Functional and immunological analysis of glycoproteins gC and gE of equine herpesvirus type 1

馬ヘルペスウイルス1型糖蛋白gCとgEの機能及び免疫学的解析

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Chapter 1

General introduction of equine herpesviruses

1.1. Classification

The family *Herpesviridae* consists of the mammal, bird and reptile viruses and is classified into four subfamilies. Herpesviruses naturally infected to horses are named as equine herpesvirus (EHV) -1, -2, -3, -4, -5, -8 and -9. EHV-1, -3, -4, -8 and -9 belong to the subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. On the other hand, EHV-2 and -5 belong to the subfamily *Gammaherpesvirinae*, genus *Percavirus* (Davison et al., 2009).

EHV-1 and -4 are closely related both genetically and antigenically and show cross-reactivity in many serological tests such as virus-neutralization (VN) test, complement-fixation (CF) test and immunoblot analysis (Patel and Heldens, 2005). Until 1981, both viruses were considered to be the same virus (Studdert et al., 1981). EHV-3 is different from EHV-1 and -4 antigenically, genetically, and pathogenically (Allen et al., 1977; Baumann et al., 1986; Gutekunst et al., 1978; Hartley et al., 1999; Staczek et al., 1983). EHV-8 is called “Asinine herpesvirus 3” and more closely related to EHV-1 than EHV-4 from the result of DNA-DNA hybridization, serum neutralization and immunoprecipitation analyses (Browning et al., 1988; Crabb and Studdert, 1990; Crabb et al., 1991). EHV-9 is called “Gazelle herpesvirus” and serologically cross-reacted with EHV-1 and -4 (Fukushi et al., 1997).

1.2. Genome and structure

The genome of herpesvirus is composed of unique long (U_L) and short (U_S) regions and a set of inverted repeat sequences, internal and terminal repeat sequences flanking U_S region (IR_S and TR_S). There are 6 sequence arrangement patterns of the double-stranded DNA genomes in herpesviruses, based on the location of repeats of terminal sequences and EHV-1 and -4 have a type D DNA genomes. Complete genomic maps and sequences of EHV-1 and -4 had been reported (Cullinane et al., 1988; Nagesha et al., 1992; Telford et al., 1992, 1998; Whalley et al., 1981) and the complete genome of EHV-l strain Ab4 and EHV-4 strain NS80567 were composed of 150,223 bp and 145,597 bp, respectively (Telford et al., 1992, 1998). EHV-1 genome contains 80 ORFs encoding proteins, two ORFs are duplicated in the IRs and TRs, so EHV-1 is considered to contain 76 unique genes (Telford et al., 1992, 1998).

Herpesvirus virion consists of three major structures, core, capsid and envelope (Wildy et al., 1960). Viral genome DNA is contained in the core as the form of torus (Furlong et al., 1972). Core and capsid form a nucleocapsid and surrounded by tegument proteins, and enclosed by an envelope derived from the host cell lipid bilayer (Miyamoto and Morgan, 1971). Many glycoproteins are located on the surface of envelope and playing important roles in binding to the receptor, penetration into the cell, virus egress and cell-to-cell spreading. Until now, glycoprotein B (gB), gC, gD, gE, gG, gH, gI, gK, gL, gM, gN and gp2 have been reported for EHV-1.

1.3. Epidemiology

EHV-1 and -4 are major causative agents of equine respiratory disease in horse, and show a wide geographic distribution, which cause serious economic losses worldwide (Allen and Bryans, 1986). EHV infection occurs through inhalation of the virus (Patel et al., 1982). In addition, respiratory secretions, fetuses, and placentas discharged from infected horses also transmit the viruses. It is reported that horses experimentally infected with EHV-1 shed the virus as early as day 1 post infection (Gardiner et al., 2012) and that most horses infected with EHV-1 cease shedding the virus within approximately 1-2 weeks post infection (Gibson et al., 1992). In Japan, EHV-1 infections have occurred predominantly in winter season at the training centers. On the other hand, EHV-4 mainly infects to young horses in breeding farms throughout the year (Matsumura et al., 1992).

1.4. Pathogenesis and clinical presentation

The respiratory tract is the natural portal of entry for EHV-1 and -4, and the respiratory mucosal epithelium is the primary target tissue for infection (Allen and Bryans, 1986; Allen et al., 1999; Gibson et al., 1992), resulting in cytolytic destruction of the nasopharyngeal respiratory epithelium, secretions and inflammatory response (Gibson et al., 1992; Kydd et al., 1994). Following the destruction, pneumonia and mucopurulent nasal discharge are induced by a secondary bacterial infection (Crabb and Studdert, 1995). Following the first infection, EHV-1 infects dendritic cells and

macrophages in the local lymph nodes, resulting in leukocyte-associated viraemia (Dutta and Myrup, 1983; Edington et al., 1986; Patel et al., 1982; Scott et al., 1983). The cell-associated viraemia has an important role in the pathogenicity of EHV-1 (Kydd et al., 2012) and it is reported that the highly pathogenic strains of EHV-1 show severe cell-associated viraemia than lower pathogenic strains (Goodman et al., 2007). The cell-associated viraemia has been shown to be an essential process for abortion and central nervous system (CNS) disorders, because replication of EHV-1 in endothelial cell lining of blood vessels in the CNS and pregnant uterus causes severe vasculitis and multifocal thrombosis, resulting in EHV-1-specific symptoms, like abortion and paresis (Edington et al., 1986, 1991; Patel et al., 1982; Smith et al., 1992, 1993). In contrast to EHV-1, leukocyte-associated viraemia, abortions and CNS disorder are not a consistent feature of EHV-4 (Edington et al., 1986; Matsumura et al., 1992; Tewari et al., 1993). EHV-4 replicates predominantly in the upper respiratory tract and rarely occurs viraemia (Dunowska, 2014).

These differences in cell-associated viraemia are thought to be one of the important differences between EHV-1 and EHV-4. EHV-1 infects vascular endothelial cells following viraemia, resulting in cause of vasculitis in blood vessels of the CNS or gravid uterus that is a trigger for the abortion or neurological disease (Allen et al., 2002; Gibson et al., 1992; Osterrieder and Van de Walle, 2010). However, it is known that there are very tight barrier to pass through the utero-placental barrier and blood-brain barrier, indicating that EHV-1 might have the other functions

to get across these barriers. The mechanism of EHV-1 to pass through these barriers and reach at the target tissue must play key roles in EHV-1 pathogenesis.

1.5. Diagnosis

Virus isolation, polymerase chain reaction (PCR), detection of viral antigens by immunofluorescence assay (IFA) and serological test to detect antibody to EHVs are used for the diagnosis of EHVs infection (Allen, 2000; Crabb and Studdert, 1995). However, since there are no perfect diagnostic methods, at least two methods are required for definitive diagnosis. In serological tests, paired serum samples, that are collected at the acute and convalescent phases of the disease, have to be compared (Thomson et al., 1976). Virus isolation from nasopharyngeal secretions, blood leukocytes and internal organs of aborted fetus are the most reliable method to detect EHVs. The cytopathic effect of EHV-1 and EHV-4 is characteristic, and sero-identification of two herpesviruses can be made with (Yeargan et al., 1985). However, virus isolation procedures also have the disadvantage on time-consuming and concerning on biosafety. Furthermore, since there is no available cell line originated from horse before this thesis and primary cells prepared from kidney of fetus have been required for virus isolation, virus isolation of EHVs can be carried out only in special facilities. To detect viral DNA in the tissues, PCR is rapid and sensitive assay (Borchers et al., 1993; Lawrence et al., 1994; Varrasso et al., 2001; Wagner et al., 1992). In addition, loop-mediated

isothermal amplification (LAMP) and real time PCR assays were also reported from several groups (Nemoto et al., 2010; Tewari et al., 2013). Antigen detection methods are also available for the rapid diagnosis of EHVs infection. Cells from nasopharyngeal secretions can be stained using immunofluorescent-labelled antibodies. Recently, type-specific antibody detection enzyme-linked immunosorbent assay (ELISA) using gG as antigens was developed and used for the serological diagnosis (Crabb and Studdert, 1993; Crabb et al., 1995; Hartley et al., 1999; Maeda et al., 2003; Yasunaga et al., 1998, 2000).

1.6. Latency

Latent infection is novel strategy of herpesviruses for surviving in the natural host and spreading among the natural host (Whitley and Gnann, 1993). Natural host organs where EHV-1 and EHV-4 exist latently are sensory neurons of the trigeminal ganglia and the T lymphocytes within lymphoid tissue (Baxi et al., 1995; Borchers et al., 1999; Chesters et al., 1997; Edington et al., 1994; Gibson et al., 1992; Slater et al., 1994; Welsh et al., 1992). In the cell latently infected with EHV, the viral genome is present without producing any structural proteins. The latent herpesvirus genome persists in a non-integrated, transcriptionally restricted state.

1.7. Vaccination

Vaccination to control respiratory diseases by EHV-1 infection began in the early 1940s in the U.S.A. using virus antigens prepared *in vivo,* but turned out to be ineffective (Doll et al., 1955; Doll and Bryans, 1963). EHV-1 isolates propagated in tissue culture led to an inactivated vaccine containing an adjuvant in the 1970s (Mayr et al., 1978). Experimental findings in hamsters and horses with the latter vaccine formed the basis of some currently licensed products in Europe. In parallel, tissue cultured inactivated vaccine was developed in the USA and initially appeared to be effective against EHV-1 challenge (Bryans, 1978). In later years, it became apparent that none of these vaccines could protect against EHV-1 mediated abortions (Burki et al., 1990; Burrows et al., 1984; Mumford et al., 1991). Following the realization that EHV-1 and EHV-4 were distinct viruses, newer inactivated vaccines included both viruses (Heldens et al., 2001). Currently, many vaccines including inactivated virus antigens are marketed. These vaccines mainly claim protection against respiratory disease due to EHV-1 and EHV-4, but not against abortion. Although inactivated vaccine elicits humoral immune response and very low cellular immune responses, suggesting that only neutralizing antibody is insufficient to protect horses from EHV-1 pathogenesis. Since cytotoxic T lymphocyte (CTL) plays an important role in clearance of virus-infected cells, CTL activity is thought to be critical for the clearance of EHV-1 infected leukocyte, indicating that cellular immune response is essential to prevent leukocyte-associated viraemia. It is reported that EHV-1 UL56 downregulates the expression of major histocompatibility complex class I (MHC-I) (Fuang et al.,

2014). In addition, it is also reported that EHV-1 and -4 use MHC-I as the cell receptor (Azab et al., 2014; Kurtz et al., 2010; Sasaki et al., 2011). These reports suggest that EHVs may interfere MHC-I to downregulate the cellular immune response. Therefore, a new vaccine is desired to induce high level of CTL response.

Recently, genetic engineering of a viral genome has been applied for the design of new vaccines. Using this gene modification technique, the attenuation of alphaherpesviruses has been achieved by the deletion of gene, which contributes to viral virulence. In Japan, EHV-1 mutant defective in gE gene, which plays an important role for EHV-1 pathogenesis, is under development in Japan Racing Association (JRA) (Tsujimura et al., 2006). This new gene modified live vaccine might be able to control cell-associated viraemia.

1.8. Glycoproteins

The genome of alphaherpesviruses encodes 9-11 glycoproteins and these glycoproteins play very important roles in the virus lifecycle. Glycoproteins are located on the surface of virus particle and infected cell surface, resulting in contribution for virus-host cell interactions such as adsorption, penetration, release and cell fusion. From the study of herpes simplex virus (HSV), gB, gC, gD and gH-gL are important for the virus attachment to cell receptors (Arii et al., 2010; Atanasiu et al., 2010; Montgomery et al., 1996). Furthermore, it is reported that EHV-1 gG binds to

chemokines and act as a viral chemokine binding protein, resulting in blocking function of chemokines (Bryant et al., 2003). Therefore, these glycoproteins are the principle targets for host immune responses and become the candidates for the subunit vaccine antigens (Awasthi et al., 2011; Keadle et al., 1997).

1.8.1. gB

Function of EHV-1 gB is virus attachment to the host cell, penetration and cell-to-cell spreading (Wellington et al., 1996; Neubauer et al., 1997). The gB has been identified as a major target protein for neutralizing antibody (Allen et al., 1992). Some groups reported about subunit vaccine using gB in small animal models (Foote et al., 2006; Kukreja et al., 1998; Osterrieder et al., 1995). Furthermore, it is also reported that recombinant vaccinia virus expressing EHV-1 gB showed the protection efficacy (Bell et al., 1990; Guo et al., 1990). EHV-1 gB-specific receptor is still unknown. Recently, paired immunoglobulin (Ig)-like type 2 receptor α (PILRα) was identified as the HSV gB specific receptor (Arii et al., 2010).

