMC were found from 1 till 21 d pi (Table 2).

For the immune ponies, expression of viral antigens on the surface of EHV-1-infected PBMC was only observed in pony A in 1 out of 8 examined EHV-1-infected PBMC (14 d pi). In the immune ponies B, C and D, all examined EHV-1-infected PBMC lacked visually detectable levels of plasma membrane-anchored viral antigens (0/7, 0/8 and 0/8 infected PBMC, respectively). Also in the non-immune pony, none of the EHV-1-infected PBMC showed surface expression of viral antigens (0/18 infected PBMC).

Cell surface expression of MHC class I molecules was found in 80 to 100 % of the EHV-1-infected PBMC in the immune ponies (4/5, 4/4, 4/4 and 6/6 infected PBMC in pony A, B, C and D, respectively) as well as in 88 % of the EHV-1-infected PBMC in the non-immune pony (15/17 infected PBMC). This resembled the percentage of MHC class I-positive cells found in non-infected PBMC (83 to 100 %). Figure 4.2.1 (see page 111) presents a confocal image of 2 non-infected and 1 EHV-1-infected PBMC showing MHC class I expression.

Serological response to experimental inoculation

The complement-dependent SN titres are shown in Table 3. For the immune ponies, a clear seroconversion was observed 7 days after experimental inoculation, confirming their immune-positive status. In the non-immune pony E, antibodies were first detected at 14 d pi.

Table 3. Serological response in ponies following experimental inoculation with EHV-1

Pony	Status at inoculation	SN (IPMA) titre at inoculation	Complement-dependent SN titre at … d pi				
			0	7	14	21	28
A	immune	48	48	96	256	≥ 384	256
B	immune	32	32	96	≥ 384	≥ 384	≥ 384
C	immune	4	4	24	192	192	128
D	immune	< 2 (10)	< 2	48	≥ 384	256	256
E	non-immune	< 2 (< 10)	< 2	< 2	32	≥ 384	≥ 384

4.2.5 Discussion

The inability of the currently available vaccines to provide a full protection against EHV-1 has serious economic implications and stimulated the search for alternative vaccination approaches (reviewed by van Maanen, 2002). So far, relatively little research has been performed on the actual mechanism that enables EHV-1 to circumvent recognition and destruction by the immune system of the horse. The present *in vivo* study demonstrates that the vast majority of EHV-1-infected PBMC present in the blood during viremia in experimentally inoculated horses lacks visually detectable levels of viral antigens on their cell surface. Absence of viral antigen expression on the surface of EHV-1-infected PBMC during viremia represents a potential strategy for the infected cells to circumvent the antibody-mediated immune response in the horse.

Besides via the antibody-mediated immune response, virus-infected cells can be recognized and destroyed via the cell-mediated immune response as well. O'Neill *et al.* (1999) already demonstrated high frequencies of EHV-1-specific, genetically restricted CTL presursors after multiple experimental inoculation of ponies. Breathnach (2001) found that CTL are elicited by experimental infection of horses with EHV-1. Notwithstanding the presence of high frequencies of EHV-1-specific CTL, infected

PBMC can be detected in the blood circulation of horses upon challenge, suggesting that their recognition by CTL is impaired (O'Neill *et al.*, 1999). The results from the present study show that the impaired recognition is not arising from an absence of MHC class I molecules on the surface of EHV-1-infected PBMC, since 80 to 100 % of the infected PBMC showed surface expression of MHC class I molecules. From our study, it cannot be concluded whether the MHC class I molecules are effectively presenting EHV-1 peptides. Inhibition of viral peptide supply to MHC class I may act as another potential strategy to avoid recognition of infected cells by CTL (reviewed by Favoreel *et al.*, 2000). Also, mechanisms of herpesvirus-induced inhibition of CTL activity independent on MHC class I may be responsible for impaired recognition of infected cells. This was already described for Epstein-Barr virus, which encodes an interleukin-10 homologue, BCRF1. BCRF1 exhibits a variety of effects on the T-helper cell type response, resulting in down-regulation of CTL activity (Hsu *et al.*, 1990; Vieira *et al.*, 1991). A similar interleukin-10 homologue, E7, is encoded by EHV-2 (Telford *et al.*, 1994).

Our results, together with those of O'Neill *et al.* (1999) and Breathnach (2001), indicate that EHV-1 infection not only induces a normal antibody response but also a normal cell-mediated immune response. This emphasizes that immune escape of EHV-1 during viremia is not the effect of an impaired response of the immune system but rather a gain of the virus that can hide itself from recognition by the immune system. In this perspective, it will be very difficult to design a vaccine that can fully prevent EHV-1-infected PBMC to spread to internal organs and to induce abortion or nervous system disorders. Therefore, one may suggest that future approaches to prevent EHV-1-associated disease should focus on improving vaccines that induce local immunity in such a way that replication of EHV-1 in the respiratory tract and subsequent viremia are prevented. Even though such vaccines may be sufficient to prevent re-infection, they will not be helpful to prevent reactivation of latent EHV-1 from PBMC.

The total number of infected PBMC was rather low in the present study when compared to the studies of Scott *et al.* (1983) and Gibson *et al.* (1992). This may be due to a difference in virulence between the EHV-1 strains used. Mumford *et al.* (1994) already demonstrated that the number of infected leukocytes varies depending on the EHV-1 strain. In their study, the maximum number of infected leukocytes was 25-fold

higher following an infection with the highly virulent EHV-1 strain Ab4, than following an infection with the less virulent strain V592/2.

The percentages of infected PBMC showing cell surface expression of viral antigens are somewhat different between the present *in vivo* study and an earlier *in vitro* study. *In vitro*, 37 % of the EHV-1-infected PBMC showed expression of viral antigens on their surface (van der Meulen *et al.*, 2003). *In vivo*, surface expression was found in only 1 infected PBMC in immune ponies and in none of the infected PBMC in the non-immune pony. It is not likely that infected PBMC showing cell surface expression are selectively destroyed *in vivo* by means of EHV-1-specific antibody-mediated immune responses, since the non-immune pony E lacked infected PBMC with surface expression as well even though no EHV-1-specific antibodies were present in its blood circulation. Destruction of PBMC showing surface expression via innate immune responses, like the antibody-independent, complement-mediated pathway, may be a more reliable explanation for the low percentage of EHV-1-infected PBMC showing cell surface expression *in vivo*. Previous studies already demonstrated that the antibody-independent, complement-mediated pathway can vouch for the destruction of approximately 10 % of EHV-1-infected PBMC showing surface expression *in vitro* within 1 hour of exposure to 20 % unheated equine serum (van der Meulen *et al.*, 2003). Besides this, it cannot be excluded that cell surface expression of viral antigens is absent on a higher number of cells *in vivo*, because of so far unknown viral and/or cellular factors influencing the process of surface expression.

We can conclude from the present study that, by far, the most EHV-1-infected PBMC lack viral antigen expression on their cell surface during viremia, thereby enabling these cells to avoid recognition and destruction by the antibody-mediated immune response. The percentage of infected PBMC showing surface expression of MHC class I molecules during viremia was 80 to 100 % and, therefore, absence of cell surface expression of MHC class I molecules will not account for the inefficient recognition of EHV-1-infected PBMC by CTL.

Acknowledgements

We thank Carine Boone, Chantal Vanmaercke, Chris Bracke, Inge Paryn, Fernand De Backer and Dieter Defever for their excellent technical assistance.

References

Allen, G. P., Bryans, J. T. (1986). Molecular epizootiology, pathogenesis and prophylaxis of equine herpesvirus-1 infections. *Progress in Veterinary Microbiology and Immunology* **1**, 78-144.

Breathnach, C. C. (2001). Mucosal humoral and cellular immune defense mechanism of the horse's upper respiratory tract against equine herpesvirus-1 infection. PhD thesis. University of Kentucky, Lexington, Kentucky.

Bryans, J. T., Allen, G. P. (1982). Application of a chemically inactivated, adjuvanted vaccine to control abortigenic infection of mares by equine herpesvirus I. *Developments in Biological Standardization* **52**, 493-498.

Bürki, F., Rossmanith, W., Nowotny, N., Pallan, C., Möstl, K., Lussy, H. (1990). Viraemia and abortions are not prevented by two commercial equine herpesvirus-1 vaccines after experimental challenge of horses. *Veterinary Quarterly* **12**, 80-86.

Burrows, R., Goodridge, D., Denyer, M. S. (1984). Trials of an inactivated equid herpesvirus 1 vaccine: challenge with a subtype 1 virus. *Veterinary Record* **114**, 369-374.

Doll, E. R. (1961). Immunization against viral rhinopneumonitis of horses with live virus propagated in hamsters. *Journal of the American Veterinary Medical Association* **139**, 1324-1330.

Doll, E. R., Bryans, J. T. (1963). Immunization of young horses against viral rhinopneumonitis. *Cornell Veterinarian* **53**, 24-41.

Favoreel, H. W., Nauwynck, H. J., Pensaert, M. B. (2000). Immunological hiding of herpesvirus-infected cells. *Archives of Virology* **145**, 1269-1290.

Frymus, T., Kita, J., Woyciechowska, S., Ganowicz, M. (1986). Foetal and neonatal foal losses on equine herpesvirus type 1 (EHV-1) infected farms before and after EHV-1 vaccination was introduced. *Polskie Archiwum Weterynaryjne* **26**, 7-14.

Gibson, J. S., Slater, J. D., Awan, A. R., Field, H. J. (1992). Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. *Archives of Virology* **123**, 351-366.

Gleeson, L. J., Coggins, L. (1980). Response of pregnant mares to equine herpesvirus 1 (EHV1). *Cornell Veterinarian* **70**, 391-400.

Harper, D. R. (1994). Viral interactions with the immune system, *In:* Molecular Virology. BIOS Scientific Publishers Ltd., Oxford, pp 51-73.

Heldens, J. G. M., Hannant, D., Cullinane, A. A., Prendergast, M. J., Mumford, J. A., Nelly, M., Kydd, J. H., Weststrate, M. W., van den Hoven, R. (2001). Clinical and virological evaluation of the efficacy of an inactivated EHV1 and EHV4 whole virus vaccine (Duvaxyn $EHV_{1,4}$). Vaccination/challenge experiments in foals and pregnant mares. *Vaccine* **19**, 4307-4317.

Hsu, D. H., de Waal-Malefyt, R., Fiorentino, D. F., Dang, M. N., Vieira, P., de Vries, J., Spits, H., Mosmann, T. R., Moore, K. W. (1990). Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. *Science* **250**, 830-832.