1.8.2. gC

EHV-1 gC, originally referred to as gp13 (Allen and Coogle, 1988; Matsumura et al., 1993), was shown to be a glycosylated component of the virion and a major target of the humoral

immune response after EHV-1 infection or vaccination (Allen and Yeargan, 1987) and is involved in virus adsorption to cells (Osterrieder, 1999). gCs of HSV-1, pseudorabies virus (PRV) and bovine herpesvirus 1 (BHV-1) mediate the initial stage of viral attachment by interacting with cellular heparan sulfate determinants on the surface of proteoglycans (Herold et al., 1991; Li et al., 1995; Mettenleiter and Rauh, 1990; Okazaki et al., 1991; WuDunn and Spear, 1989). EHV-4 gC also reported that playing an important role in viral binding to cell surface via the heparan sulfate (Azab et al., 2010). Studies on early HSV-cell interactions have revealed that heparan sulfate moieties of the cell plasma membrane serve as a receptor for initial binding of HSV. This assertion is based on findings that heparin, a related glycosaminoglycan, inhibits virus attachment (WuDunn and Spear, 1989), HSV adsorption to heparan sulfate-deficient mutant Chinese hamster ovary (CHO) cells is severely impaired (Shieh et al., 1982), and enzymatic digestion of cell surface heparan sulfate reduces HSV binding (WuDunn and Spear, 1989). In addition, it is reported that haemagglutination (HA) activity of HSV-1 gC against murine red blood cell (RBC) is inhibited by treatment with heparin (Noda et al., 1993). Furthermore, herpesviral gC can bind to the complement component C3b (Eisenberg et al., 1987; Friedman et al., 1984), and EHV-1 and EHV-4 gCs also perform a homologous complement receptor function (Huemer et al., 1995). Binding to this complement factor may protect herpesvirus-infected cells from complement-mediated lysis (Fries et al., 1986). EHV-1, HSV-1 and PRV gCs have been shown to induce cytotoxic T cell for cellular immune response

(Rosenthal et al., 1987; Soboll et al., 2003; Stokes et al., 1996; Zuckermann et al., 1990).

1.8.3. gD

EHV-1 gD is also involved in virus entry (Whittaker et al., 1992). Therefore, gD is also the major immunogen. It is reported that EHV-1 entry via endocytosis is facilitated by interaction between alpha V integrins and gD (Van de Walle et al., 2008). Furthermore, it is reported that EHV-1 gD enables EHV-4 to infect a non-permissive cell line (Whalley et al., 2007). This report indicated that gD is involved in cell tropism. Recent studies have revealed that the MHC-I molecule serves as the entry receptor for EHV-1 and -4 and that the binding glycoprotein is gD (Azab et al., 2014; Kurtz et al., 2010; Sasaki et al., 2011)

1.8.4. gG

EHV-1 and -4 gGs have interesting features. They were expressed as a secreted protein after proteolytic processing. It is reported that EHV-1 gG binds to chemokines of human and mouse origin as a viral chemokine binding receptor (virokine or viroceptor), resulting in blocking interaction between chemokines and chemokine-specific receptors (Bryant et al., 2003). It is reported that inflammatory response was exacerbated by EHV-1 with deletion of gG (Von Einem et al., 2007). Furthermore, it is also reported that EHV-1 gG inhibits chemotaxis of equine neutrophils *in vitro*

(Van de Walle et al., 2007). EHV-4 gG is also secreted into the medium of infected cells. In contrast to EHV-1, EHV-4 gG has no chemokine binding activity (Bryant et al., 2003; Crabb and Studdert, 1993; Van de Walle et al., 2007). Therefore, these activities of EHV-1 gG may be involved in EHV-1 pathogenesis.

1.8.5. gE and gI

EHV-1 gE and gI are encoded by ORF 74 and 73, respectively (Audonnet et al., 1990). gE and gI of herpesviruses are displayed on the surface of infected cells as heterodimer. It is reported that gE and gI of HSV-1 have a Fc receptor function for the immunoglobulin G (IgG) (Johnson et al., 1987, 1988). The function as the Fc receptor mediates clearance of antibodies and antigens from infected cell surface (Ndjamen et al., 2014). Furthermore, it is also reported that gE and gI are responsible for HSV-1 movement inside neuronal cells, cell-to-cell spreading and neurovirulence (Dingwell et al., 1998; Farnsworth et al., 2006; Howard et al., 2013; Polcicova et al., 2005; Snyder et al., 2008; Wang et al., 2010). In EHVs, some groups reported that gE/gI of EHV-1 and -4 are responsible for the virus virulence (Damiani et al., 2000; Matsumura et al., 1998).

1.9. The aim of our study

Although EHV-1 and -4 are closely related both genetically and antigenically, there are

many differences between both viruses. Especially, it is still unknown what factor is responsible for the EHV-1-specific pathogenesis, abortion and CNS disorder. Furthermore, there is no complete vaccine to protect horses from abortion by EHV-1 infection.

The aim of our study is clarifying the virulence factor of EHV-1 and contributing to the vaccine development. In this study, we established a new equine derived cell line for research on EHVs and analyzed the haemagglutination activity by EHV-1, but not by EHV-4. Furthermore, a novel ELISA using peptide was established for differentiation between EHV-1 and EHV-4 infections and between vaccination and infection with EHV-1 field strain.

1.10. Figure legends

Fig. 1. 1 A schematic diagram of major glycoproteins of EHVs and its functions. Functions seem to be related in immune-evasion are shown in red. Some glycoproteins have the activity to bind cytokine, complement component and immunoglobulin. These functions may be involved in the immune suppression.

Fig. 1. 2 A schematic diagram of EHV spreading in the horse body. EHVs firstly infect endothelial cells, following spreading to lymphoid tissues and ganglia. After infection, EHV-1 causes leukocyte associated viraemia, resulting in vasculitis. On the other hand, EHV-4 predominantly infects locally.

Fig. 1. 1

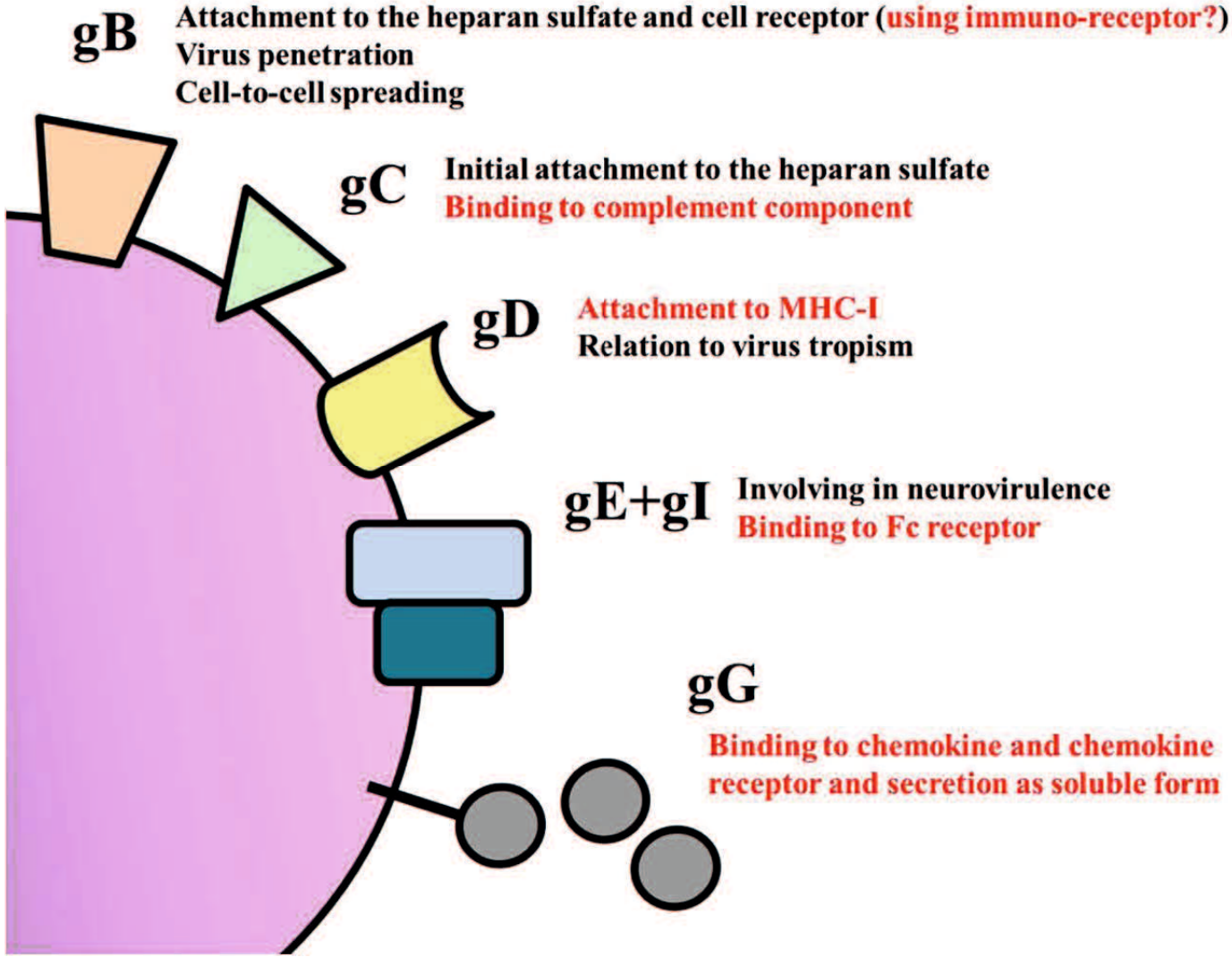
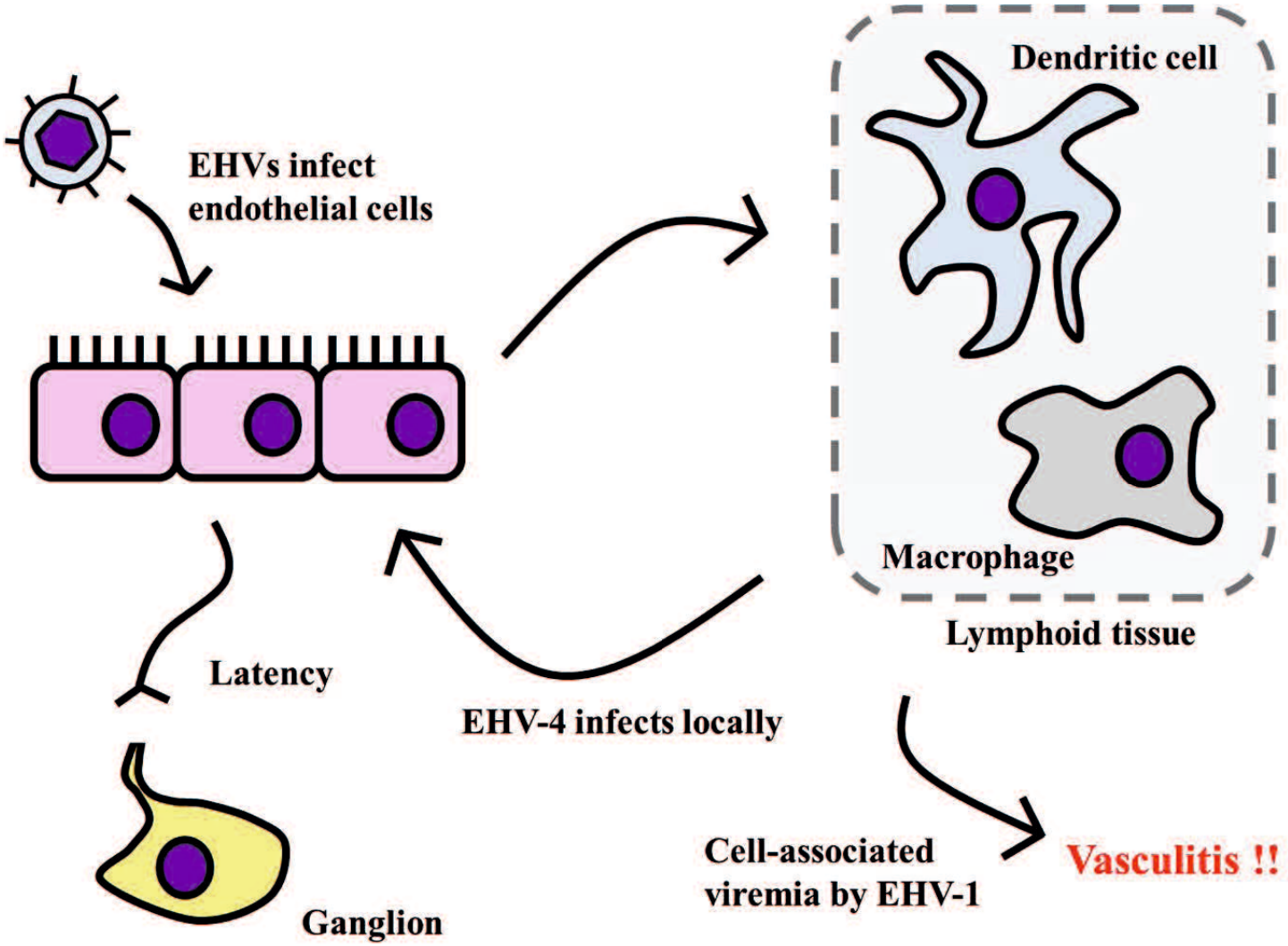

gB
Attachment to the heparan sulfate and cell receptor (using immuno-receptor?)
Virus penetration
Cell-to-cell spreading
gC
Initial attachment to the heparan sulfate
Binding to complement component
gD
Attachment to MHC-I
Relation to virus tropism
gE+gI
Involving in neurovirulence
Binding to Fc receptor
gG
Binding to chemokine and chemokine receptor and secretion as soluble form

Fig.1. 2


Dendritic cell
EHVs infect
endothelial cells
Macrophage
Lymphoid tissue
Latency
EHV-4 infects locally
Cell-associated
viremia by EHV-1
Vasculitis !!
Ganglion

Chapter 2

Establishment of equine cell line “FHK-Tcl3.1”.

2.1. Summary

Cell lines originated from horses are necessary for isolation and propagation of EHVs. In the previous study, we established the equine-derived cell, FHK-Tcl3. However, the growth of FHK-Tcl3 cells ceased after less than 40 passages (Maeda et al., 2007). In this study, FHK-Tcl3 cell kept growing after 40 passages and was propagated over 100 times. Therefore, FHK-Tcl3 cell was cloned by limiting dilution at 100th passage and cloned cell line, FHK-Tcl3.1, was established. FHK-Tcl3.1 cell grew well and was propagated every 3-4 days by splitting 1:5. In addition, EHV-1, -2 and -4 showed clear CPE on the FHK-Tcl3.1 cell and these CPE were very similar to those on parental FHK-Tcl3 and primary fetal horse kidney (FHK) cells. The FHK-Tcl3.1 cell continues to grow and the passage history is over 300 times after cloning. Therefore, this cell seemed to be immortalized. To our knowledge, this is the first report of equine cell line that could be propagated over 100 times. This FHK-Tcl3.1 cell should be useful for growth and diagnosis of the other equine viruses as well as EHVs.

2.2. Introduction

EHV-1 can grow well in many cell lines, equine-, rabbit- and bovine-derived cell lines and so on. However, it has been reported that EHV-1 propagated in non-equine cells possesses a mutation in the gC gene and became more susceptible to heparin (Sugahara et al., 1997). On the other hand, EHV-4 has more restricted host-specificity and grows well only in the cell derived from horses such as primary FHK cells. Furthermore, EHV-4 which was adapted to Madin-Darby bovine kidney (MDBK) cells possessed mutations in the gB gene and became more susceptible to heparin (our unpublished data). Therefore, cell lines originated from horses are necessary for isolation and propagation of equine herpesviruses.

Since cell lines originated from horse are very rare, it was difficult for many laboratories to propagate, isolate and diagnose EHV-4. Previously a new equine cell, FHK-Tcl3, was established in our laboratory (Maeda et al., 2007). The FHK-Tcl3 cell could support viral growth of EHV-1 and EHV-4 as well as primary FHK cells and were useful for isolation of EHV-2 and EHV-4 from horses. In addition, EHV-3 could also grow in FHK-Tcl3 cells. However, the cells could not be propagated over 40 times at three trials.

In this study, we succeeded in propagation of FHK-Tcl3 cell over 100 times and the cloned cell line, FHK-Tcl3.1, could still support growth of EHV-1, -2 and -4. This is the first report for equine cell, which could be propagated over 100 times.

2.3. Materials and methods

2.3.1. Viruses

EHV-1 strain 89c25 was isolated from a racehorse with respiratory disease in the epizootic of EHV-1 respiratory infection (Matsumura et al., 1992, 1994). EHV-4 strain TH20 was isolated from a colt suffering from respiratory disease (Kawakami et al., 1962). Both 89c25 and TH20 were plaque-purified three times in primary FHK cells and termed as 89c25p and TH20p, respectively (Damiani et al., 2000; Matsumura, 1998). EHV-2 strain E5-1129 was isolated from nasal discharge of a healthy riding horse in Yamaguchi in 2007.

2.3.2. Cells

FHK-Tcl3 cell was established by transfection of primary FHK cell with plasmid pLNCLT (kindly provided by Dr. S. Yasumoto at Kanagawa Cancer Institute) and maintained in Dulbecco's modified Eagle's medium (DMEM) (InVitrogen, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 100 units penicillin and 100 μg of streptomycin per ml (Maeda et al., 2007).

2.3.3. Cloning of FHK-Tcl3 cells by limiting dilution

At 100th passage of FHK-Tcl3, the FHK-Tcl3 cell was cloned by limiting dilution method. Cells were dispensed into 96-well multiplates (Sumitomo Bakelite, Tokyo, Japan) at a concentration of 0.1 to 10 cells per well. After limiting dilution, three cell clones, termed as FHK-Tcl3.1, FHK-Tcl3.2 and FHK-Tcl3.3, were selected, expanded and maintained.

2.3.4. Growth of cells

Approximately 2.5×10^5 of cloned cells were seeded in 35 mm dishes. Cells were collected by treatment with phosphate-buffered saline (PBS) containing 0.2% trypsin (Nacalai tesque, Kyoto, Japan) and 0.02% ethylenediamine tetraacetic acid (EDTA) (Wako, Osaka, Japan) every 24 hr and counted by using Burker-Turk hemocytometer after staining with trypan blue.

2.3.5. Observation of CPE of EHV infected cells

FHK-Tcl3.1 cell was infected with EHV-1 and EHV-4 at 100 plaque forming unit (PFU) and 200 μl of EHV-2 viral solution. FHK-Tcl3.1 cell was washed once with DMEM supplemented with 2% FCS, and then inoculated with EHVs. After adsorption at 37℃ for 1 hr, the cell was washed twice with DMEM supplemented with 2% FCS and incubated in DMEM supplemented with 10% FCS in a humidified atmosphere with 5% CO_2 at 37℃. Cells infected with EHVs were observed every day and CPE was recorded using Motic Images plus Ver.2.1 (Shimazu-rika, Tokyo, Japan).

2.4. Results

2.4.1. Establishment of horse-originated cell lines

FHK-Tcl3 cells were previously established in our laboratory (Maeda et al., 2007), but its growth ceased after less than 40 passages. Although several trials of cell passages for longer period were carried out, any immortalized cell line had not been obtained. In this study, FHK-Tcl3 cell kept growing after 40 passages at 4th trial and was propagated over 100 times. Therefore, this cell line certainly seemed to be immortalized. Next, FHK-Tcl3 cell was cloned by limiting dilution at 100th passage. Three cloned cell lines were obtained and termed as FHK-Tcl3.1, FHK-Tcl3.2 and FHK-Tcl3.3, respectively.

2.4.2. Comparison of the cell growth

For comparison of cell growth, approximately 2.5×10^5 cloned cells were seeded in 35 mm dishes and the number of cells was counted every 24 hr. FHK-Tcl3.1 and FHK-Tcl3.2 cells grew well and the cell number plateaued at 120 hrs (approximately 1.7×10^6). FHK-Tcl3.3 cell grew a little slower than the other two cell lines (Fig. 2. 1). For further analysis, FHK-Tcl3.1 cell was selected and maintained.

2.4.3. Sensitivity of FHK-Tcl3.1 cells against EHVs infection

The sensitivity of FHK-Tcl3.1 cell against EHVs was examined. The result showed that EHV-1, -2 and -4 showed clear CPE on FHK-Tcl3.1 cells (Fig. 2. 2). These CPE were very similar to those on parental FHK-Tcl3 cells and primary FHK cells. In particular, EHV-2 also showed clear CPE on FHK-Tcl3.1 cells, indicating that this cell line support the growth of gammaherpesviruses whose host range is very restricted.

2.5. Discussion

In this study, the new equine cell line, FHK-Tcl3.1 cell, was established. FHK-Tcl3.1 cell was cloned at the 100th passage, and still keeps growing and the passage history is over 100 times after cloning. Therefore, these cells seemed to be immortalized.

Parental FHK-Tcl3 cell and the other equine cell line, E. Derm (NBL-6) cell (ATCC number: CCL-57), have a limited propagation ability (less than 40 passages) and E.Derm cell grows slowly. Our established FHK-Tcl3.1 cell grew well and its cell growth is similar to that of parental FHK-Tcl3 cell. Although it is unknown why the FHK-Tcl3 cell kept growing after 40th passage, this is the first report of the equine cell that could be propagated over 100 times.

In the previous study, FHK-Tcl3 cell could support growth of EHV-1, -2 -3 and -4, and EHV-2 and -4 could be isolated from horses by using FHK-Tcl3 cells (Maeda et al., 2007). In this study, FHK-Tcl3.1 cell could also support growth of EHV-1, -2 and -4 and showed clear CPE (Fig. 2. 2). These characters of FHK-Tcl3.1 cell were very similar to those of parental FHK-Tcl3 cell, indicating that immortalization and cloning of cells might not significantly influence the character of FHK-Tcl3.1 cell. Recently, another group reported that FHK-Tcl3.1 cell could propagate many viruses affected with horse, including EHV-3 (Oguma et al., 2013). Oguma et al. (2013) indicated that equine adenovirus, coronavirus, getahvirus and Japanese encephalitis virus could not be propagated. Although it is unknown whether FHK-Tcl3.1 cell support the growth of other equine

herpesviruses, EHV-5, -8 and -9, these data indicate that FHK-Tcl3.1 cell would be useful for the serological test and virus isolation.

In conclusion, the novel equine cell, FHK-Tcl3.1, was established and could be used for isolation and propagation of equine herpesviruses. In addition, the FHK-Tcl3.1 cell must be useful for propagation, diagnosis and research of many equine viruses including equine herpesviruses.

2.6. Figure legends

Fig. 2. 1 Growth of cloned cells. Cells were counted every 24 hr and vertical error bars indicate standard deviation of means.

Fig. 2. 2 CPE in FHK-Tcl3.1 cells infected with EHV-1 (A), -2 (C), -4 (B) or Mock (D). CPE was observed after 32 hr (A), 153 hr (C) and 72 hr (B), respectively.

Fig. 2. 1

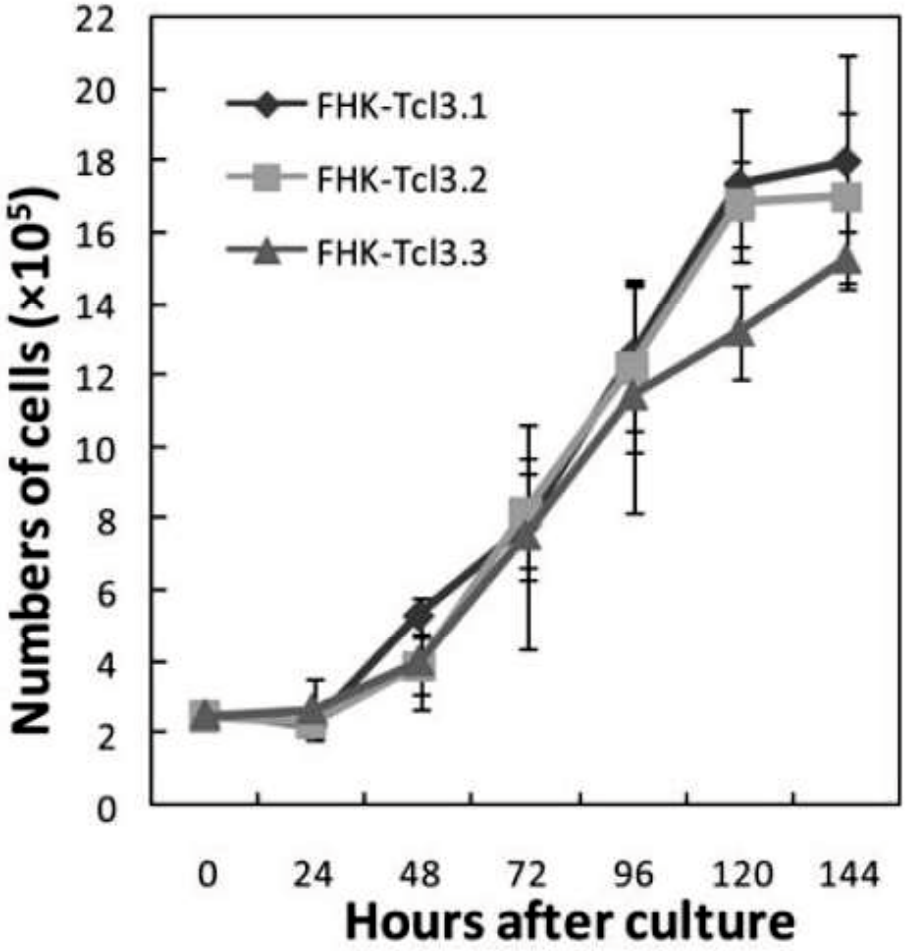
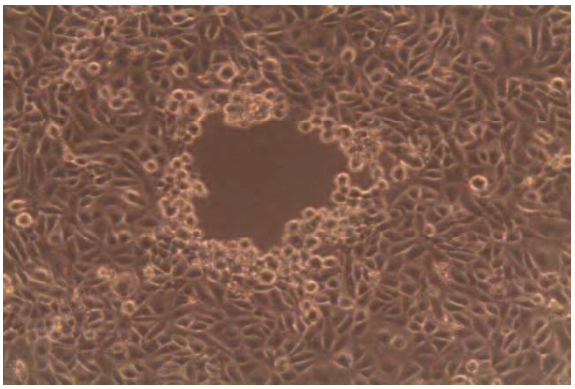
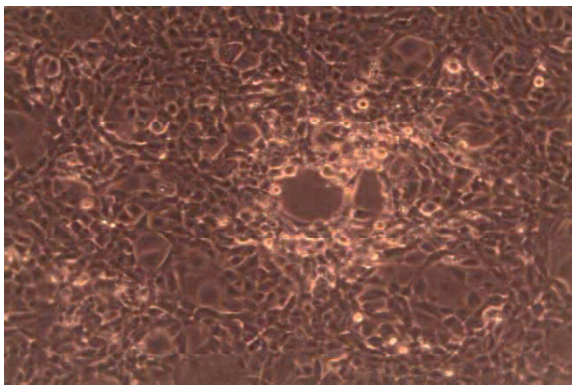