Kydd, J. H., Smith, K. C., Hannant, D., Livesay, G. J., Mumford, J. A. (1994a). Distribution of equid herpesvirus-1 (EHV-1) in respiratory tract of ponies: implications for vaccination strategies. *Equine Veterinary Journal* **26,** 466-469.

Kydd, J. H., Smith, K. C., Hannant, D., Livesay, G. J., Mumford, J. A. (1994b). Distribution of equid herpesvirus-1 (EHV-1) in respiratory tract associated lymphoid tissue: implications for cellular immunity. *Equine Veterinary Journal* **26**, 470-473.

McCartan, C. G., Russell, M. M., Wood, J. L. N., Mumford, J. A. (1995). Clinical, serological and virological characteristics of an outbreak of paresis and neonatal foal disease due to equine herpesvirus-1 on a stud farm. *Veterinary Record* **136**, 7-12.

Mumford, J. A., Rossdale, P. D., Jessett, D. M., Gann, S., Ousey, J., Cook, R. F. (1987). Serological and virological investigations of an equid herpesvirus 1 (EHV_1) abortion storm on a stud farm in 1985. *Journal of Reproduction and Fertility Supplement* **35**, 509-518.

Mumford, J. A., Hannant, D., Jessett, D. M., O'Neill, T., Smith, K. C., Ostlund, E. N. (1994). Abortigenic and neurological disease caused by experimental infection with equid herpesvirus-1. Equine Infectious Diseases VII, Proceedings of the Seventh International Conference of Equine Infectious Diseases. Tokyo, pp 261-275.

O'Neill, T., Kydd, J. H., Allen, G. P., Wattrang, E., Mumford, J. A., Hannant, D. (1999). Determination of equid herpesvirus 1-specific, $CD8^+$, cytotoxic T lymphocyte precursor frequencies in ponies. *Veterinary Immunology and Immunopathology* **70**, 43-54.

Patel, J. R., Edington, N., Mumford, J. A. (1982). Variation in cellular tropism between isolates of equine herpesvirus-1 in foals. *Archives of Virology* **74**, 41-54.

Scott, J. C., Dutta, S. K., Myrup, A. C. (1983). *In vivo* harboring of equine herpesvirus-1 in leukocyte populations and subpopulations and their quantitation from experimentally infected ponies. *American Journal of Veterinary Research* **44**, 1344-1348.

Slater, J. D., Borchers, K., Thackray, A. M., Field, H. J. (1994). The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. *Journal of General Virology* **75**, 2007-2016.

Telford, E. A., Watson, M. S., Aird, H. C., Perry, J., Davison, A. J. (1995). The DNA sequence of equine herpesvirus 2. *Journal of Molecular Biology* **249**, 520-528.

Thein, P., Brown, K. (1988). Infection mit equinen Herpesviren und Manifestation am Zentralnervensystem beim Pferd. *Tierärztliche Praxis* **16**, 295-302.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2000a). Equine herpesvirus type 1 abortion, neonatal foal death and nervous system disorders diagnosed in Belgium in 1999. *Flemish Veterinary Journal* **69**, 38-41.

van der Meulen, K. M., Nauwynck, H. J., Buddaert, W., Pensaert, M. B. (2000b). Replication of equine herpesvirus type 1 in freshly isolated equine peripheral blood mononuclear cells and changes in susceptibility following mitogen stimulation. *Journal of General Virology* **81**, 21-25.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2003). Absence of viral antigens on the surface of equine herpesvirus 1-infected blood mononuclear cells: a strategy to avoid complement-mediated lysis. *Journal of General Virology* **84**, 93-97.

van Maanen, C. (2002). Equine herpesvirus 1 and 4 infections: an update. *Veterinary Quarterly* **24**, 58-78.

Vieira, P., de Waal-Malefyt, R., Dang, M. N., Johnson, K. E., Kastelein, R., Fiorentino, D. F., de Vries, J. E., Roncarolo, M. G., Mosmann, T. R., Moore, K. W. (1991). Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 1172-1176.

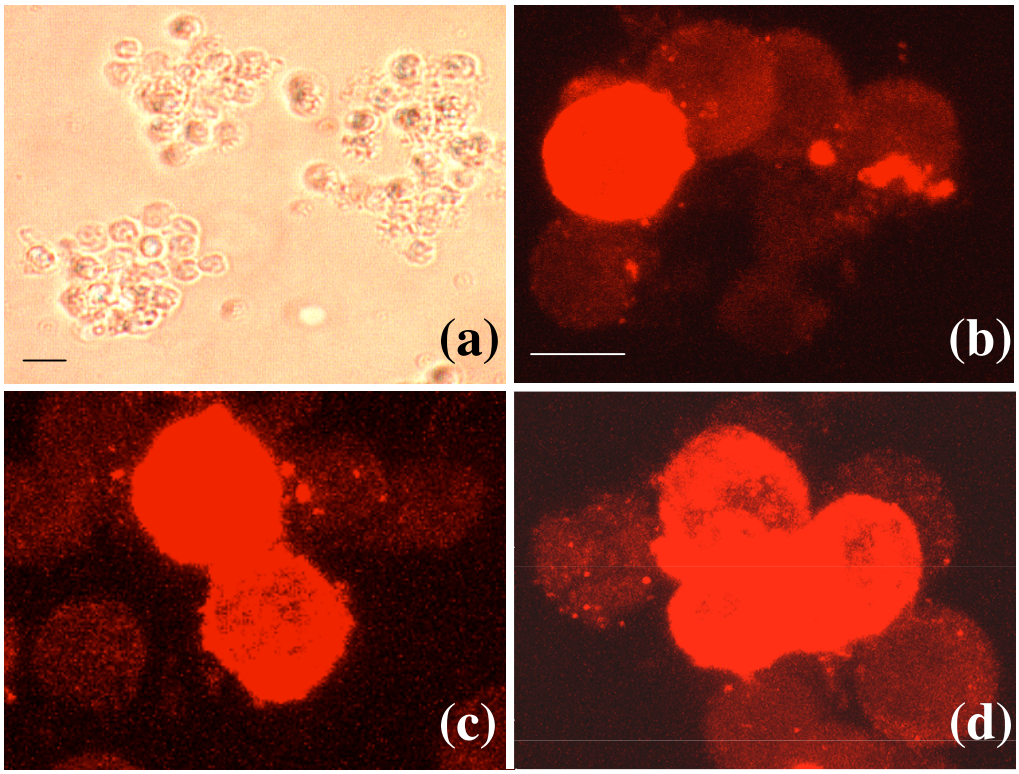

Figure 3.2.1. Formation of clusters of equine PBMC, induced by stimulation with IONO/PDB, facilitates cell-to-cell transmission of EHV-1

PBMC were stimulated during 24 hours with IONO/PDB and infected afterwards. At 8, 17 and 24 h pi an immunofluorescence staining using biotinylated horse anti-EHV-1 polyclonal antibodies and streptavidine-Texas Red® was performed to detect viral antigen-positive cells. Clusters of PBMC are shown in Figure (a) as detected by light microscopy. Bar, 10 μm. Figures (b), (c) and (d) show the 3-dimensional images of a cluster containing 1, 2 and three adjacent infected cells, respectively, constructed by super-imposing the images obtained at 20 different sections throughout the cell by confocal microscopy. Bar, 5 μm.

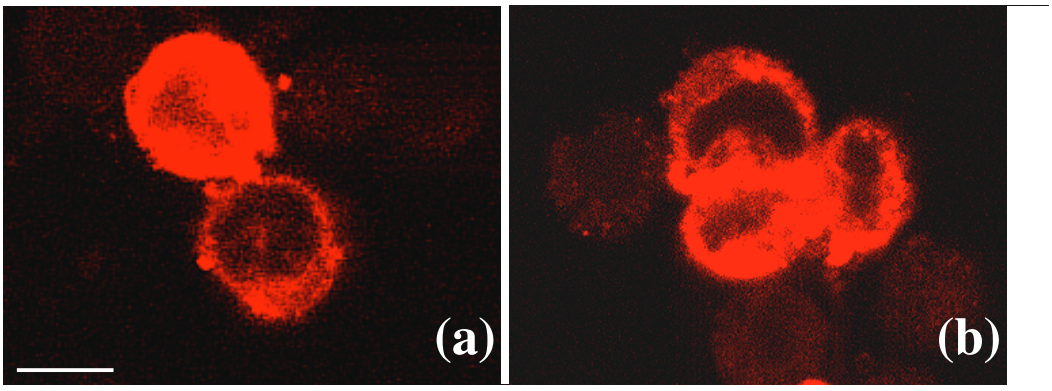

Figure 3.2.2. Close intercellular contacts are present between adjacent infected PBMC

Figures (a) and (b) show a single section throughout the infected cells in the clusters shown in Figure 1 (c) and (d), respectively. Bar, 5 μm.

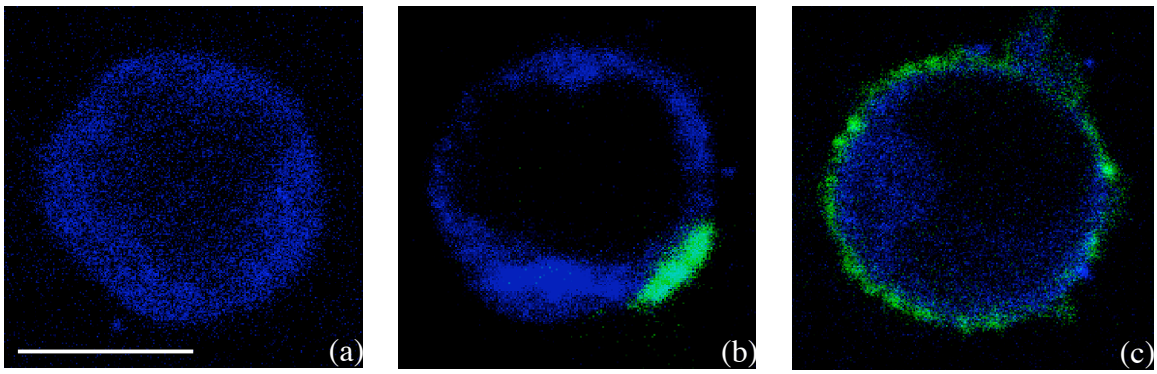

Figure 4.1.1. Expression patterns of viral antigens on the cell surface of EHV-1-infected PBMC

PBMC were stimulated during 24 hours with ionomycin/phorbol dibutyrate and infected afterwards. At 6, 9, 12 and 24 h pi, cells were fixed and an immunofluorescence staining was performed using FITC-labeled horse anti-EHV-1 IgG to detect cell surface expression of viral antigens and, after permeabilisation, biotinylated horse anti-EHV-1 IgG and streptavidin-Alexa Fluor® 350 to determine whether a cell was infected. Figures (a), (b) and (c) represent confocal images of an EHV-1-infected PBMC without surface expression, with expression on part of the cell surface ('focal expression'), and with expression on the entire cell surface ('general expression'), respectively. Bar 5 □m.