FHK-Tcl3.1
FHK-Tcl3.2
FHK-Tcl3.3
Numbers of cells (×10⁵)
0
2
4
6
8
10
12
14
16
18
20
22
0
24
48
72
96
120
144
Hours after culture

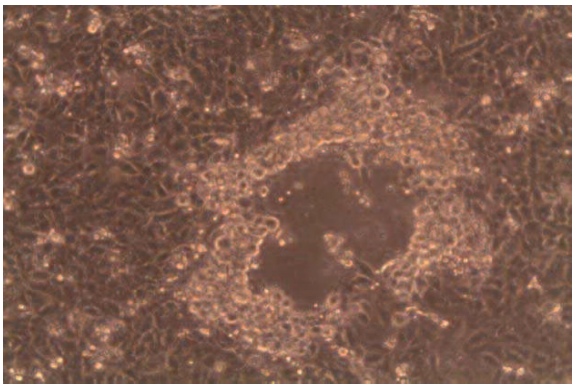
Fig. 2. 2



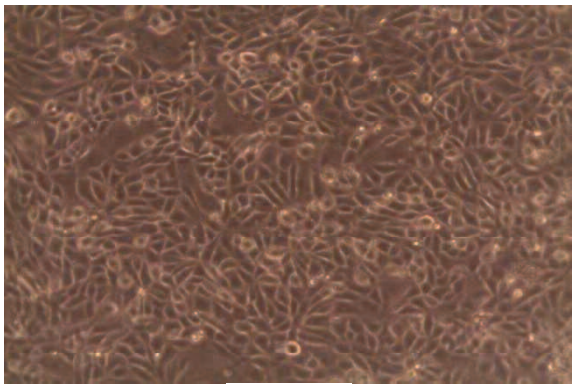
EHV-1



EHV-2



EHV-4



Mock

Chapter 3

The haemagglutination activity of equine herpesvirus type 1 glycoprotein C.

3.1. Summary

EHV-1 has HA activity towards equine RBC, but the identity of its haemagglutinin is unknown. To identify the haemagglutinin of EHV-1, major glycoproteins of EHV-1, gp2, gB, gC, gD, gG, gE, gI, gM and gN, were expressed in 293T cells, and the cells or cell lysates were mixed with equine RBCs. The results showed that only EHV-1 gC-producing cells adsorbed equine RBCs, and that the lysate of EHV-1 gC-expressing cells agglutinated equine RBCs. EHV-1 lacking gC did not show HA activity. HA activity was inhibited by monoclonal antibodies (MAbs) specific for gC, but not for other glycoproteins. In addition, HA activity was not inhibited by the addition of heparin. These results indicate that EHV-1 gC can bind equine RBCs irrespective of heparin, in contrast to other herpesvirus gC proteins.

3.2. Introduction

EHV-1 and EHV-4 are genetically and antigenically similar, but differ in some characteristics, including their pathogenesis. Notably, EHV-1 can grow in some non-equine cells, whereas the growth of EHV-4 appears to be restricted to equine cells. Therefore, it has been difficult to compare both viruses in vitro. In chapter 1, we established the equine cell line, FHK-Tcl3.1, that is able to propagate both viruses, resulting in permitting a more direct comparison of their biology (Andoh et al., 2009; Maeda et al., 2007).

Several herpesviruses possess HA activities (Gillespie et al., 1971; Nemoto et al., 1990; Prokofieva and Babkin, 1965; Tetsu et al., 1989. Trepanier et al., 1985; Trybala et al., 1990). Feline herpesvirus-1 (FHV-1), canine herpesvirus (CHV) and infectious laryngotracheitis virus (ILTV) agglutinate RBCs from their respective host species. For both FHV-1 and CHV, the gD protein has been identified as the haemagglutinin (Maeda et al., 1994, 1997a). However, multiple viruses [including FHV-1, CHV, HSV-1, BHV-1 and PRV] also exhibit HA activity against murine RBCs; for each of these viruses, the murine-specific haemagglutinin has been assigned to gC (Maeda et al., 1998; Matsuda et al., 1991; Noda et al., 1993; Okazaki et al., 1991, 1993, 1994; Tetsu et al., 1989; Trybala et al., 1990, 1993). HA activities of these herpesviral gCs can be inhibited by heparin (Maeda et al., 1998; Noda et al., 1993; Okazaki et al., 1991, 1993; Trybala et al., 1993).

Although EHV-1 is reported to have HA activity against equine RBCs (McCollum et al., 1956),

the identity of its haemagglutinin remains unknown. In this study, we confirm that EHV-1 is capable of equine RBC agglutination, and that its haemagglutinin is gC. Furthermore, we show that the HA activity of EHV-1 is not inhibited by treatment with heparin.

3.3. Materials and methods

3.3.1. Cells

FHK-Tcl3.1 (Andoh et al., 2009; Maeda et al., 2007), 293T, Crandel–Ree feline kidney (CRFK) and cloned porcine kidney (CPK) cell lines were maintained in DMEM (Invitrogen, CA, USA) supplemented with 10% heat-inactivated FCS, 100 units of penicillin and 100 μg/mL streptomycin (Invitrogen, CA, USA). Cells were grown at 37℃ in 5% CO2. CPK cells were kindly provided by the National Institute of Animal Health, Japan. Equine dermal (ED) cells (ATCC CCL-57) were maintained in DMEM supplemented with 10% foetal bovine serum (FBS).

3.3.2. Viruses

EHV-1 89c25p and EHV-4 TH20p were previously described in chapter 2. The EHV-1 HH1 and EHV-4 H45 strains were originally isolated in Japan from aborted equine foetuses (Kawakami et al., 1970; Shimizu et al., 1959). The EHV-1 5089 strain, which has lost gC expression because of a reduced level of gC mRNA (Kirisawa et al., 2005), was propagated in ED cells, whereas all other EHV strains were propagated in FHK-Tcl3.1 cells. Isolates of FHV-1 (strain C7301) and PRV (Indiana strain) were propagated in CRFK and CPK cells, respectively.

3.3.3. RBCs

Defibrinated equine blood was purchased from Nippon Bio Test Laboratories (Tokyo, Japan). Murine, feline and canine RBCs were collected from healthy C57BL/6N Sea mice (Kyudo, Saga, Japan) and a healthy cat and dog, respectively. RBCs were preserved in Alsever's solution (Biological Industries, Israel) until use.

3.3.4. Construction of expression plasmids

For expression of the genes encoding the major glycoproteins (gp2, gB, gC, gD, gG, gE, gI, gM and gN) of EHV-1, each gene was amplified from the genome of 89c25p by PCR and cloned (Mahmoud et al., 2013). In brief, amplified genes were cloned into the expression plasmid pCAGGS, which was kindly provided by Dr. Miyazaki (Osaka University). The resulting plasmids were designated pCAGGS-gp2, -gB, -gC, -gD, -gG, -gE, -gI, -gM and -gN. For transfection, all expression plasmids were purified using a QIAprep Spin Miniprep Kit (QIAGEN, Tokyo, Japan) or a QIAfilter plasmid Midi Kit (QIAGEN, Maryland, USA).

3.3.5. MAbs to EHV-1 gC

MAbs to EHV-1 gCs were established from BALB/c mice inoculated with EHV-1 (Mahmoud et al., 2013). Hybridoma cells secreting EHV-1-specific MAbs were cloned twice by the limiting dilution method and were then inoculated into BALB/c mice pretreated with pristane

(Sigma) for the preparation of ascites. All four MAbs to EHV-1 gCs (5F8, 1G10, 8B2 and 8H4) have a virus-neutralizing activity against EHV-1.

3.3.6. Transfection of expression plasmids into 293T cells

293T cells were transfected with the expression plasmids using Lipofectamine 2000 or polyethylenimine (PEI). Transfection using Lipofectamine 2000 was performed according to the manufacturer's protocol (Invitrogen, CA, USA), and 8 μg of the plasmid was transfected into approximately 5×10^5 293T cells. In PEI transfection, 3.2 μg of plasmid was mixed with 8 μL of PEI (2 mg/mL) and then transfected into approximately 5×10^5 293T cells according to the protocol given by Boussif et al. (1995). Transfected cells were harvested at 72 hr post-transfection.

3.3.7. Haemadsorption (HAD) test

FHK-Tcl3.1 cells were infected with EHVs at a MOI of 0.2, and HAD test was performed 20–48 hr post-infection. Transfected cells were also tested 45 hr post-transfection. Transfected or infected cells were washed two times with PBS and fixed by PBS supplemented with 4% paraformaldehyde for 45 min at room temperature. After fixation, cells were washed three times with ice-cold FA buffer, which consists of PBS supplemented with 2% FCS and 0.1% sodium azide (Wako, Osaka, Japan). Following the wash, the cells were incubated with 0.2% equine RBC in FA

buffer for 1 hr at 4°C. After incubation, cells were washed two times with FA buffer and observed.

3.3.8. HA test

FHK-Tcl3.1 cells were infected with EHVs at a MOI of 0.2 and FHK-Tcl3.1 cells infected with EHV-1 or -4 and 293T cells transfected with the expression plasmids were harvested at 72 hr post-infection or post-transfection. These cells were scraped off the petri dishes, washed three times with PBS, suspended in PBS containing 0.25% Tween 80 (Wako, Osaka, Japan) and mixed with one-fifth volume of diethyl ether for 2 hr at 4℃. The solution was then centrifuged to remove the cell debris. The aqueous layers were harvested and used as HA antigens. The aqueous layer was serially two-fold diluted with dilution buffer [PBS containing 0.1% (w/v) bovine serum albumin (Fraction V; Sigma, Steinheim, Germany) and 0.002% gelatin (BioRad, Tokyo, Japan)] in 96-well U-bottom microtiter plates. An equal volume of 0.2% equine, murine, feline or canine RBCs in the dilution buffer was then added to the diluted antigen. The HA titer was defined as the reciprocal of the highest dilution showing complete HA after incubation for 2 hr at room temperature.

3.3.9. Inhibition of HA by MAbs and heparin

To examine the inhibition of HA activity by MAbs and heparin, 25 μL of serial two-fold diluted MAbs or ten-fold diluted heparin (Wako, Osaka, Japan) was added to 96-well U-bottom microtiter

plates and mixed with 25 μL of a solution containing 8 HA units of haemagglutinin. After incubation for 1 hr at 37℃, 50 μL of a 0.2% equine RBC suspension was added and the plates were placed at room temperature for 2 hr. HA inhibition (HI) activity was defined as the reciprocal of the highest dilution of MAbs or heparin showing a complete HI pattern.

3.4. Results

3.4.1. HAD and HA activities of EHV-1

To examine the HAD activities of EHV-1 and EHV-4, infected cells were mixed with equine RBCs. Our results showed that only EHV-1-infected FHK-Tcl3.1 cells adsorbed equine RBCs; mock- or EHV-4-infected cells did not reveal any adsorption (Fig. 3. 1A). Next we conducted an HA test to confirm the HA activity of EHV-1. Our results revealed that EHV-1 89c25p, but not EHV-4 TH20p infected cells, agglutinated equine RBCs (Fig. 3. 2A). The aqueous extracts of EHV-1 did not agglutinate feline or canine RBCs (data not shown). In addition, the aqueous extracts of another EHV-1 isolate (strain HH1) agglutinated equine RBCs, whereas a separate EHV-4 isolate (strain H45) did not exhibit agglutination (Table 3. 1). Furthermore, we performed an HA test using the variant EHV-1 strain 5089, which lacks gC expression (Kirisawa et al., 2005). This strain failed to produce any detectable HA activity (Table 3. 1).

3.4.2. Identification of EHV-1 haemagglutinin

To determine the haemagglutinin of EHV-1, expression plasmid pCAGGS encoding EHV-1 gp2, gB, gC, gD, gG, gE, gI, gM or gN (Mahmoud et al., 2013) was transfected into 293T cells. HA tests using lysates of the transfected cells showed that only pCAGGS-gC transfected cells adsorbed and agglutinated equine RBCs (Fig. 3. 1B, Fig. 3. 2B).

3.4.3. Inhibition of HA activity by MAbs to gC

To confirm the HI activity of MAbs, HI test using lysates from EHV-1 infected or EHV-1 gC transfected cells was performed. The results indicated that two anti-gC MAbs were able to inhibit HA activity (Table 3. 2).

3.4.4. Inhibition of HA activity by heparin

Our characterisation of the HA activities of EHV-1 and EHV-1 gC included the testing of heparin sensitivity. The HA activities of EHV-1 and EHV-1 gC in equine RBCs were not inhibited by the addition of 100 U/mL heparin (Fig. 3. 3). However, the HA activity of FHV-1 in murine RBCs was inhibited by the addition of 0.1 U/mL heparin.

3.5. Discussion

In this study, we have demonstrated that the gC of EHV-1 possesses HA activity against equine RBCs and that this activity is inhibited by anti-gC MAbs. We confirmed this finding by demonstrating that an EHV-1 variant that lacks the expression of gC, also lacks HA activity. Furthermore, we showed that the HA activity of EHV-1 gC is not inhibited by heparin.

The ability of viruses to agglutinate RBCs can be used as a simple assay for viral attachment to cellular receptors. FHV-1, CHV and ILTV agglutinate RBCs from their respective hosts, and in FHV-1 and CHV the gD proteins serve as the haemagglutinins (Maeda et al., 1994, 1997a, 1998; Prokofieva and Babkin, 1965). Although EHV-1 also possesses HA activity against equine RBCs, we have shown that this activity is associated with gC, not gD. Additional tests demonstrated that this HA activity was specific for equine RBCs and did not apply to feline or canine RBCs. To the best of our knowledge, this represents the first report of a herpesviral gC capable of agglutinating the RBCs of its respective host.

Notably, the gCs of FHV-1, CHV, HSV-1, BHV-1 and PRV are able to agglutinate non-host (murine) RBCs (Maeda et al., 1998; Matsuda et al., 1991; Noda et al., 1993; Okazaki et al., 1991, 1993, 1994; Tetsu et al., 1989; Trepanier et al., 1985; Trybala et al., 1990, 1993). For each of these published examples, HA of murine RBCs was inhibited by the addition of heparin, suggesting that the heparin-binding domain of gC has structural similarity to the domain for binding to murine

RBCs (Inaba et al., 1990; Maeda et al., 1997b, 1998; Noda et al., 1993; Okazaki et al., 1993; Tetsu et al., 1989; Trybala et al., 1990, 1993). However, the HA activity of EHV-1 gC was not inhibited by heparin. These results suggest that EHV-1 gC may bind to an unknown receptor on equine RBCs.

An EHV-1 gC mutant was previously shown to have decreased heparin susceptibility and reduced viral penetration and infectivity in cells with suppressed or defective glycosaminoglycan biosynthesis (Kirisawa et al., 2005; Osterrieder, 1999). Furthermore, the adaptation of EHV-1 to an unnatural host reportedly results in gC mutation and an increased susceptibility to heparin (Sugahara et al., 1997). Together, these reports suggest that EHV-1 gC interacts with heparin. Therefore, we speculate that EHV-1 gC contains two different domains; a heparin-interacting domain and an unknown receptor binding domain that permits binding to equine RBCs. EHV-1 gC has a different character from gCs of the other herpesviruses including EHV-4, suggesting that EHV-1 gC may have an important role for EHV-1-specific virulence, like abortion and neurological disorder. Therefore, it is hypothesized that EHV-1 bound to equine RBC may distribute throughout the body of horses. Further studies will be required to clarify the details of the interactions between EHV-1 gC and its host's cells.

Our study has shown that EHV-1 gD is not responsible for HA activity. This is in contrast to other herpesviruses (e.g. CHV and FHV-1), where gD has been identified as the haemagglutinin and suggests a possible absence of receptors for EHV-1 gD on the surface of equine RBCs. Recent

studies have revealed that the equine MHC-I molecule serves as the entry receptor for EHV-1 and that the binding glycoprotein is gD (Kurtz et al., 2010; Sasaki et al., 2011). However, MHC-I is not displayed on the cell surface of RBCs. These results are thus consistent with our hypothesis.

In conclusion, we determined that EHV-1 gC was responsible for HA activity against equine RBCs. Furthermore, we have demonstrated that the HA activity of EHV-1 gC against equine RBCs is not inhibited by heparin. To the best of our knowledge, this is the first report of heparin-insensitive HA activity associated with a herpes viral gC protein.

3.6. Figure legends

Fig. 3. 1 HAD of (A) EHV-1 and EHV-4-infected FHK-Tcl3.1 cells or (B) EHV-1 gC-transfected cells to equine RBCs.

Fig. 3. 2 HA of equine RBCs by extracts from (A) FHK-Tcl3.1 cells infected with EHV-1 and -4 or (B) 293T cells transfected EHV-1 gC. The extracts were serially two-fold diluted with dilution buffer in 96-well U-bottom microtiter plates. An equal volume of 0.2% equine RBC suspension was added to each well. The HA activity was judged after incubation for 2 hr at room temperature. Numbers on the tops of wells represent the dilution titers.

Fig. 3. 3 HI activity by heparin. Twenty-five microliters of serially ten-fold diluted heparin was mixed with 50 μL of a solution containing 8 HA units of haemagglutinin of EHV-1-infected cells or EHV-1 gC-transfected cells and 25 μL of 0.4% equine RBC suspension. After 2 hr incubation at room temperature, HI activities were judged as the reciprocal of the highest heparin dilution showing complete HI pattern. As controls, FHV-1-infected CRFK cells and murine RBCs were used as controls.

Table 3. 1 HA activity of EHVs

EHVs-infected FHK-Tcl3.1 cells		HA activity		
		1st test	2nd test	3rd test
EHV-1	89c25p	1:320	NT	1:256
	HH1	NT	1:512	NT
	5089	NT	<1:2	NT
EHV-4	TH20p	<1:10	NT	<1:2
	Mock	<1:10	<1:2	<1:2

NT: Not tested

Table 3. 2 HI activity of MAbs

MAbs	Isotype[a]	Proteins[a]	Virus neutralization titer[a]	HI titer	
				EHV-1[b]	gC [b]
8H11	IgG2a	gp2	<1:10	<1:100	<1:100
5F8	IgM	gC	1:80	<1:100	<1:100
1G10	IgM	gC	1:640	<1:100	<1:100
8H4	IgM	gC	1:1280	1:1600	1:3200
8B2	IgM	gC	1:3200	1:3200	1:12800
5F12	IgM	gB	<1:10	<1:100	<1:100
7E11	IgM	gB	<1:10	<1:100	<1:100
6F4	IgG2a	gB	<1:10	<1:100	<1:100
6G12	IgM	gB	<1:10	<1:100	<1:100

a; These data were shown in our previous report (Mahmoud et al., 2013).

b; HA antigens used for the HI test.

Fig. 3. 1

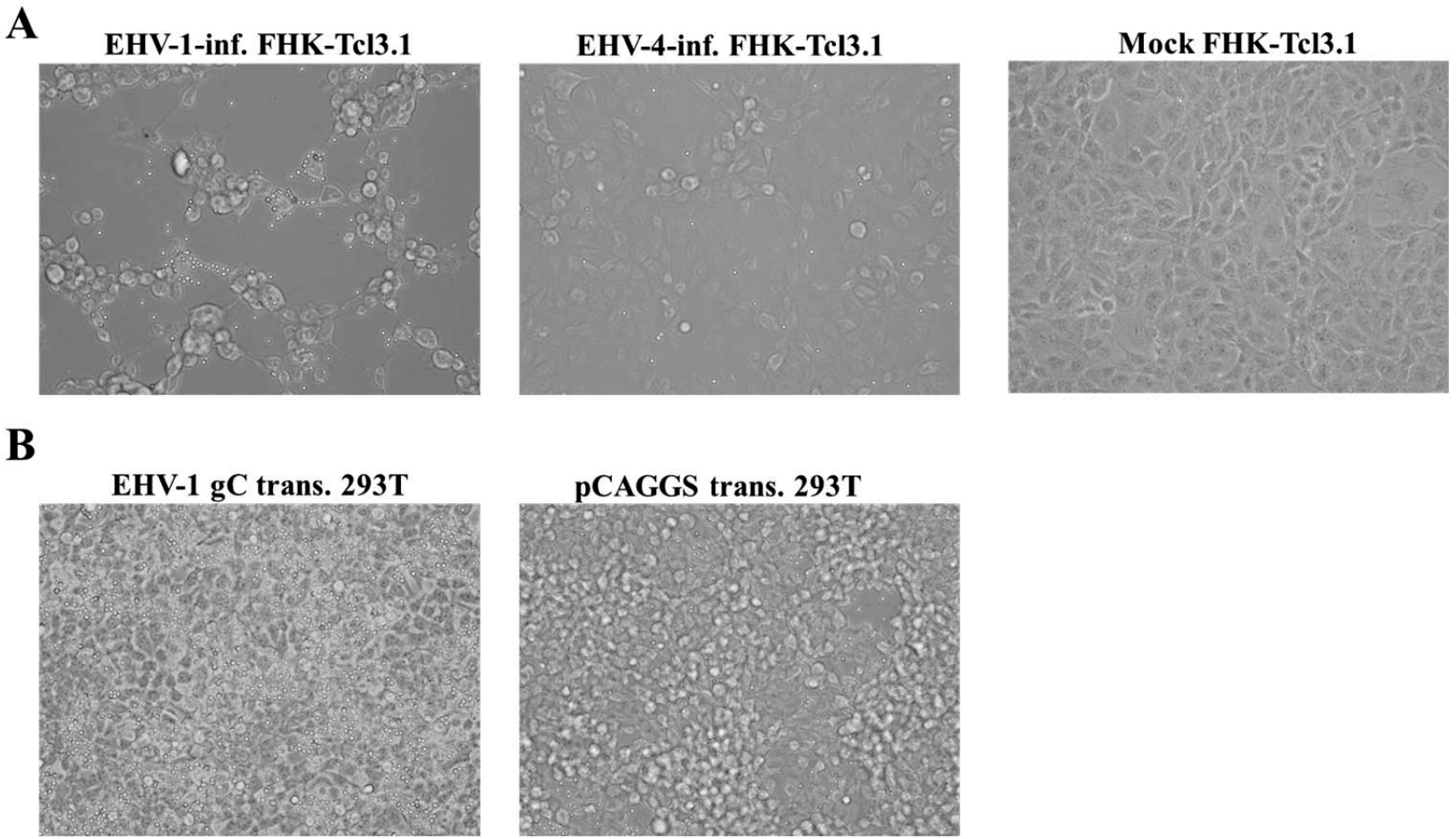
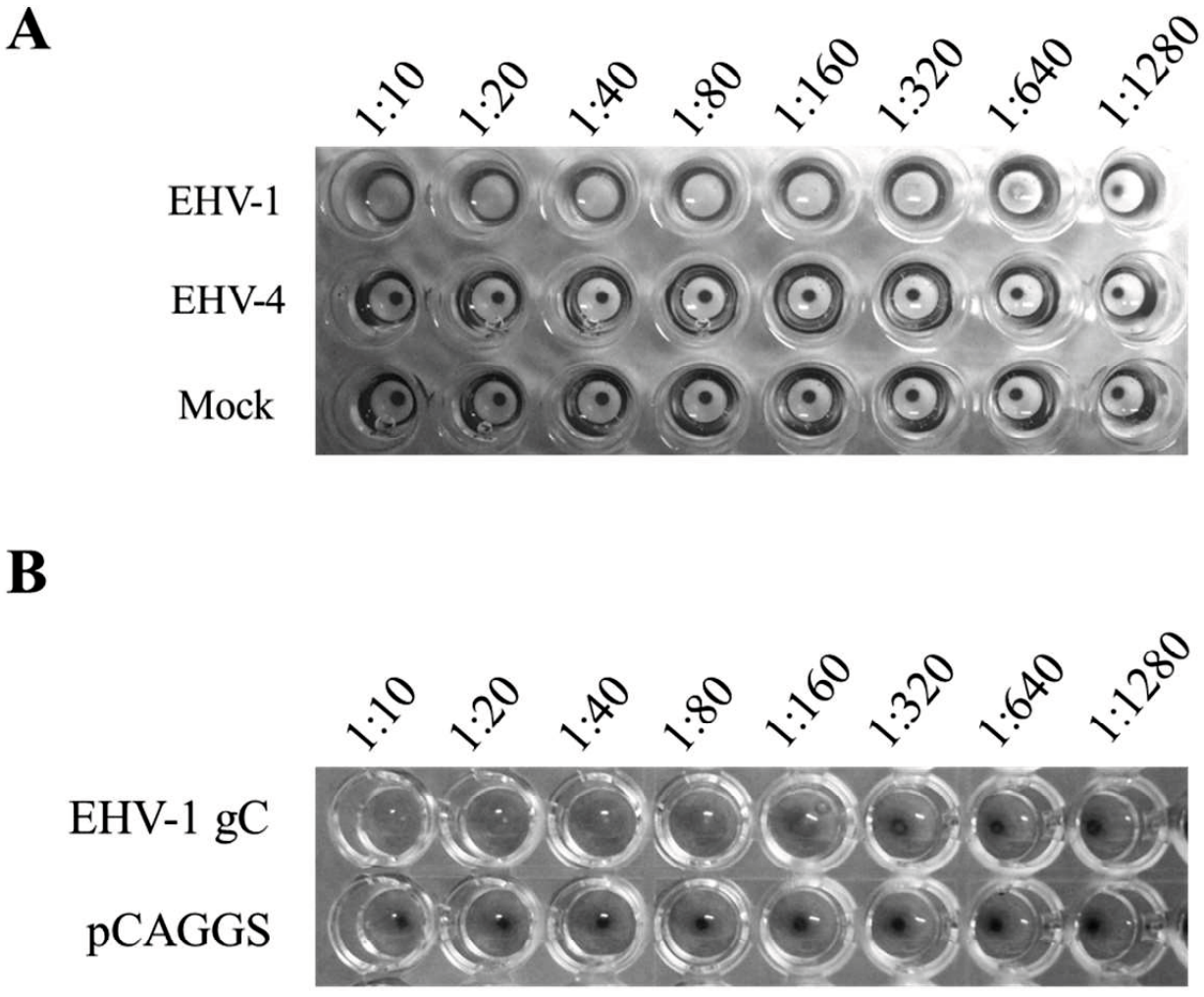