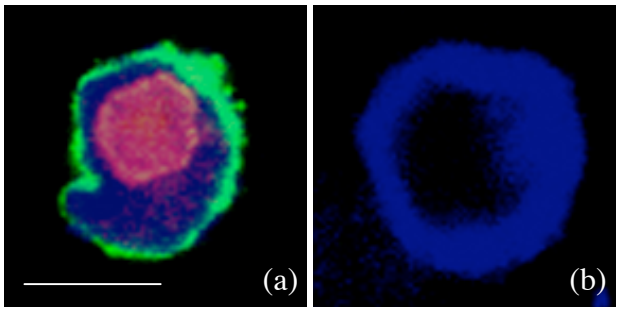

Figure 4.1.2. Absence of cell surface expression protects EHV-1-infected PBMC from complement-mediated cell lysis

PBMC were stimulated during 24 hours with ionomycin/phorbol dibutyrate and infected afterwards. At 24 h pi, a complement-mediated cell lysis assay was performed. Cells were successively incubated with protein G-purified and FITC-labeled horse anti-EHV-1 IgG (green), with non-inactivated EHV-1-negative horse serum as a source of complement and with ethidium monoazide bromide to determine the viability of the cells (red). After fixation and permeabilisation, biotinylated horse anti-EHV-1 IgG and streptavidin-Alexa Fluor® 350 were added to determine whether a cell was infected (blue). Pictures (a) and (b) are confocal images of a lysed, EHV-1-infected PBMC showing general cell surface expression and of a viable, EHV-1-infected PBMC without cell surface expression, respectively. Bar 5 □m

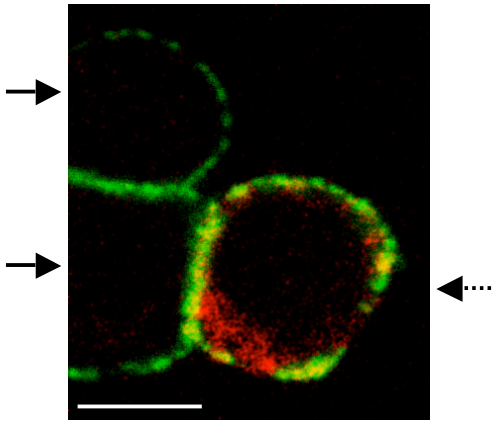

Figure 4.2.1. MHC class I is expressed on the surface of EHV-1-infected PBMC

PBMC were isolated from a pony experimentally inoculated with EHV-1. After fixation, cells were incubated with PT85A, a monoclonal mouse anti-MHC class I antibody, followed by goat anti-mouse-FITC (green). After subsequent permeabilisation, cells were incubated with biotinylayed horse anti-EHV-1 IgG followed by streptavidin-Texas Red□ (red). The picture shows a confocal image 2 non-infected PBMC (full arrow) and 1 EHV-1-infected (dashed arrow) PBMC showing a clear surface expression of MHC class I. Bar 5 □m

5. GENERAL DISCUSSION

GENERAL DISCUSSION

The first purpose of the studies described in this thesis was to closely examine the replication kinetics of equine herpesvirus 1 (EHV-1) and to identify the infected cells in freshly isolated as well as in mitogen-stimulated peripheral blood mononuclear cells (PBMC) *in vitro*. It was found that monocytes as well as T- and B-lymphocytes were susceptible to EHV-1 infection. The susceptibility of freshly isolated lymphocytes to EHV-1 was extremely low, but increased by stimulation of the cells with mitogens. Our finding that freshly isolated lymphocytes were highly refractory to EHV-1 infection *in vitro* is not fully in agreement with earlier findings in PBMC from experimentally inoculated ponies (Scott *et al.*, 1983). Scott *et al.* (1983) found that during viremia, T-lymphocytes were the predominant cell type that harboured virus even before culturing with mitogens *in vitro*. The disparity between the results of Scott *et al.* and our results may be explained as follows. It is known that an experimental EHV-1 infection of ponies results in an increase in the percentage of circulating blastic cells up to 40 % during viremia (McCulloch *et al.*, 1993). It is very well possible that the PBMC collected by Scott *et al.* (1983) had already undergone blastic transformation in the ponies as a consequence of infection, thereby replacing the effect of mitogen stimulation *in vitro*. We, on the other hand, collected PBMC from healthy horses which were likely to contain almost no blastic cells prior to mitogen stimulation, giving a probable explanation for the very low susceptibility of lymphocytes to EHV-1 in the freshly isolated samples.

Our *in vitro* studies demonstrated that the influence of mitogen stimulation on the susceptibility of PBMC to EHV-1 was 2-fold. First, stimulation initiated the entry of a limited number of lymphocytes into the cell cycle thereby enabling EHV-1 infection of these cells and, second, mitogens induced the formation of close intercellular contacts between PBMC thereby facilitating the cell-to-cell transmission of EHV-1. The positive role of the cell cycle on EHV-1 replication is most likely attributed to proliferation events active from late G1 onward, since an increase in the percentage of EHV-1-infected PBMC was highly correlated with an increase in the percentage of cells in the S-phase of the cell cycle. It seems rather surprising that EHV-1 benefits from factors active during the cell cycle, knowing that herpesviruses themselves contain a large array of genes,

which encode viral DNA-metabolizing enzymes. Nevertheless, the replication of several other herpesviruses depends on these factors as well. For example, herpes simplex virus 1 (HSV-1) replication requires the cyclin-dependent kinases 1, 2 or 7 which are involved in DNA transcription and synthesis while passing through the S-phase of the cell cycle (Schang *et al.*, 1998, 1999, 2000, 2002). Also, the replication of HSV-2 (Schang *et al.*, 1998), human cytomegalovirus (HCMV) (Bresnahan *et al.*, 1997) and varicella-zoster virus (VZV) (Taylor *et al.*, 2001) seems to benefit from cellular cyclin-dependent kinases, since blocking the activity of these kinases results in an inhibition of viral replication. Upon experimental EHV-1 infection of ponies, McCulloch *et al.* (1993) demonstrated an increase in the percentage of circulating blastic cells from 10 up to 40 % during viremia. Whether EHV-1 benefits from these proliferation events in PBMC *in vivo* remains unclear.

Beside via activation of specific cell cycle events, mitogens also influenced the replication of EHV-1 via the formation of clusters of PBMC. Mitogen stimulation induces the surface expression of several families of cell adhesion molecules, resulting in a cascade of binding events which lead to the formation of aggregates or clusters of PBMC (reviewed by Hogg and Landis, 1993). We found that the close intercellular contacts formed between PBMC within these clusters facilitated the transmission of EHV-1 from infected cells to uninfected, adjacent cells. Addition of virus-neutralizing antibodies in the medium during infection did not affect this transmission of EHV-1, proving that it occurred via direct cell-to-cell spread (van der Meulen *et al.*, 2002). Our findings that direct cell-associated transmission of EHV-1 is facilitated as a result of close intercellular contacts initiated by cell adhesion molecules together with the knowledge that adherence of equine leukocytes to equine endothelial cells requires the expression of certain adhesion molecules (Smith *et al.*, 2002) may suggest that adhesion molecules play a role in the transfer of virus from infected circulating PBMC to endothelial cells of the target organs, such as the pregnant uterus and the nervous system.

From the studies presented in this thesis, it was concluded that, in freshly isolated PBMC, monocytes are the most important cell fraction in which EHV-1 replicates. Our studies also allowed to conclude that lymphocytes become more susceptible following stimulation with mitogens because of the initiation of certain proliferation events in

stimulated cells and because of the facilitated cell-associated transmission of EHV-1 within stimulated cell clusters.

The second purpose of the studies described in the present thesis was to obtain more insights in the mechanism by which EHV-1-infected PBMC are able to circulate in the immune horse without efficiently being eliminated by the host's immune system. From our *in vitro* studies, it became clear that EHV-1-infected PBMC were able to express viral antigens on their cell surface, as described in Chapter 3.1. However, the PBMC that showed cell surface expression of viral antigens only represented one-third of all EHV-1-infected PBMC, as demonstrated by the studies described in Chapter 4.1. The remaining two-thirds lacked visually detectable levels of EHV-1 antigens on their cell surface. Since absence of EHV-1 antigens on the surface may prevent the binding of virus-specific antibodies to the infected cell, we hypothesized that PBMC without surface expression remain unrecognizable for the antibody-dependent components of the immune system. Indeed, it was demonstrated in our *in vitro* studies that infected PBMC without viral antigens on their surface, were insensitive to antibody-dependent, complement-mediated cell lysis and, most likely, to antibody-dependent, cell-mediated cytotoxicity as well. Focal surface expression rendered infected PBMC much less susceptible to antibody-dependent lysis in comparison to infected PBMC showing generalized surface expression. From these results, we concluded that absence of viral antigens on the cell surface, either partial or complete, represents a potential strategy of herpesvirus-infected PBMC to avoid antibody-dependent lysis, in addition to the strategies already described in Chapter 1.4.1 of the present thesis.

Amongst all EHV-1-infected PBMC *in vitro*, approximately 10 % showed a generalized expression of viral antigens on their surface. It is striking that despite the extensive presence of viral antigens on their surface, 70 % of these cells survived in the presence of antibody and complement. Two likely explanations may be put forward. First, the generally low efficiency of equine complement, as described by Ish *et al.* (1993), may have accounted for the high number of surviving cells showing generalized surface expression. Second, it is possible that certain EHV-1 glycoproteins expressed on the cell surface interfered with the activation of the complement cascade by displaying Fc receptor activity. For suid herpesvirus 1 (SuHV-1) (Favoreel *et al.*, 1999b) and varicella-

zoster virus (VZV) (Litwin *et al*., 1992), the glycoprotein complex gE/gI has been shown to possess such Fc receptor activity and expression of these glycoproteins has already been demonstrated on porcine blood monocytes (Favoreel *et al*., 2000) and human PBMC (Mainka *et al*., 1998). Moreover, Van de Walle *et al*. (in press) confirmed that expression of gE/gI on SuHV-1-infected monocytes helps to avoid efficient antibody-dependent, complement-mediated lysis. Homologues of gE and gI are also expressed by EHV-1 (Audonnet *et al*., 1990; Elton *et al*., 1991; Telford *et al*., 1992), but it remains the subject of future investigation to determine whether they possess Fc receptor activity.