A
EHV-1-inf. FHK-Tcl3.1
EHV-4-inf. FHK-Tcl3.1
Mock FHK-Tcl3.1
B
EHV-1 gC trans. 293T
pCAGGS trans. 293T

Fig. 3. 2


A
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
EHV-1
EHV-4
Mock
B
1:10
1:20
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1:80
1:160
1:320
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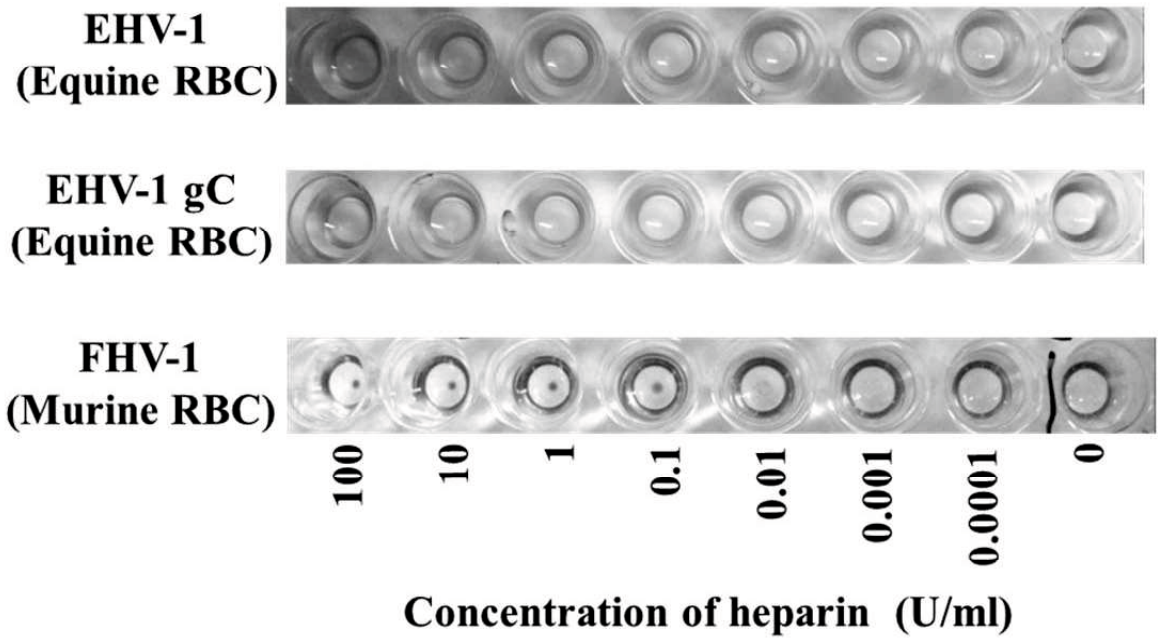
1:1280
EHV-1 gC
pCAGGS

Fig. 3. 3


EHV-1
(Equine RBC)
EHV-1 gC
(Equine RBC)
FHV-1
(Murine RBC)
100
10
1
0.1
0.01
0.001
0.0001
0
Concentration of heparin (U/ml)

Chapter 4

Identification of a major immunogenic region of equine herpesvirus type 1 glycoprotein E and its application to enzyme-linked immunosorbent assay.

4.1. Summary

In this chapter, a major immunogenic region of EHV-1 gE was identified. Firstly, the various fragments of EHV-1 gE were expressed as fusion proteins with glutathione S-transferase (GST) in *Escherichia coli* (*E. coli*) and their antigenicities were compared by immunoblot analysis using sera from horses experimentally infected with EHV-1. Thirty-three amino acids of gE (a.a. 169–201) specifically and sensitively reacted with the antibodies induced by EHV-1 but not EHV-4 infection. The corresponding region of EHV-4 gE (a.a. 169–199) did not react with antibodies to EHV-1, indicating that this region is specific for each virus. In addition, when the antigenicities of three 20-mer synthetic peptides of EHV-1 gE, gE1 (169–188), gE1 (176–195) and gE1 (182–201) were compared by ELISA, gE1 (169–188) was found to contain a major B-cell epitope. ELISA using two synthetic peptides, gE1 (169–188) and gG4 (319–330), previously identified as the major EHV-4-specific epitope in gG, was developed and could specifically detect antibodies to EHV-1 and EHV-4, respectively. In Japan, the EHV-1 deleted in gE gene (EHV-1 ΔgE) virus is expected to be introduced in the field as a new modified live vaccine. This ELISA did not react with antibodies induced by inoculation with EHV-1 ΔgE, indicating that it is a useful method to differentiate between EHV-1 infection and EHV-1 ΔgE inoculation. In conclusion, the ELISA described herein, using synthetic peptides, is a simple method to distinguish between EHV-1 and EHV-4 infections and will be suitable as a vaccine marker after introduction of EHV-1 ΔgE into field horses.

4.2. Introduction

EHV-1 causes acute upper respiratory disease, occasional central nervous system disorder and contagious virus abortion in horses and EHV-4 also causes pyrexia and respiratory disease (Crabb and Studdert, 1995). Both virus strains have shown a wide geographic distribution and cause serious economic losses worldwide (Allen and Bryans, 1986). Vaccination is considered to be the most effective method to control the diseases induced by EHV-1. However, only one inactivated vaccine is available in Japan, whose efficacy seems to be limited. Recently, Tsujimura et al. (2006) reported the development of EHV-1 with a deletion of the gE gene (EHV-1 ΔgE) and showed that the virus was avirulent and immunogenic in experimental animal models. Furthermore, inoculation with EHV-1 ΔgE in thoroughbred foals deprived of colostrum did not induce any clinical signs of respiratory disease, and protected the horses from disease inducible by EHV-1 challenge (Tsujimura et al., 2009). Currently, the EHV-1 ΔgE is part of ongoing attempts to introduce a new vaccine in Japan. The gE deletion mutants of BHV-1 and PRV, which belongs to the same subfamily as EHV-1, *Alphaherpesvirinae*, have been available as modified live virus vaccines in the field (Mars et al., 2001; Muller et al., 2003; Pensaert et al., 2004). The deletion of gE in these vaccines makes it possible to differentiate between natural infection and vaccination using ELISA to detect antibodies to gE (Van Orischot et al., 1988, 1997). Therefore, a serological assay to detect antibodies to EHV-1 gE is anticipated for the introduction of the EHV-1 ΔgE vaccine. In previous studies, we established

an ELISA using type specific regions of gG as antigens. This ELISA using Japanese isolates of EHV-1 and -4 referred to the reports of Crabb and Studdert (1993) and Crabb et al. (1995) and was useful for the sero-epizootiology and diagnosis of EHVs in Japan (Maeda et al., 2004, 2005; Mizukoshi et al., 2002; Yasunaga et al., 1998, 2000). In the assay the antigens were expressed as fusion proteins with GST in *E. coli* and then purified. In general, if synthetic peptides are available for ELISA, the ELISA should be more useful for many diagnostic applications. Until now, we identified two immunogenic domains in EHV-4 gG and the derived synthetic peptides were useful for detection of antibodies to EHV-4 as ELISA antigens (Maeda et al., 2004, 2005). On the other hand, the immunogenic domain in EHV-1 gG has not been identified yet due to EHV-1 gG being less immunogenic to horses than EHV-4 gG. Other immunogenic epitopes of EHV-1 need to be identified to establish ELISA using synthetic peptides. In this study, the type-specific epitope of EHV-1 gE was determined and a simple ELISA using synthetic peptides was developed to differentiate EHV-1 and EHV-4 infections.

4.3. Materials and methods

4.3.1. Viruses and cells

All viruses and cells are previously described in Chapter 1 and 2.

4.3.2. Construction of expression plasmids

For expression of various gE fragments of EHV-1 and EHV-4, genes were amplified from the genome of 89c25p or TH20p by PCR using Takara LA PCR kit (Takara, Japan). Primers were listed in Table 4. 1. Viral DNA was extracted from the infected FHK-Tcl3.1 cells using the DNeasy tissue kit (Qiagen, Germany). The forward primers contain an *Eco*RI site at the 5'-end and the reverse primers contain a *Sal*I site at the 5'-end. The amplified fragments were purified by QIAquick PCR purification kit (Qiagen), digested with *Eco*RI and *Sal*I, and then cloned into the bacterial expression plasmid pGEX6p-1 digested with *Eco*RI and *Sal*I.

4.3.3. Expression of GST-fusion proteins

Various fragments of EHV-1 and EHV-4 gE were expressed as fusion proteins with GST. *E. coli* JM109 containing the recombinant plasmid was inoculated into LB medium containing 50 μg/ml ampicillin and incubated overnight at 37 ℃. The culture sample was inoculated into a ten-fold volume of LB medium containing 50 μg/ml ampicillin, incubated for 2 hr at 37 ℃, and then

isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at 1 mM final concentration. At 4 hr post-induction, cells were harvested. For identification of immunogenic domains, the cells were directly dissolved in sample buffer (62.5 mM Tris–HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS) and 0.001% bromophenol blue) and then analyzed by immunoblot analysis using horse sera. For purification of the expressed GST-fusion proteins, the cells were suspended in sonication buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) and sonicated. The sonicated samples were mixed with Triton X-100 at a final concentration of 1%, and then centrifuged at 15,000 rpm for 30 min. The supernatant was mixed with glutathione sepharose 4B (GE Healthcare, USA) and incubated for 30 min at 4 ℃. The supernatant was discarded and the glutathione sepharose was washed with sonication buffer, and then mixed with elution buffer (10 mM glutathione). After incubation for 10 min at 4 ℃, supernatant was harvested.

4.3.4. Immunoblot analysis

Samples were dissolved in sample buffer and electrophoretically separated by SDS-15% polyacrylamide gel electrophoresis. The polypeptides were electrophoretically transferred to polyvinylidene difluoride membranes (Immobillon; Millipore, USA) and incubated with blocking buffer containing 3% gelatin (BIO-RAD, USA) for 1 hr at 37 ℃. The membranes were washed three times with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.05% Tween 20 and

incubated with diluted horse serum at 37 ℃ for 30 min. After washing three times, membranes were incubated with peroxidase-conjugated anti-horse immunoglobulins (EY Laboratories, USA) at 37 ℃ for 30 min. The reaction was visualized with the substrate diaminobenzidine-hydrogen peroxidase (Wako, Japan).

4.3.5. Synthetic peptides

Peptides gG4 (319–330), gE1 (169–188), gE1 (176–196) and gE1 (182–201) were synthesized by Biosynthesis (USA) and dissolved in dimethyl sulfoxide at 10 mg/ml final concentration and kept in -80 ℃ until use.

4.3.6. ELISA

The purified GST-fusion proteins and synthetic peptides were diluted to 5 μg/ml with adsorption buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6), and 50 μl was added to each well of 96-well microplates (Maxisorp; Nunc, Denmark). As controls, only GST or adsorption buffer without peptide was added. After incubation at 37 ℃ for 2 hr, the plates were placed at 4 ℃ overnight. After washing three times with PBS containing 0.05% Tween 20 (PBS-T), 100 μl of 0.1% bovine serum albumin (Sigma, USA) in PBS was added and incubated at 37 ℃ for 1 hr. After washing with PBS-T, 100 μl of sera diluted in PBS-T containing 10% FCS was added to the wells.

After incubation at 37 ℃ for 1 hr, the wells were washed three times with PBS-T and incubated with 100 µl of peroxidase-conjugated antihorse immunogloblins (EY Laboratories) at 37 ℃ for 30 min. Following three washes with PBS-T, 50 µl of peroxidase substrate kit (BIO-RAD) was added to each well. After incubation for 30 min, the enzymatic reaction was stopped by adding 50 µl of 2% oxalic acid to each well. The absorbance was measured by a spectrophotometer (BIORAD) with a 405-nm filter.

4.3.7. Serum samples

4.3.7.1. Sera from foals experimentally infected with EHV-1 or EHV-4

Sera were sequentially collected from three foals (A-1, A-2, and A-3), which were experimentally infected with EHV-1 in 2002. These foals were deprived of colostrum and intramuscularly inoculated twice with 10^6 PFU of EHV-1 ΔgE at an interval of 3 weeks and then challenged with EHV-1 89c25p at 4 weeks after the second inoculation (Tsujimura et al., 2009).

Sera were collected from three foals (#4–#6). Three foals were challenged with the EHV-4 TH20 revertant (Damiani et al., 2000).

4.3.7.2. Paired sera from racehorses with pyrexia

Paired sera at acute and convalescent phases were collected from 44 racehorses, in which a

tentative serological diagnosis of EHV-1 infection was made by CF test and gG-ELISA.

4.4. Results

4.4.1. Identification of immunogenic regions in gE of EHV-1 by immunoblot analysis

Various regions of EHV-1 gE were expressed as fusion proteins with GST. Since many recombinant proteins precipitated in the inclusion bodies after sonication, *E.coli*-expressing fusion proteins were dissolved directly in sample buffer and analyzed to identify the immunogenic domains in the gE. At first, 10 recombinant proteins, GST-egE1 (1–551), -egE1 (1–135), -egE1 (136–272), -egE1 (273–407), -egE1 (408–551), -egE1 (1–272), -egE1 (136–407), -egE1 (273–551), -egE1 (1–407) and -egE1 (136–551) were examined by immunoblot analysis using sera from EHV-1- infected horses, and we found that all recombinant proteins containing two regions, egE1 (136–272) or egE1 (408–551), reacted strongly with EHV-1 antibodies (Fig. 4. 1A). To specify the antigenic region in egE1 (136–272), further deletion fragments of GST-egE1 (1–272) and -egE1 (136–407) were expressed and analyzed by immunoblot analysis, resulting in the major immunogenic region being located in egE1 (169–201) (Fig. 4. 1B and C). Furthermore, the major immunogenic region in egE1 (408–551) was also analyzed by immunoblot analysis using five deletion fragments of egE1 (273–551) and found to be located in egE1 (480–515) (Fig. 4. 1D). To compare the antigenicities of these immunogenic regions, four regions, egE1 (169–201), egE1 (202–235), egE1 (169–235) and egE1 (480–515) were expressed as GST-fusion proteins and then purified using glutathione sepharose 4B. Three-recombinant proteins, GST-egE1 (169–201), -egE1 (169–235) and -egE1 (480–515) reacted

with antibodies to EHV-1, but the reaction of proteins containing egE1 (169–201) was stronger than that of GST-egE1 (480–515) (Fig. 4. 2B). These immunogenic regions did not cross-react with the serum from EHV-4-infected horse (data not shown).

4.4.2. Analysis of the homologous region of EHV-4

For further analysis of the type-specificity of gE1 (169–201), EHV-4 gE (169–199), which is homologous to EHV-1 gE (169–201), was expressed and purified as a fusion protein with GST, GST-egE4 (169–199). Amino acid sequences of EHV-1 gE (169–201) and EHV-4 gE (169–199) are shown in Fig. 4. 2A. Type-specificity of GST-egE1 (169–201) and GST-egE4 (169–199) were compared by immunoblot analysis (Fig. 4. 2C) and ELISA (Fig. 4. 3). The result showed that GST-egE1 (169–201) reacted only with antibodies to EHV-1, but not with those to EHV-1 ΔgE and EHV-4 (Figs. 4. 2C and 4. 3). In contrast, GST-egE4 (169–199) reacted only with those to EHV-4, but did not show any reaction with antibodies to EHV-1 and EHV-1 ΔgE (Figs. 4. 2C and 4. 3).

4.4.3. Identification of the peptide containing a major epitope in EHV-1 gE

To identify the major antigenic region in gE1 (169–201), three 20-mer peptides, gE1 (169–188), gE1 (176–195) and gE1 (182–201) (Fig. 4. 4A) were synthesized and examined by ELISA using sera collected from EHV-1-infected foals. ELISA using the peptide gE1 (169–188) detected

antibodies to EHV-1 (Fig. 4. 4B). Although peptide gE1 (176–195) also detected EHV-1 antibodies, the reactivity was less than that of gE1 (169–188). The peptide gE1 (182–201) did not react with EHV-1 antibodies. Hence, the peptide gE1 (169–188) was used for further analysis.

4.4.4. Establishment of ELISA using synthetic peptides

To confirm the reactivity of the synthetic peptides, an ELISA using the synthetic peptides, gE1 (169–188) and gG4 (319–330), previously reported as the antigenic and specific epitopes in EHV-4 gG (Maeda et al., 2004), was established (Fig. 4. 5). Sera collected from three foals that were inoculated twice each with EHV-1 ΔgE (No. 1–No. 3) followed by a EHV-1 89c25p challenge, and other three foals challenged with EHV-4 TH20 were examined. The peptide gE1 (169–188) detected antibodies induced after EHV-1 infection in all foals, but did not react with antibodies induced by EHV-1 ΔgE and EHV-4. Peptide gG4 (319–330) did not show any cross-reactivity with antibodies induced by EHV-1 and EHV-1 ΔgE.

4.4.5. Application of the ELISA for diagnosis in the field

To confirm whether ELISA using synthetic peptides gE1 (169–188) and gG4 (319–330) could be useful for serological diagnosis of EHV-1 and EHV-4 infections in the field, paired sera collected from 44 racehorses with pyrexia and diagnosed to be infected with EHV-1 by CF test and

gG-ELISA (Yasunaga et al., 1998) were examined. Sera were diluted to 1:100 or 1:10 and significant increases of over 0.5 in absorbance were tentatively judged as seroconversion. As shown in Table 4.2, antibodies to peptide gE1 (169–188) increased between acute and convalescent phases in all foals. Three foals showed increased antibodies to peptide gG4 (319–330).

4.5. Discussion

In this study, we identified a major antigenic region in EHV-1 gE and established an ELISA using synthetic peptides, EHV-1 gE (169–188) and EHV-4 gG (319–330), to distinguish between EHV-1 infection and EHV-1 ΔgE vaccination, and between EHV-1 and EHV-4 infections. Crabb and Studdert (1993) and Crabb et al. (1995) reported that type-specific regions are located in gG, and developed an ELISA in which the recombinant proteins of gG were used as ELISA antigens. In previous studies, we modified this ELISA using Japanese isolates of EHV-1 and -4, and found that this method could be useful for the sero-epizootiology and diagnosis of EHVs (Maeda et al., 2004, 2005; Mizukoshi et al., 2002; Yasunaga et al., 1998, 2000). Currently, EHV-1 ΔgE is in ongoing development for introduction in the field as a new vaccine in Japan, but this ELISA using a type-specific region of EHV- 1 gG may not be able to differentiate between natural infection and vaccination with EHV-1 ΔgE due to fact that the modified live vaccine, EHV-1 ΔgE, contains the intact EHV-1 gG gene. Therefore, a new serological method to differentiate between natural infection and vaccination with EHV-1 ΔgE is required. In this study, it was revealed that the region of EHV-1 gE, a.a. 169–201, was highly antigenic (Figs. 4. 1 and 4. 2B). Immunoblot analysis and ELISA using GST-fusion proteins, GST-egE1 (169–201) and GST-egE4 (169–199), showed that these regions of gE were type-specific (Figs. 4. 2C and 4. 3). However, the reactivity of GST-egE4 (169–199) was weaker in some horses (data not shown). Therefore, the synthetic peptide gG4 (319–

330) was chosen as the target antigen for detection of EHV-4 specific antibodies. A major antigenic region in gE1 (169–201) was identified by three synthetic peptides (Fig. 4. 4). The results show that the peptide gE1 (169–188) was the most antigenic and a candidate ELISA antigen. Birch-Machin et al. (2000) reported that a short amino acid sequence, homologous to an EHV-1 gE fragment, had a potential antibody binding region as determined using phage random peptide display libraries, and showed that gE1 (175–191) was a type specific region of EHV-1 gE. Previously, in an ELISA using synthetic peptide fragments, gE1 (175–191) specifically reacted with antibodies to EHV-1 but not EHV-4 (Birch-Machin et al., 2000), the results of which were similar to ours. ELISA using the synthetic peptides gE1 (169–188) and gG4 (319–330) did not detect any antibodies induced by inoculation with EHV-1 ΔgE (Fig. 4. 5), revealing that our established ELISA could distinguish not only between EHV-1 and EHV-4, but also between EHV-1 and EHV-1 ΔgE infections. In serological diagnosis of EHV-infections in the field, the peptide gE1 (169–188) could detect antibodies to EHV-1 in all 44 horses with pyrexia, while the peptide gG4 (319–330) detected an increase of antibodies in three horses (Table 4. 2), suggesting that ELISA using synthetic peptides was specific and sensitive for detecting antibodies in the field. In conclusion, we have developed an ELISA using easily prepared antigens based on synthetic peptides that could be useful to differentiate between EHV-1 and EHV-4 infections, and between EHV-1 infection and vaccination with EHV-1 ΔgE.

4.6. Figure legends

Fig. 4. 1. (A) Schematic of EHV-1 gE segments expressed as GST-fusion proteins. Black boxes indicate immunogenic regions. Reactivity shows the result of immunoblot analysis using sera from horses experimentally infected with EHV-1 (+; positive, -; negative). (B, C) Identification of the antigenic region in gE (a.a. 136-272) by immunoblot analysis using sera from horses experimentally infected with EHV-1. Various gE fragments deleted from the C-terminal of egE1 (1-272) (B) or N-terminal of egE1 (136-407) (C) were expressed as GST-fusion proteins. (D) Identification of the antigenic region in gE (a.a. 408-551) by immunoblot analysis. Various gE fragments deleted from the C-terminal of egE1 (273-551) were expressed. All expressed proteins in this figure were unpurified.

Fig. 4. 2. (A) Comparison of amino acid sequences between egE1 (169-201) and egE4 (169-199). (B) Confirmation of immunogenic regions of EHV-1 gE by immunoblot analysis using sera from EHV-1-infected horses. (C) Comparison of reactivities between egE1 (169-201) and egE4 (169-199) by immunoblot analysis using sera from EHV-1-infected horses. All fusion proteins were purified using glutathione sepharose 4B.

Fig. 4. 3. ELISA using purified GST-fusion proteins, GST-egE1 (169-201) (filled triangles) and GST-egE4 (169-199) (open triangles). Primary antibodies were serum samples collected from foals experimentally infected with EHV-1 and EHV-1 ΔgE (A-1, A-2 and A-3) or EHV-4 (#4-#6). Sera were diluted 1:100 and reactivities are shown as the absorbance at 405nm. Black and white arrows show inoculation of EHV-1 ΔgE and EHV-1, respectively.