We extended our studies to experimentally inoculated ponies to determine whether absence of viral antigens on the cell surface, as observed *in vitro*, also arises in EHV-1-infected PBMC during viremia. It was clearly demonstrated that lack of viral antigen expression on the surface of infected PBMC also occurred during the viremic phase of an acute EHV-1 infection, emphasizing the relevance of this potential strategy to avoid antibody-mediated lysis. However, infected PBMC may not only be destroyed via antibody-mediated lysis, but also via major histocompatibility complex (MHC) class I-dependent, cytotoxic T-lymphocyte (CTL)-mediated lysis. That this CTL response, like the antibody-dependent response, is not fully capable of eliminating EHV-1-infected PBMC in the horse is illustrated by the study of O'Neill *et al*. (1999). They demonstrated the presence of viremia in ponies despite high frequencies of EHV-1-specific CTL precursors which have the potency to kill EHV-1-infected cells. In the present studies, we examined whether absence of MHC class I molecules on the surface of EHV-1-infected PBMC could be responsible for inefficient elimination of the cells by CTL during viremia in experimentally inoculated ponies. It was found that 80 to 100 % of the EHV-1-infected PBMC showed surface expression of MHC class I molecules, which was similar as observed in non-infected PBMC. Therefore, we concluded that the impaired elimination of circulating infected PBMC by CTL during an EHV-1 infection in the horse does not arise from the absence of MHC class I molecules on the cell surface.

Lack of viral antigens on the surface of EHV-1-infected PBMC during viremia was observed independently of the immune status of the ponies, indicating that not the components of the immune system but the virus itself or virus - cell interactions are responsible for the lack of surface expression. This is different from the clearance of viral

antigens from the surface of SuHV-1-infected monocytes, which results from an antibody-dependent endocytosis (Favoreel *et al.*, 1999a). As already mentioned while describing the replication cycle of EHV-1 (Chapter 1.2.1), viral proteins are co-translationally transported into the endoplasmatic reticulum (ER) and, subsequently, transported via the Golgi apparatus to the cell surface. For VZV (Wang *et al.*, 1998) and SuHV-1 (Favoreel, personal communication), which show a very similar replication cycle as EHV-1, it was found that certain glycoproteins play an important role in this transport process. For example, expression of the VZV glycoproteins gE and gI is necessary for the other VZV glycoproteins gB, gC, gH and gL to reach the *trans*-Golgi network suggesting that gE and gI serve as "navigators" that guide the other glycoproteins to the *trans*-Golgi network (Wang *et al.*, 1998). For SuHV-1, it was demonstrated that cells infected with a gE^-/gI^- mutant displayed a reduced surface expression of gB suggesting that the gE/gI complex has a navigating role for at least some of the other viral glycoproteins in SuHV-1-infected cells (Favoreel, personal communication). We hypothesize that the cell surface expression in EHV-1-infected PBMC may also be mediated by one or more viral glycoprotein(s) that serve(s) as a "navigator" for all viral glycoproteins and, consequently, we suggest that absence of all viral glycoproteins on the cell surface may be the result of an impaired or even absent expression of that(those) glycoprotein(s) in the infected cell. Preliminary experiments carried out in our laboratory indicated that EHV-1 glycoprotein gC may be such a "navigator" protein. It was found that approximately two-thirds of the EHV-1-infected PBMC completely lacked expression of gC at 24 hours after inoculation, which is similar to the number of infected PBMC that lack expression of all glycoproteins on their cell surface at that time.

Even though absent surface expression of viral antigens on EHV-1-infected PBMC, as observed in our studies, may help in avoiding antibody-mediated lysis and even though other, yet unknown, strategies may help in avoiding CTL-mediated lysis, it is clear that a natural EHV-1 infection is able to induce a short-lived protective immune response against EHV-1-induced viremia. Indeed, horses do not develop viremia upon re-infection within 5 months after a previous EHV-1 infection despite the occurrence of viral replication in the respiratory tract (Edington *et al.*, 1990; Gibson *et al.*, 1992; Slater *et al.*,

1993; Tewari *et al.*, 1993). Also, challenge experiments performed in 2 immune ponies at our laboratory showed that at least 1 animal was still protected against viremia at 6 months after a previous infection (van der Meulen, unpublished results). EHV-1 re-infection at later time points following prior infection may induce viremia, but our *in vivo* study demonstrated that the number of circulating EHV-1-infected PBMC in the infection-immune ponies following challenge was 1.5- to 20-fold lower when compared to that in the non-immune pony. The relevance of the latter finding in view of protection against EHV-1-induced abortion or neurological disease remains to be confirmed, but it is logical to suggest that a reduction in the number of infected PBMC reduces the chance of interaction with endothelial cells of the target organs and, consequently, decreases the chance for EHV-1-induced abortion or neurological disease.

In contrast to the immunity evoked by natural EHV-1 infection, the immunity induced by vaccines which are commercially available for the prevention of EHV-1-induced abortion, is much less efficient to protect against EHV-1-induced viremia upon challenge (Burrows *et al.*, 1984; Bürki *et al.*, 1990; Heldens *et al.*, 2001). To find out which immunological factors are of key importance in determining the degree of viremia remains the subject for future research. We believe that the inability of EHV-1 to induce viremia is the result of an efficient local immune response that minimizes virus replication in the respiratory epithelia and, importantly, reduces invasion of the virus into the deeper tissues, thereby preventing EHV-1 from reaching the draining lymph nodes and the blood circulation. Whereas replication of wild type virus in the respiratory tract may be sufficient to induce such a local immune response, the restricted replication of the attenuated EHV-1 vaccine strains or the lack of replication of the inactivated EHV-1 vaccine strains in the respiratory tract may, in turn, be insufficient to induce such a local immune response.

The knowledge that the immunity induced by a natural infection is capable of protecting against EHV-1-induced viremia for at least 5 months after primary infection holds a promising message. Apparently, the immunity evoked by EHV-1 can prevent viremia for a short period of time, which strongly suggests that EHV-1-induced abortion and nervous system disorders may also be prevented. For future vaccine development, it seems indispensable to find out what components of the immune system achieve this

protective response against EHV-1-induced viremia. Efforts should be put in the development of vaccines that induce a strong local immunity. In this manner, replication of EHV-1 in the epithelium of the respiratory tract may be hindered and, subsequently, viremia may be prevented.

References

Audonnet, J. C., Winslow, J., Allen, G., Paoletti, E. (1990). Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI and gE. *Journal of General Virology* **71**, 2969-2978.

Bresnahan, W. A., Boldogh, I., Chi, P., Thompson, E. A., Albrecht, T. (1997). Inhibition of cellular Cdk2 activity blocks human cytomegalovirus replication. *Virology* **231**, 239-247.

Bürki, F., Rossmanith, W., Nowotny, N., Pallan, C., Möstl, K., Lussy, H. (1990). Viraemia and abortions are not prevented by two commercial equine herpesvirus-1 vaccines after experimental challenge of horses. *Veterinary Quarterly* **12**, 80-86.

Burrows, R., Goodridge, D., Denyer, M. S. (1984). Trials of an inactivated equid herpesvirus 1 vaccine: challenge with a subtype 1 virus. *Veterinary Record* **114**, 369-374.

Edington, N., Bridges, C. G. (1990). One way protection between equid herpesvirus 1 and 4 *in vivo*. *Research in Veterinary Science* **48**, 235-239.

Elton, D. M., Bonass, W. A., Killington, R. A., Meredith, D. M., Halliburton, I. W. (1991). Location of open reading frames coding for equine herpesvirus type-1 glycoproteins with homology to gE and gI of herpes simplex virus. *American Journal of Veterinary Research* **52**, 1252-1257.

Favoreel, H. W., Nauwynck, H. J., Halewyck, H. M., Van Oostveldt, P., Mettenleiter, T. C., Pensaert, M. B. (1999a). Antibody-induced endocytosis of viral glycoproteins and major histocompatibility complex class I on pseudorabies virus-infected monocytes. *Journal of General Virology* **80**, 1283-1291.

Favoreel, H. W., Nauwynck, H. J., Pensaert, M. B. (1999b). Role of the cytoplasmic tail of gE in antibody-induced redistribution of viral glycoproteins expressed on pseudorabies virus-infected cells. *Virology* **259**, 141-147.

Favoreel, H. W., Nauwynck, H. J., Van Oostveldt, P., Pensaert, M. B. (2000). Role of anti-gB and -gD antibodies in antibody-induced endocytosis of viral and cellular cell surface glycoproteins expressed on pseudorabies virus-infected monocytes. *Virology* **267**, 151-158.

Gibson, J. S., Slater, J. D., Awan, A. R., Field, H. J. (1992). Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. *Archives of Virology* **123**, 351-366.

Heldens, J. G. M., Hannant, D., Cullinane, A. A., Prendergast, M. J., Mumford, J. A., Nelly, M., Kydd, J. H., Weststrate, M. W., van den Hoven, R. (2001). Clinical and virological evaluation of the efficacy of an inactivated EHV1 and EHV4 whole virus vaccine (Duvaxyn $EHV_{1,4}$). Vaccination/challenge experiments in foals and pregnant mares. *Vaccine* **19**, 4307-4317.

Hogg, N., Landis, R. C. (1993). Adhesion molecules in cell interactions. *Current Opinions in Immunology* **5**, 383-390.

Ish, C., Ong, G. L., Desai, N., Mattes, M. J. (1993). The specificity of alternative complement pathway-mediated lysis of erythrocytes: a survey of complement and target cells from 25 species. *Scandinavian Journal of Immunology* **38**, 113-122.

Litwin, V., Jackson, W., Grose, C. (1992). Receptor properties of two varicella-zoster virus glycoproteins, gpI and gpIV, homologous to herpes simplex virus gE and gI. *Journal of Virology* **66**, 3643-3651.

Mainka, C., Fuss, B., Geiger, H., Hofelmayr, H., Wolff, M. H. (1998). Characterization of viremia at different stages of varicella-zoster virus infection. *Journal of Medical Virology* **56**, 91-98.

McCulloch, J., Williamson, S. A., Powis, S. J., Edington, N. (1993). The effect of EHV-1 infection upon circulating leucocyte populations in the natural equine host. *Veterinary microbiology* **37**, 147-161.

O'Neill, T., Kydd, J. H., Allen, G. P., Wattrang, E., Mumford, J. A., Hannant, D. (1999). Determination of equid herpesvirus 1-specific, $CD8^+$, cytotoxic T lymphocyte precursor frequencies in ponies. *Veterinary Immunology and Immunopathology* **70**, 43-54.

Schang, L. M., Phillips, J., Schaffer, P. A. (1998). Requirement for cellular cyclin-dependent kinases in herpes simplex virus replication and transcription. *Journal of Virology* **72**, 5626-5637.

Schang, L. M., Rosenberg, A., Schaffer, P. A. (1999). Transcription of herpes simplex virus immediate-early and early genes is inhibited by roscovitine, an inhibitor specific for cellular cyclin-dependent kinases. *Journal of Virology* **73**, 2161-2172.

Schang, L. M., Rosenberg, A., Schaffer, P. A. (2000). Roscovitine, a specific inhibitor of cellular cyclin-dependent kinases, inhibits herpes simplex virus DNA synthesis in the presence of viral early proteins. *Journal of Virology* **74**, 2107-2120.

Schang, L. M. (2002). Cyclin-dependent kinases as cellular targets for antiviral drugs. *Journal of Antimicrobial Chemotherapy* **50**, 779-792.

Scott, J. C., Dutta, S. K., Myrup, A. C. (1983). *In vivo* harboring of equine herpesvirus-1 in leukocyte populations and subpopulations and their quantitation from experimentally infected ponies. *American Journal of Veterinary Research* **44**, 1344-1348.

Smith, D., Hamblin, A., Edington, N. (2002). Equid herpesvirus 1 infection of endothelial cells requires activation of putative adhesion molecules: an *in vitro* model. *Clinical and Experimental Immunology* **129**, 281-287.

Slater, J. D., Gibson, J. S., Field, H. J. (1993). Pathogenicity of a thymidine kinase-deficient mutant of equine herpesvirus 1 in mice and specific pathogen-free foals. *Journal of General Virology* **74**, 819-828.

Taylor, S. L., Moffat, J. F. (2001). VZV replication *in vitro* is prevented by roscovitine, an inhibitor of the cell cycle. *In:* Proceedings of the Fourth International Conference on VZV. La Jolla, California, Abstract A35.

Telford, E. A., Watson, M. S., McBride, K., Davison, A. J. (1992). The DNA sequence of equine herpesvirus-1. *Virology* **189**, 304-316.

Tewari, D., Gibson, J. S., Slater, J. D., O'Neill, T., Hannant, D., Allen, G. P., Field, H. J. (1993). Modulation of the serological response of specific pathogen-free (EHV-free) foals to EHV-1 by previous infection with EHV-4 or a TK-deletion mutant of EHV-1. *Archives of Virology* **132**, 101-120.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2002). Increased susceptibility of peripheral blood mononuclear cells to equine herpes virus type 1 infection upon mitogen stimulation: a role of the cell cycle and of cell-to-cell transmission of the virus. *Veterinary Microbiology* **86**, 157-163.

van de Walle, G. R., Favoreel, H. W., Nauwynck, H. J., Pensaert, M. B. (2003). Antibody-induced internalization of viral glycoproteins and gE-gI receptor activity protect pseudorabies virus-infected monocytes from efficient complement-mediated lysis. *Journal of General Virology* **84** (in press).

Wang, Z., Gershon, M. D., Lungu, O., Panagiotidis, C. A., Zhu, Z., Hao, Y., Gershon, A. A. (1998). Intracellular transport of varicella-zoster glycoproteins. *Journal of Infectious Diseases* **178** *Supplement 1*, 7-12.

6. SUMMARY / SAMENVATTING

6.1 SUMMARY

6.2 SAMENVATTING

6.1 SUMMARY

Equine herpesvirus 1 (EHV-1) is recognized worldwide as a major pathogen of horses causing serious economic losses due to abortion, neonatal foal death and, less frequently, nervous system disorders. Peripheral blood mononuclear cells (PBMC) play a key role in the pathogenesis of an EHV-1 infection, both in naive and in immune horses, transporting EHV-1 from the primary site of replication to the target organs such as the pregnant uterus and the nervous system.

In Chapter 1, the current knowledge on the characteristics of EHV-1 and on the interaction of EHV-1 with its host cell as well as with the naive and the immune horse were reviewed, with emphasis on the role of infected PBMC. Furthermore, the different strategies that are exploited by herpesviruses to circumvent elimination of infected PBMC by the immune system of the host were described.

In Chapter 2, the aims of the studies described in the present thesis were put forward. Our first aim was to obtain more detailed information on the kinetics of EHV-1 replication and on the identity of the infected cells in freshly isolated PBMC as well as in mitogen-stimulated PBMC. Subsequently, we wanted to unravel the effect of mitogens on the susceptibility of PBMC to EHV-1. Our second aim was to obtain some insights in how EHV-1-infected PBMC are able to avoid efficient elimination by humoral and cellular components of the host's immune system. Studies were performed using *in vitro* infected PBMC as well as infected PBMC isolated from experimentally inoculated ponies.

The first aim was addressed in Chapter 3:

In Chapter 3.1, a detailed study was performed on the replication of EHV-1 in freshly isolated, equine PBMC and the effect of mitogen stimulation on the replication kinetics of EHV-1 was investigated. Therefore, cytoplasmic and plasma membrane expression of viral antigens, intra- and extracellular virus titres and plaque formation in co-culture were determined in a population of freshly isolated and of mitogen-stimulated PBMC following inoculation with the Belgian EHV-1 strain 97P70. EHV-1 replicated in freshly isolated monocytes, although in a highly restricted way. Viral antigens were found in a maximum number of 8.7 % of the monocytes at 12 hours post inoculation (h

pi). The infection was productive in only 0.16 % of the monocytes, with a virus yield of $10^{0.7}$ tissue culture infectious dose $_{50}$ ($TCID_{50}$) per productive cell. In a population of resting lymphocytes, 0.9 % of the cells were infected and less than 0.05 % produced infectious virus. The virus yield was $10^{0.1}$ $TCID_{50}$ per productive cell. However, after prestimulation with different mitogens, the number of EHV-1-infected lymphocytes increased 4 to 12 times. The susceptible lymphocytes were T-lymphocytes. No infected B-lymphocytes were detected due to a loss of these cells during subsequent stimulation, inoculation and incubation. In mitogen-stimulated PBMC, a clear expression of viral antigens was found on the plasma membrane of 8 % of all cells. In conclusion, it can be stated that in freshly isolated PBMC, monocytes are the most important cell fraction in which EHV-1 replicates. After mitogen stimulation of the PBMC, T-lymphocytes become more susceptible.

In the first part of Chapter 3.2, the identity of the EHV-1-infected, mitogen-stimulated PBMC was examined more thoroughly. It was found that among the viral antigen-positive PBMC, 38.4 ± 4.5 % were $CD5^+$ T-lymphocytes (18.1 ± 3.2 % $CD4^+$; 13.6 ± 1.8 % $CD8^+$), 18.1 ± 5.4 % were B-lymphocytes, 8.5 ± 3.9 % were monocytes and 35 % remained unidentified. This composition of EHV-1-infected PBMC reflected well the composition of the total population of PBMC. In the second part of Chapter 3.2, the mechanism(s) by which mitogen stimulation affected the susceptibility of PBMC to EHV-1 were characterized. The role of the cell cycle in the increased susceptibility to EHV-1 upon stimulation was examined by stimulating PBMC during 0, 12, 24 and 36 hours prior to inoculation. A high correlation was found between the increase of cells in the S- ($r = 0.974$) and G2/M-phase ($r = 0.927$) at the moment of inoculation and the increase of infected cells at 12 h pi. This suggests that a specific stage of the S-phase or S- and G2/M-phase facilitates viral replication. At 24 h pi, lower correlations were found suggesting that other effects are involved. From 12 hours after addition of ionomycin/ phorbol dibutyrate (IONO/PDB), formation of clusters or aggregates of PBMC became manifest. It was examined if close intercellular contacts in these clusters facilitated cell-to-cell transmission of EHV-1. Between 8 and 17 h pi, the percentage of clusters containing adjacent infected cells increased from 1.6 to 13.4 % and the maximal number of adjacent infected cells increased from 2 to 4. Confocal microscopy visualized close

intercellular contacts between adjacent infected cells. It can be concluded that mitogen stimulation positively influences an EHV-1 infection in 2 ways: (i) by initiating specific cell cycle events and (ii) by inducing the formation of clusters of PBMC, thereby facilitating transmission of EHV-1 from infected cells to uninfected adjacent cells.

The second aim of the studies described in the present thesis was addressed in Chapter 4:

In Chapter 4.1, an *in vitro* study was performed to investigate how EHV-1-infected PBMC are able to avoid elimination by the antibody-dependent immune responses. It was found that the majority of EHV-1-infected PBMC (approximately 70 %) lacked expression of viral antigens on their surface. Absence of surface expression was observed independently of the subtype of PBMC. Among the remaining 30 % of the infected PBMC that did express viral antigens on their surface, two-thirds expressed antigens on part of the cell surface only, a status designated 'focal expression', and one-third expressed EHV-1 antigens on the entire surface, a status designated 'general expression'. When EHV-1-inoculated PBMC were incubated in the presence of antibody and of serum as a source of complement, it was found that between 1.2 and 3.6 % of the infected PBMC without cell surface expression were lysed. This percentage was similar as observed for non-infected PBMC suggesting that the PBMC without cell surface expression are insensitive to complement-mediated lysis. In contrast, for infected PBMC showing general surface expression, between 19.4 and 31.2 % of the cells were lysed in the presence of antibody and complement, which was significantly higher when compared to non-infected PBMC. When complement was added in the absence of antibodies, 10.5 % of PBMC showing general surface expression were lysed, whereas addition of antibodies in the absence of complement resulted in 5.4 % lysed cells. PBMC showing general surface expression are apparently destroyed by three different immune mechanisms. Binding of antibodies to the surface of these infected PBMC in the absence of complement triggered destruction most likely via antibody-dependent, cell-mediated cytotoxicity (natural killer (NK) cells and phagocytes). Lysis by complement in the absence of antibodies most likely occurred via the antibody-independent, complement-mediated pathway, whereas addition of antibodies increased the percentage of lysed cells by activation of the antibody-dependent, complement-mediated pathway. For infected

PBMC showing focal surface expression, the percentage of lysed cells was only significantly higher compared to non-infected PBMC when an antibody concentration of 1.6 mg/ml and a serum concentration of 20 % were used (8.3 % lysed cells). In summary, at least two-thirds of EHV-1-infected PBMC lack cell surface expression of viral antigens. Absence of surface expression was observed independently of the subtype of PBMC. It protects infected PBMC from antibody-dependent and antibody-independent lysis by equine complement and may, therefore, represent a potential strategy of immune evasion.

Based on the findings of the *in vitro* study, it was examined in Chapter 4.2 whether EHV-1-infected PBMC also lacked surface expression of viral antigens during viremia upon experimental inoculation of immune ponies. Moreover, surface expression of major histocompatibility complex (MHC) class I molecules was examined on infected PBMC, since MHC class I molecules present viral peptides to cytotoxic T-lymphocytes (CTL) and thus also play a role in making infected cells recognizable for the immune system. Four infection-immune ponies and 1 EHV-1-negative control pony were inoculated with the Belgian EHV-1 strain 97P70. PBMC were collected at various time points between 0 and 28 days post inoculation (d pi). Surface expression of viral antigens and MHC class I molecules on EHV-1-infected PBMC was determined by immunofluorescence labeling. For the immune ponies, expression of viral antigens on the surface of EHV-1-infected PBMC was only observed in 1 pony in 1 out of 8 examined EHV-1-infected PBMC (14 d pi). In the other three immune ponies, all examined EHV-1-infected PBMC lacked visually detectable levels of plasma membrane-anchored viral antigens (0/7, 0/8 and 0/8 infected PBMC, respectively). Also in the non-immune control pony, none of the EHV-1-infected PBMC showed surface expression of viral antigens (0/18 infected PBMC). Cell surface expression of MHC class I molecules was found in 80 to 100 % of the EHV-1-infected PBMC in the immune ponies as well as in 88 % of the EHV-1-infected PBMC in the non-immune pony. This resembled the percentage of MHC class I-positive cells found in non-infected PBMC (83 to 100 %). We concluded from the *in vivo* study that, by far, the most EHV-1-infected PBMC lack viral antigen expression on their cell surface during viremia, thereby enabling these cells to avoid recognition and destruction by the antibody-mediated immune response. The percentage of EHV-1-infected PBMC showing

surface expression of MHC class I during viremia was 80 to 100 %. Therefore, absence of cell surface expression of MHC class I molecules will not account for the inefficient recognition of EHV-1-infected PBMC by CTL.

Following general conclusions can be made from the studies performed in the present thesis:

(1) T-lymphocytes, B-lymphocytes and monocytes are susceptible to EHV-1 infection. Lymphocytes become more susceptible following stimulation with mitogens because of the initiation of certain proliferation events in stimulated cells and the facilitated cell-associated transmission of EHV-1 within stimulated cell clusters.

(2) Absence of viral antigens on the cell surface, either partial or complete, represents a potential strategy of herpesvirus-infected PBMC to avoid antibody-dependent lysis.

(3) The impaired elimination of circulating infected PBMC by CTL during an EHV-1 infection in the horse does not arise from an absence of MHC class I molecules on the cell surface.

(4) The number of circulating EHV-1-infected PBMC in the infection-immune ponies following challenge is reduced when compared to that in the non-immune pony. It is logical to suggest that a reduction in the number of infected PBMC during viremia reduces the chance of their interaction with endothelial cells of the target organs and, consequently, reduces the incidence of EHV-1-induced abortion or neurological disease.

6.2 SAMENVATTING

Het equine herpesvirus 1 (EHV-1) is een belangrijk pathogeen voor paarden. Wereldwijd veroorzaken EHV-1-geïnduceerde uitbraken van abortus, neonatale sterfte en, in mindere mate, zenuwstoornissen voor grote economische verliezen. Perifere bloed mononucleaire cellen (PBMC) spelen een sleutelrol in de pathogenese van een EHV-1 infectie, zowel in naïeve paarden als in immune paarden. Ze vervoeren het virus van de plaats van primaire vermeerdering naar de doelwitorganen zoals de drachtige baarmoeder en het zenuwstelsel.

In hoofdstuk 1 werd een overzicht gegeven van de huidige kennis over het EHV-1, over zijn interactie met de gastheercel en over zijn interactie met het naïeve en het immune paard. Er werd hierbij vooral aandacht besteed aan de rol van de PBMC. Daarnaast werden de verschillende immuno-evasieve mechanismen besproken die herpesvirus-geïnfecteerde PBMC in staat stellen om te ontsnappen aan de antistof-afhankelijke en de cel-afhankelijke immuunreacties van de gastheer.

In hoofdstuk 2 van deze thesis werden de doelstellingen van de verschillende studies uiteengezet. Een eerste doelstelling bestond er uit om de kinetiek van vermeerdering van EHV-1 te bestuderen en om de identiteit van geïnfecteerde cellen te bepalen, enerzijds in een populatie van vers geïsoleerde PBMC en anderzijds in een populatie van PBMC gestimuleerd met mitogenen. Bijkomend wilden we nagaan wat het effect is van mitogene stimulatie op de gevoeligheid van PBMC voor een EHV-1 infectie. De tweede doelstelling bestond er uit om enkele inzichten te verwerven in de wijze waarop EHV-1-geïnfecteerde PBMC hun uitschakeling door antistof- en major histocompatibility complex (MHC) klasse I-afhankelijke immuunreacties kunnen ontlopen. Dit werd onderzocht aan de hand van *in vitro*-geïnfecteerde PBMC en geïnfecteerde PBMC geïsoleerd uit experimenteel geïnoculeerde pony's.

In hoofdstuk 3 kwam de eerste doelstelling aan bod:

In hoofdstuk 3.1 werd een gedetailleerde studie uitgevoerd van de vermeerderingskinetiek van EHV-1 in vers geïsoleerde equine PBMC en in equine PBMC gestimuleerd met verschillende mitogenen. Diverse parameters werden bestudeerd

zoals de expressie van virale antigenen in het cytoplasma en op de plasmamembraan, intracellulair en extracellulair geproduceerde virushoeveelheden en de vorming van plaques in een co-cultuur van PBMC en konijnenniercellen (RK13). Vermeerdering van EHV-1 vond plaats in vers geïsoleerde monocyten, zij het in beperkte mate. Virale antigenen werden teruggevonden in maximum 8,7 % van de monocyten op 12 uren na inoculatie. Een productieve infectie was slechts aanwezig in 0,16 % van de monocyten en de virusopbrengst per productief geïnfecteerde cel bedroeg $10^{0,7}$ weefselcultuur infectieuze dosis. In een populatie van vers geïsoleerde lymfocyten waren slechts 0,9 % van de cellen geïnfecteerd en minder dan 0,05 % produceerde infectieus virus. De virusopbrengst per productief geïnfecteerde lymfocyt bedroeg $10^{0,1}$ weefselcultuur infectieuze dosis. Werden de lymfocyten daarentegen gestimuleerd met mitogenen voorafgaande aan de infectie, dan nam het aantal geïnfecteerde lymfocyten 4 tot 12 maal toe. De geïnfecteerde cellen in de populatie van gestimuleerde lymfocyten waren vooral T lymfocyten. Er werden geen geïnfecteerde B lymfocyten waargenomen, maar dit was te wijten aan een verlies van B lymfocyten tijdens de achtereenvolgende handelingen van stimulatie, inoculatie en incubatie. In een populatie van gestimuleerde PBMC konden bij 8 % van de cellen virale antigenen worden aangetoond op de plasmamembraan. Uit deze eerste studie werd besloten dat vermeerdering van EHV-1 in vers geïsoleerde PBMC vooral plaatsvindt in de monocyten. Worden de PBMC gestimuleerd met mitogenen voorafgaande aan de infectie, dan worden de T lymfocyten meer gevoelig voor een EHV-1 infectie. Over een eventuele toename in de gevoeligheid van de B lymfocyten na stimulatie kon geen uitspraak gedaan worden.

In het eerste deel van hoofdstuk 3.2 werd de identiteit van de met mitogenen gestimuleerde en met EHV-1-geïnoculeerde PBMC meer in detail uitgewerkt. De populatie van geïnfecteerde, gestimuleerde PBMC was als volgt samengesteld: 38,4 ± 4,5 % $CD5^+$ T lymfocyten (18,1 ± 3,2 % $CD4^+$; 13,6 ± 1,8 % $CD8^+$), 18,1 ± 5,4 % B lymfocyten, 8,5 ± 3,9 % monocyten en 35 % ongeïdentificeerde cellen. Deze samenstelling van de geïnfecteerde PBMC was zeer gelijkaardig aan de samenstelling van de totale populatie van PBMC. In het tweede deel van hoofdstuk 3.2 werd getracht te ontrafelen hoe mitogenen de gevoeligheid van PBMC voor een EHV-1 infectie kunnen doen toenemen. Eerst werd de rol van de celcyclus bestudeerd door PBMC gedurende

verschillende tijdsintervallen te stimuleren en vervolgens te infecteren. Er bleek een zeer goede correlatie te bestaan tussen de toename van het aantal cellen in de S-fase na stimulatie en de toename van het aantal geïnfecteerde cellen tussen 0 en 12 uren na inoculatie (r = 0,974). Ook de toename van het aantal cellen in de G2/M-fase na stimulatie was nauw gecorreleerd met de toename van het aantal geïnfecteerde cellen tussen 0 en 12 uren na inoculatie (r = 0,927). Deze bevinding suggereert dat welbepaalde processen die optreden tijdens de S-fase of de S- en G2/M-fase, de vermeerdering van EHV-1 in PBMC kunnen bevorderen. Op 24 uren na inoculatie werden lagere correlaties teruggevonden, wat er op wijst dat ook andere factoren een invloed uitoefenen op de vermeerdering van EHV-1. Vanaf 12 uren na het toedienen van mitogenen aan PBMC begonnen de cellen aggregaten of clusters te vormen. Er werd nagegaan of de intercellulaire contacten tussen PBMC in deze clusters de celgeassocieerde overdracht van EHV-1 konden vergemakkelijken. Het aantal clusters met aanééngesloten geïnfecteerde cellen nam toe van 1,6 % op 8 uren tot 13,4 % op 17 uren na inoculatie. Daarenboven nam het aantal aanééngesloten geïnfecteerde cellen binnen de clusters toe van maximaal 2 tot maximaal 4. Met behulp van confocale microscopie konden de nauwe, intercellulaire contacten tussen aanééngesloten geïnfecteerde cellen duidelijk in beeld gebracht worden. Er werd geconcludeerd dat mitogenen een EHV-1 infectie van PBMC op 2 manieren beïnvloeden: (i) mitogenen stimuleren het optreden van welbepaalde processen verbonden aan de celcyclus die op hun beurt de virusvermeerdering vergemakkelijken en (ii) mitogenen induceren nauwe intercellulaire contacten tussen PBMC die het celgeassocieerd spreiden van het virus van geïnfecteerde cellen naar niet-geïnfecteerde aanéénsluitende cellen ten goede komen.

De tweede doelstelling werd nader uitgewerkt in hoofdstuk 4:

In hoofdstuk 4.1 werd een *in vitro* studie verricht die tot doel had te onderzoeken hoe EHV-1-geïnfecteerde PBMC kunnen ontkomen aan antistof-afhankelijke immuunreacties. Er werd gevonden dat de meerderheid van de EHV-1-geïnfecteerde PBMC (ongeveer 70 %) geen virale antigenen tot expressie brengt op de plasmamembraan. Dit fenomeen werd zowel bij T lymfocyten, B lymfocyten als monocyten waargenomen. De resterende 30 % van de EHV-1-geïnfecteerde PBMC vertoonde wel plasmamembraanexpressie, hetzij op het volledige celoppervlak (10 %),

hetzij op slechts een deel van het celoppervlak (20 %). Om na te gaan of de afwezigheid van plasmamembraanexpressie de geïnfecteerde cellen onherkenbaar kan maken voor antistof-afhankelijke, complement-gemedieerde cellyse, werden EHV-1-geïnoculeerde PBMC geïncubeerd in de aanwezigheid van EHV-1-specifieke antistoffen en EHV-1-negatief paardenserum als bron van complement. Het percentage lyse in de populatie van geïnfecteerde PBMC zonder plasmamembraanexpressie bedroeg nooit meer dan 3,6 %, onafhankelijk van de gebruikte antistof- en serumconcentraties. Dit percentage was gelijk aan het percentage lyse gevonden in een populatie van niet-geïnfecteerde PBMC. Een dergelijke bevinding wijst er op dat geïnfecteerde PBMC zonder plasmamembraanexpressie niet gevoelig zijn aan complement-gemedieerde cellyse. Daarentegen werden in de populatie van geïnfecteerde PBMC met volledige plasmamembraanexpressie 19,4 tot 31,2 % gelyseerde cellen teruggevonden. Werd paardenserum toegevoegd zonder antistoffen, dan bedroeg het percentage gelyseerde PBMC met volledige plasmamembraanexpressie nog altijd 10,5 % en toevoeging van antistoffen zonder paardenserum resulteerde in 5,4 % gelyseerde cellen. Blijkbaar kunnen PBMC met volledige plasmamembraanexpressie op drie verschillende manieren vernietigd worden. Binding van antistoffen aan de virale antigenen op de plasmamembraan in de afwezigheid van complement veroorzaakt waarschijnlijk een vernietiging van de geïnfecteerde cel door antistof-afhankelijke, celgemedieerde cytotoxiciteit (door natural killer (NK) cellen en fagocyten). Lyse in de aanwezigheid van complement maar in de afwezigheid van antistoffen verloopt waarschijnlijk via de antistof-onafhankelijke complement cascade. Tenslotte zal de gelijktijdige aanwezigheid van antistoffen èn complement bijkomend resulteren in antistof-afhankelijke, complement-gemedieerde cellyse. Het percentage lyse in de populatie van geïnfecteerde PBMC met slechts een gedeeltelijke plasmamembraanexpressie was enkel significant hoger dan de niet-geïnfecteerde PBMC wanneer 1,6 mg antistof/ml en 20 % paardenserum werden gebruikt (8,3 % gelyseerde cellen). Samengevat kunnen we stellen dat tenminste 70 % van de EHV-1-geïnfecteerde PBMC geen virale antigenen tot expressie brengt op de plasmamembraan. Afwezigheid van plasmamembraanexpressie beschermt de geïnfecteerde cellen tegen antistof-afhankelijke en antistof-onafhankelijke

lyse door paardencomplement en vormt daarom een mogelijk immuno-evasief mechanisme van EHV-1-geïnfecteerde PBMC.

In hoofdstuk 4.2 werd vervolgens nagegaan of afwezigheid van plasmamembraanexpressie van EHV-1 antigenen op geïnfecteerde PBMC ook voorkomt tijdens de viremie in experimenteel geïnoculeerde, immune pony's. Bovendien werd de expressie van MHC klasse I moleculen op de EHV-1-geïnfecteerde PBMC aanwezig tijdens de viremie, nader bestudeerd. Immers, ook MHC klasse I moleculen spelen een belangrijke rol bij de herkenning en vernietiging van geïnfecteerde cellen. Ze presenteren virale peptiden aan cytotoxische T-lymfocyten (CTL) die op hun beurt de geïnfecteerde cel lyseren. Afwezigheid van MHC klasse I moleculen zou mede verantwoordelijk kunnen zijn voor de inefficiënte herkenning van EHV-1-geïnfecteerde PBMC door het immuunsysteem van het paard. Voor de *in vivo* studie werden 4 infectie-immune pony's en 1 EHV-1-negatieve controle pony geïnoculeerd met het Belgische EHV-1 isolaat 97P70 en vervolgens werden PBMC verzameld tussen 0 en 28 dagen na inoculatie. De expressie van virale antigenen en MHC klasse I moleculen op de EHV-1-geïnfecteerde PBMC werd bestudeerd aan de hand van immunofluorescentiekleuringen. Bij de immune pony's werd plasmamembraanexpressie van virale antigenen enkel waargenomen bij 1 pony en slechts op 1 van de 8 onderzochte EHV-1-geïnfecteerde PBMC (14 dagen na inoculatie). Bij de andere 3 immune pony's vertoonde geen enkele van de onderzochte EHV-1-geïnfecteerde PBMC zichtbare expressie van virale antigenen op hun plasmamembraan (0 op 7, 0 op 8 en 0 op 8 geïnfecteerde PBMC respectievelijk). Ook bij de EHV-1-negatieve controle pony werden geen PBMC met plasmamembraanexpressie van virale antigenen waargenomen (0 op 18 geïnfecteerde PBMC). Plasmamembraanexpressie van MHC klasse I was daarentegen aanwezig op 80 tot 100 % van de EHV-1-geïnfecteerde PBMC bij de immune pony's en op 88 % van de EHV-1-geïnfecteerde PBMC bij de EHV-1-negatieve controle pony. Deze percentages waren gelijkaardig aan de percentages geobserveerd in niet-geïnfecteerde PBMC (83 tot 100 %). Hieruit kunnen we besluiten dat de overgrote meerderheid van de EHV-1-geïnfecteerde PBMC aanwezig tijdens de viremie, geen virale antigenen tot expressie brengt op de plasmamembraan. Aldus hebben deze geïnfecteerde cellen een mogelijke immuno-evasieve strategie om te ontsnappen aan de antistof-afhankelijke immuunreacties van het

paard. Het percentage EHV-1-geïnfecteerde PBMC met plasmamembraanexpressie van MHC klasse I moleculen was 80 tot 100 %. Een inefficiënte herkenning van EHV-1-geïnfecteerde PBMC door CTL zal dus niet te wijten zijn aan afwezigheid van MHC klasse I moleculen op de plasmamembraan.

Op basis van de studies uitgevoerd in deze thesis kunnen volgende algemene conclusies naar voren worden gebracht:

(1) Zowel T lymfocyten, B lymfocyten als monocyten zijn gevoelig voor een infectie met EHV-1. Lymfocyten worden gevoeliger door voorafgaande stimulatie met mitogenen als gevolg van het optreden van bepaalde proliferatieprocessen in gestimuleerde cellen en als gevolg van een vergemakkelijkt celgeassocieerd spreiden van het virus binnen clusters van gestimuleerde cellen.

(2) Een gehele of gedeeltelijke afwezigheid van virale antigenen op de plasmamembraan van geïnfecteerde PBMC vormt een mogelijke verklaring waarom EHV-1-geïnfecteerde PBMC kunnen ontsnappen aan de antistof-afhankelijke componenten van het immuunsysteem.

(3) De inefficiënte herkenning van circulerende geïnfecteerde PBMC wordt niet veroorzaakt door een afwezigheid van MHC klasse I moleculen op de plasmamembraan.

(4) Bij de infectie-immune pony's is het aantal EHV-1-geïnfecteerde PBMC aanwezig tijdens de viremie na herinfectie, lager dan dat in de EHV-1-negatieve pony. Het lijkt logisch dat een reductie in het aantal circulerende geïnfecteerde PBMC leidt tot een verminderde kans op interactie tussen deze PBMC en de endotheelcellen van de doelwitorganen en, bijgevolg, tot een verminderd aantal gevallen van EHV-1-geïnduceerde abortus of zenuwstoornissen.

7. CURRICULUM VITAE

CURRICULUM VITAE

Karen van der Meulen werd geboren op 9 december 1973 in Zeist, Nederland. In 1992 beëindigde zij haar secundaire opleiding aan het Elshof College te Nijmegen, Nederland, en behaalde ze het diploma van het Voorbereidend Wetenschappelijk Onderwijs (VWO). In datzelfde jaar werd de studie diergeneeskunde aangevangen aan de Universiteit van Gent in België. In 1998 behaalde ze het diploma van dierenarts met grote onderscheiding. Daarenboven werd haar tijdens de uitreiking van het diploma de Prijs van de Faculteit Diergeneeskunde toegekend, evenals de Pharmacia Upjohn Award voor haar eindejaarswerk getiteld “Computer tomografie van het straalbeen bij het paard”. Onmiddellijk na het beëindigen van haar studies trad Karen in dienst als assistent van het Laboratorium voor Virologie, Vakgroep Virologie, Parasitologie en Immunologie. Daar verrichtte ze onderzoek naar de interactie van het equine herpesvirus 1 met de witte bloedcellen van het paard en naar enkele mogelijke strategieën die de geïnfecteerde witte bloedcellen in staat stellen te ontsnappen aan de immuniteit van het paard. Dit onderzoek heeft aanleiding gegeven tot een 4-tal publicaties, waarvan er reeds 3 gepubliceerd zijn in internationale wetenschappelijke tijdschriften met zeer hoge impactfactor. Naast het verrichten van onderzoek was Karen ook betrokken bij de diagnostiek van het equine herpesvirus en het equine arteritisvirus in Belgïe, evenals bij het practische onderricht in de algemene virologie aan de studenten van de derde kandidatuur diergeneeskunde en in de optierichting paard aan de studenten van de derde proef diergeneeskunde.

Publicaties in internationale wetenschappelijke tijdschriften

van der Meulen, K. M., Nauwynck, H. J., Buddaert, W., Pensaert, M. B. (2000). Replication of equine herpesvirus type 1 in freshly isolated equine peripheral blood mononuclear cells and changes in susceptibility following mitogen stimulation. *Journal of General Virology* **81**, 21-25.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2001). Mitogen stimulation favours replication of equine herpesvirus 1 in equine blood mononuclear cells by inducing cell proliferation and formation of close intercellular contacts. *Journal of General Virology* **82**, 1951-1957.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2003). Absence of viral antigens on the surface of equine herpesvirus 1-infected blood mononuclear cells: a strategy to avoid complement-mediated lysis. *Journal of General Virology* **84**, 93-97.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2003). Expression of viral antigens and MHC class I molecules on the surface of equine herpesvirus 1-infected blood mononuclear cells during viremia in immune ponies. *Submitted.*

Proceedings

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2002). Increased susceptibility of peripheral blood mononuclear cells to equine herpes virus type 1 infection upon mitogen stimulation: a role of the cell cycle and of cell-to-cell transmission of the virus. *Veterinary Microbiology* **86**, 157-163.

Publicaties in nationale wetenschappelijke tijdschriften

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2000). Equine herpesvirus type 1 abortion, neonatal foal death and nervous system disorders diagnosed in Belgium in 1999. *Vlaams Diergeneeskundig Tijdschrift* **69**, 38-41.

van der Meulen, K. M., Caij, A., Nauwynck, H. J., Pensaert, M. B. (2001). An outbreak of equine viral arteritis abortion in Belgium. *Vlaams Diergeneeskundig Tijdschrift* **70**, 221-222.

Abstracts

van der Meulen, K. M., Nauwynck, H. J., Favoreel, H. W., Pensaert, M. B. (2000). Replication of equine herpesvirus type 1 in equine peripheral blood mononuclear cells *in vitro*: kinetics and identification of the susceptible cells. 25th International Herpesvirus Workshop, Portland, VS.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2001). Increased susceptibility of peripheral blood mononuclear cells to equine herpes virus type 1 infection upon mitogen stimulation: a role of the cell cycle and of cell-to-cell transmission of the virus. 1st ESVV Veterinary Herpesvirus Symposium, Zürich, Zwitserland.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2001). Absence of viral antigens and MHC-I on the plasma membrane of EHV-1-infected PBMC *in vitro*: A mechanism of immune evasion? 26th International Herpesvirus Workshop, Regensburg, Duitsland.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2001). Absence of viral antigens and MHC-I on the plasma membrane of EHV-1-infected PBMC *in vitro*: A mechanism of immune evasion? Symposium "Towards understanding Microbial Interactions" van de Belgische Vereniging voor Microbiologie, Louvain-La-Neuve, België.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2001). Recente gegevens over equine virale arteritis (Equine viral arteritis: an update). 18de Symposium van de Belgian Equine Practioners Society, Brussel, België.

van der Meulen, K. M., Nauwynck, H. J., Pensaert, M. B. (2002). Escape of equine herpesvirus 1-infected blood mononuclear cells from complement-mediated lysis. 27th International Herpesvirus Workshop, Cairns, Australië.

8. DANKWOORD

DANKWOORD

Na heel wat maanden van zwoegen en zweten lijkt het eindpunt van mijn doctoraat in zicht. Er wacht echter nog een belangrijke taak en dat is iedereen bedanken die geholpen heeft bij het tot stand komen van mijn doctoraatswerk.

Allereerst wil ik mijn beide promotoren bedanken. Hans, ik denk dat jij het best te omschrijven bent als de *perpetuum mobile* van de wetenschappelijke ideëen. Jij bent een onmisbare drijfveer geweest bij het maken van mijn doctoraat. Jouw vele ideëen maken dat menig jonge doctoraatsstudent, inclusief mijzelf, besmet raakt met het onderzoeksvirus. Bovendien ben ik dankzij jou nu één van de weinige Nederlanders die een klein woordje West-Vlaams spreekt. Merci!

Professor Pensaert, u zou ik graag bedanken voor de kennis die u mij de afgelopen jaren heeft bijgebracht. Uw kritische visie heeft me niet alleen veel geleerd over de wetenschap. Het heeft mij ook geleerd om de niet-wetenschappelijke wereld eerst goed te interpreteren vooraleer tot een conclusie te komen.

I would like to thank the members of the accompanying committee, Professor Mettenleiter from the Federal Research Centre for Virus Diseases of Animals in Insel Riems in Germany, Professor Thiry from the Faculty of Veterinary Medicine at the University of Liege as well as Professor Deprez and Professor Ducatelle from the Faculty of Veterinary Medicine at Ghent University. Thank you all for critically reading the manuscript and for coming forward with many inventive suggestions to improve my thesis.

De Universiteit Gent zou ik willen bedanken voor de mogelijkheid die ze mij, en vele andere Nederlandse studenten, geboden heeft om de studie diergeneeskunde aan te vangen. Daarnaast wens ik de Universiteit Gent te bedanken voor de jaren na mijn studie die ik heb kunnen invullen als assistent en die geleid hebben tot het huidige doctoraatswerk.

Al mijn collega's wil ik van harte bedanken voor de goede werksfeer en voor de steun tijdens de wetenschappelijke of persoonlijke 'ups' en 'downs'. Allereerst bedank ik mijn bureaugenoten. Herman, mijn eerste bureaugenoot, jij bent naast Hans de persoon die mij heeft binnengeloodst in de wondere wereld der immuno-evasie. Geoffrey, mijn

tweede en meest duurzame bureaugenoot, we hebben heel wat lief en leed gedeeld en ik kan niet anders zeggen dan dat ik jou heel erg waardeer. Griet, mijn jongste bureaugenoot, ik ben blij dat ik nu een 'paarden'maatje heb. Gerlinde, je bent niet alleen een fijne collega, maar ook een fijne vriendin. Kristin, bedankt voor alle gezellige thee-pauzes van de afgelopen maanden. Steven, jouw onstuitbaar enthousiasme voor de wetenschap is een voorbeeld voor menig doctoraatsstudent. Peter, dank je wel dat ik elke keer weer opnieuw jouw boek over immunologie heb mogen lenen, ook al staat het volledig op het internet. Geert, Peter en Nathalie, jullie bedank ik voor de hulp bij het oplossen van al mijn 'moleculaire' problemen. En daarenboven ... bedankt Geert, voor de vele keren dat je de lamp van de fluorescentiemicroscopen hebt vervangen, omdat ik weeral niet wist hoe het ook weer in elkaar stak, Peter, voor je aanstekelijk wetenschappelijk enthousiasme en Nathalie, voor je kritische inbreng bij het klaarstomen van bepaalde verslagen en publicaties. Kristien, jou wil ik bedanken voor je hulp bij het opstellen van de practica voor de derde kandidatuur en voor het inspringen bij de practica wanneer ik even in tijdnood zat. Romeo, thanks for spreading those lovely Eastern flavours every time you have diner. Chris en Dieter, bedankt voor de vele kleuringen die jullie hebben uitgevoerd. En ... Chris, voor mij ben je bovenal een luisterend oor op het laboratorium waar je altijd terecht kan. Chantal, bedankt voor de vele miljoenen witte bloedcellen die je hebt geïsoleerd uit het paardenbloed en Lieve, voor je geduldige uitleg over virologische testen. En jullie beiden bedank ik om altijd maar weer opnieuw uit te leggen hoe men nu een infectiviteitstiter moet bepalen. Dat wil toch moeilijk in mijn hoofd blijven hangen. Carine, ook jou ben ik veel dank verschuldigd voor alle hulp bij mijn voorbije experimenten en ik waardeer je hulp en interesse voor ons huidige serologisch onderzoek. Fernand, bedankt voor je hulp met de ponies. Ik denk dat ze het net zoveel gewaardeerd hebben als ik. En ook bedankt voor de zorg die je draagt voor de 'inboedel' van het laboratorium. Trees, jij hebt er toe bijgedragen dat de vele isolaties van witte bloedcellen altijd weer tot een goed einde gebracht konden worden door er voor te zorgen dat er altijd medium en PBS was. Gert, jij verdient een eervolle vermelding als entertainer die mij altijd wel een (glim)lach kon ontlokken. Liliane, bedankt voor alle administratieve hulp tijdens de afgelopen jaren. En hetzelfde geldt natuurlijk voor Mieke, maar dan tijdens de afgelopen weken. Dirk, op jou kon ik altijd rekenen als er weer een

weerbarstige computer bijgestuurd moest worden. Marijke, bedankt voor de ontspannende babbeltjes tijdens het kuisen van ons bureau en, bovenal, bedankt dat je het laboratorium elke dag weer piekfijn in orde te brengt. En tenslotte wil ik alle mensen bedanken die inmiddels het laboratorium hebben verlaten, want ook zij waren stuk voor stuk fijne collega's.

Behalve op mijn werk zijn er ook talrijke mensen daarbuiten die ik wil bedanken. Omdat het zwaartepunt van het doctoraat niet langer op het wetenschappelijk gedeelte, maar op het dankwoord zou komen te liggen wanneer ik iedereen persoonlijk ga bedanken, zal ik mij beperken tot mijn naasten. Papa en mama, jullie wil ik bedanken voor de onaflatende steun die jullie mij gegeven hebben tijdens de studie diergeneeskunde, tijdens mijn doctoraat en tijdens alle andere stappen in mijn leven die genomen zijn of op het punt staan genomen te worden. Peter, bedankt voor de goede zorgen en bezorgdheden voor je 'kleine' zusje. Je bent voor mij altijd een groot voorbeeld geweest, al denk ik niet dat ik ooit de wereld zal rondtrekken. En oma, ik ben blij dat ik zoveel op je lijk!

Aleid en Ester, bedankt voor jullie trouwe vriendschap en steun tijdens de studie en tijdens de afgelopen jaren. Ik hoop dat we alledrie kunnen blijven vechten voor onze dromen en dat we ze alledrie uiteindelijk kunnen realiseren.

David, jou wil ik bedanken voor je begrip en steun tijdens de afgelopen maanden als ik weer eens een heel weekend achter de computer verscholen zat, voor de soms broodnodige dosis relativeringsvermogen als ik dacht dat de wereld enkel nog maar uit virussen bestond en voor je hulp bij het afwerken van de thesis. Deze thesis is voor mij een belangrijke mijlpaal in mijn leven. Er zullen echter nog vele andere mijlpalen volgen, sommige zelfs al zeer binnenkort, en ik kijk er naar uit om ze allemaal samen met jou te bereiken.

En tenslotte, misschien wat ongebruikelijk, zou ik graag de verschillende trouwe viervoeters bedanken die toch een onmisbare bijdrage hebben geleverd aan mijn doctoraat. Bedankt Babette, Kleine, Mieke, paardjes van verloskunde, Zwarte, Vos, Hengst en Bruine. En bovenal bedankt Smokey, voor de bijdrage aan de wetenschap en voor de vele aangename borsteluurtjes na het werk.