Fig. 4. 4. (A) amino acid sequences of synthetic peptides. (B) ELISA using synthetic peptides of EHV-1 gE. Sera from horses experimentally infected with EHV-1 were diluted 1:10 and reactivities are shown as the absorbance at 405nm.

Fig. 4. 5. ELISA using synthetic peptides, gE1 (169-188) (filled squares) and gG4 (319-330) (open squares). Primary antibodies were serum samples collected from foals experimentally infected with EHV-1 and EHV-1 ΔgE (A-1, A-2 and A-3) or EHV-4 (#4-#6). Sera were diluted 1:100 and reactivities are shown as the absorbance at 405nm. Black and white arrows show inoculation of EHV-1 ΔgE and EHV-1, respectively.

Table 4. 1 Primers used in this experiment for PCR.

Primers			Sequence	Positions[a]
EHV-1	Forward	1F	5'-gcgaattcatggagctgttggccgcaag-3'	134406-134425
		136F	5'-gcgaattcctacaaatccatagggcaac-3'	134811-134830
		169F	5'-gcgaattcagaagccgcccaaacaacc-3'	134911-134929
		202F	5'-gcgaattcgcacggataccactcgcg-3'	135011-135028
		236F	5'-gcgaattccagtggtactatatgaatacatc-3'	135111-135133
		273F	5'-gcgaattctgcagcttcacatcccccat-3'	135222-135241
		408F	5'-gcgaattctttttgctggttattatctgca-3	135627-135648
		480F	5'-gcgaattcgacgaagagttggaataccc-3'	135843-135862
	Reverse	135R	5'-atgtcgactcagacgctgcgggtttcggcaa-3'	134810-134791
		168R	5'-atgtcgactcatgacggtgagcagcacgac-3'	134910-134892
		201R	5'-atgtcgactcaaccacaaaatccgtgtgggt-3'	135010-134991
		235R	5'-atgtcgactcaaatctccgcgctgaagctg-3'	135110-135092
		272R	5'-atgtcgactcaggtgtgttgttccgggtgca-3'	135223-135202
		407R	5'-atgtcgactcaggcgaggtagtgtcgagtcc-3'	135627-135607
		443R	5'-atgtcgactcagttcagcacttcatacggct-3'	135734-135715
		479R	5'-atgtcgactcatgaatcagagtcgaaggagtc-3'	135842-135822
		515R	5'-atgtcgactcaaaccttgaatccggacctgc-3'	135952-135931
		551R	5'-atgtcgactcattttagtatcgactttagct-3'	136058-136036
EHV-4	Forward	169F	5'-gcgaattcaagaaaccaccaacactacca-3'	133097-133117
	Reverse	199R	5'-atgtcgactcatatgaaatctgtatgtgcctct-3'	133189-133168

[a] Position on the genome of EHV-1 Ab4 strain (Accession No. AY665713) and EHV-4 NS80567 strain (Accession No. AF030027) are shown.

Table 4. 2. ELISA using sera from horses with pyrexia induced by EHV-1 infection.

Horse No.	egE1-ELISA				egG4-ELISA			
	1:10[b]		1:100[c]		1:10[b]		1:100[c]	
	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent
126	0.36	>3.50[a]	-0.02	0.36	0.05	0.00	0.01	0.00
156	-0.08	0.93[a]	0.10	0.07	0.04	0.04	0.18	0.06
165	0.19	>3.50[a]	0.35	1.37[a]	-0.13	-0.11	0.05	-0.01
211	0.55	2.00[a]	-0.12	0.11	0.23	0.05	0.01	0.01
227	0.26	2.22[a]	0.07	0.47	0.21	0.10	0.01	0.01
236	-0.22	>3.50[a]	-0.15	0.19	-0.01	0.21	0.01	0.06
23	0.10	1.14[a]	-0.02	2.22[a]	0.19	0.20	-0.01	-0.01
29	0.79	>3.50[a]	-0.17	0.00	1.31	1.06	0.01	0.01
31	1.56	>3.50[a]	-0.24	0.20	-0.03	0.01	-0.01	-0.02
48	1.18	2.37[a]	-0.10	-0.09	0.53	0.47	0.02	0.02
50	0.70	2.97[a]	-0.20	0.11	-0.07	-0.04	-0.01	-0.01
59	1.69	2.33[a]	0.77	>3.50[a]	0.08	0.14	0.00	-0.01

Horse No.	egE1-ELISA				egG4-ELISA			
	1:10[b]		1:100[c]		1:10[b]		1:100[c]	
	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent
125	1.16	2.24[a]	-0.21	-0.04	-0.03	0.16	0.04	0.01
129	1.58	>3.50[a]	-0.40	0.06	0.38	0.52	-0.05	-0.14
130	0.82	>3.50[a]	-0.24	-0.26	-0.09	0.00	0.01	-0.05
136	2.25	>3.50[a]	-0.15	0.17	0.22	0.08	0.24	0.15
140	0.12	2.13[a]	-0.08	0.02	0.24	0.27	0.17	0.01
146	-0.04	>3.50[a]	-0.20	0.07	0.50	0.40	0.01	0.02
150	-0.08	>3.50[a]	-0.31	0.00	0.00	0.04	-0.03	-0.01
151	0.19	>3.50[a]	-0.13	0.92[a]	0.26	0.27	0.00	-0.01
152	0.24	>3.50[a]	0.02	2.49[a]	0.01	0.02	-0.01	-0.01
155	0.38	>3.50[a]	-0.02	0.82[a]	0.18	0.15	-0.02	-0.02
158	-0.17	2.10[a]	-0.04	0.60[a]	0.78	0.81	0.04	0.04
159	1.14	2.03[a]	0.14	0.33	0.03	0.06	-0.01	0.00

Horse No.	egE1-ELISA				egG4-ELISA			
	1:10[b]		1:100[c]		1:10[b]		1:100[c]	
	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent
164	0.51	>3.50[a]	0.01	2.88[a]	1.01	0.76	0.04	0.03
166	0.33	2.56[a]	0.04	0.66[a]	1.54	1.07	0.11	0.09
178	1.14	2.36[a]	0.20	1.65[a]	0.62	0.43	0.01	0.01
179	-0.26	>3.50[a]	-0.10	0.77[a]	0.60	0.51	0.04	0.04
180	-0.02	0.59[a]	-0.02	0.08	>3.50	>3.50	0.59	1.72[a]
183	0.81	>3.50[a]	0.12	2.52[a]	0.41	0.43	0.04	0.03
185	0.02	>3.50[a]	-0.02	0.75[a]	0.25	0.13	-0.03	-0.01
187	0.10	2.43[a]	0.04	0.45	0.24	0.11	-0.01	-0.02
189	0.76	>3.50[a]	0.08	0.86[a]	>3.50	>3.50	1.40	1.13
194	0.29	2.25[a]	-0.02	0.46	0.44	0.48	0.01	0.01
196	0.40	1.02[a]	0.04	0.15	0.52	0.41	0.06	0.03
198	0.28	>3.50[a]	0.00	0.70[a]	1.86	1.47	0.06	0.04

Horse No.	egE1-ELISA				egG4-ELISA			
	1:10[b]		1:100[c]		1:10[b]		1:100[c]	
	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent
202	0.04	>3.50[a]	0.00	1.99[a]	0.22	0.77[a]	-0.02	0.06
209	0.73	>3.50[a]	0.13	1.44[a]	0.02	-0.01	-0.01	-0.01
217	0.42	>3.50[a]	0.05	0.74[a]	1.05	1.02	0.07	0.07
220	0.51	>3.50[a]	0.04	0.86[a]	0.30	1.13[a]	0.02	0.06
221	2.49	>3.50[a]	2.88	>3.50[a]	0.12	0.02	-0.04	-0.03
225	0.72	>3.50[a]	0.05	1.96[a]	0.28	0.30	-0.03	-0.01
226	0.19	>3.50[a]	0.04	1.94[a]	0.58	0.62	0.06	0.06
234	0.18	>3.50[a]	-0.02	0.76[a]	-0.20	-0.14	-0.06	-0.05

[a] Increase of over 0.5 in absorbance was tentatively judged as sero-conversion

[b] Horse sera were diluted to 1:10 and then added to each wells.

[c] Horse sera were diluted to 1:100 and then added to each wells.

Fig. 4. 1

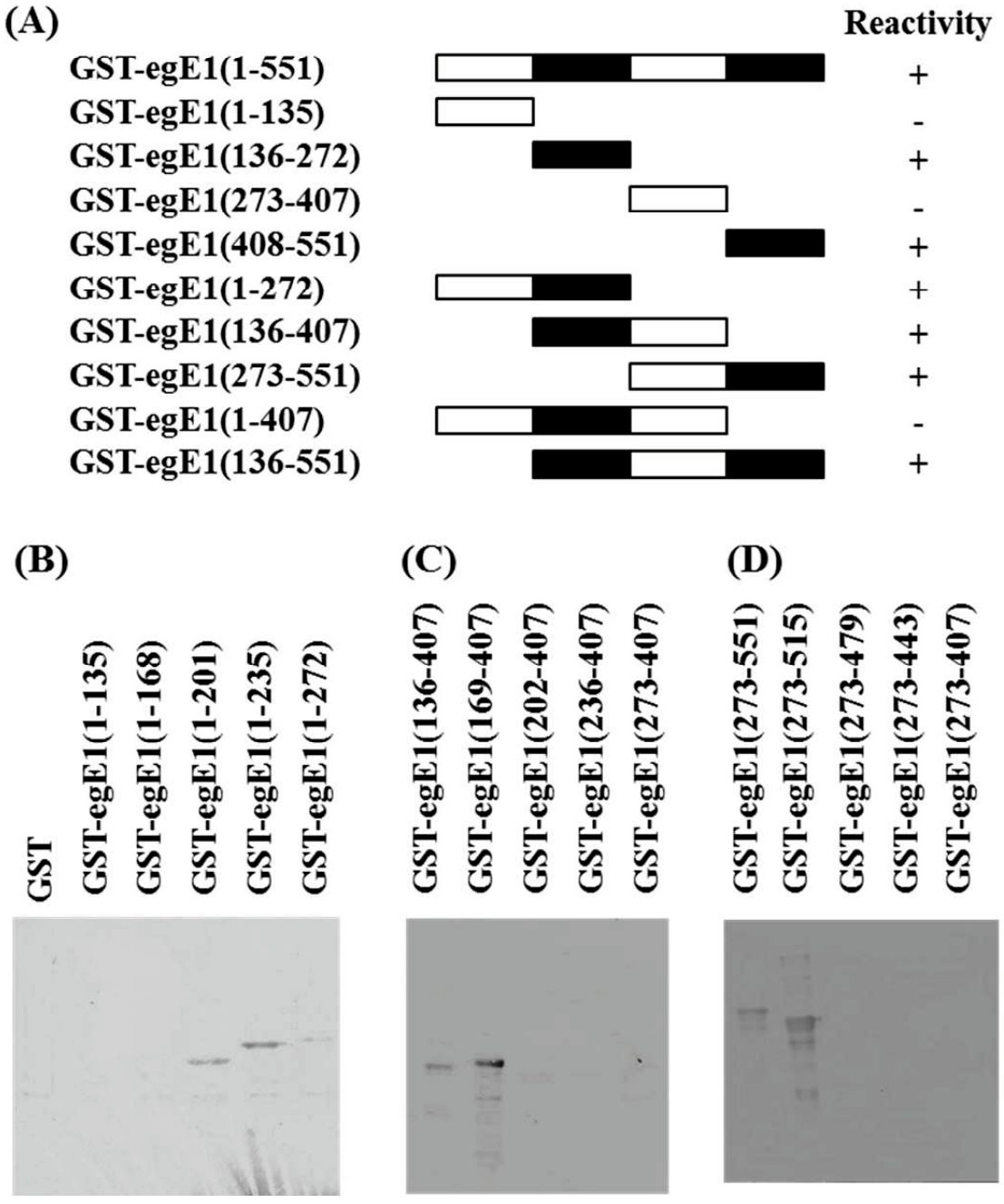

(A)
Reactivity
GST-egE1(1-551)
GST-egE1(1-135)
GST-egE1(136-272)
GST-egE1(273-407)
GST-egE1(408-551)
GST-egE1(1-272)
GST-egE1(136-407)
GST-egE1(273-551)
GST-egE1(1-407)
GST-egE1(136-551)
+
-
+
-
+
+
+
+
-
+
(B)
GST
GST-egE1(1-135)
GST-egE1(1-168)
GST-egE1(1-201)
GST-egE1(1-235)
GST-egE1(1-272)
(C)
GST-egE1(136-407)
GST-egE1(169-407)
GST-egE1(202-407)
GST-egE1(236-407)
GST-egE1(273-407)
(D)
GST-egE1(273-551)
GST-egE1(273-515)
GST-egE1(273-479)
GST-egE1(273-443)
GST-egE1(273-407)

Fig. 4. 2.

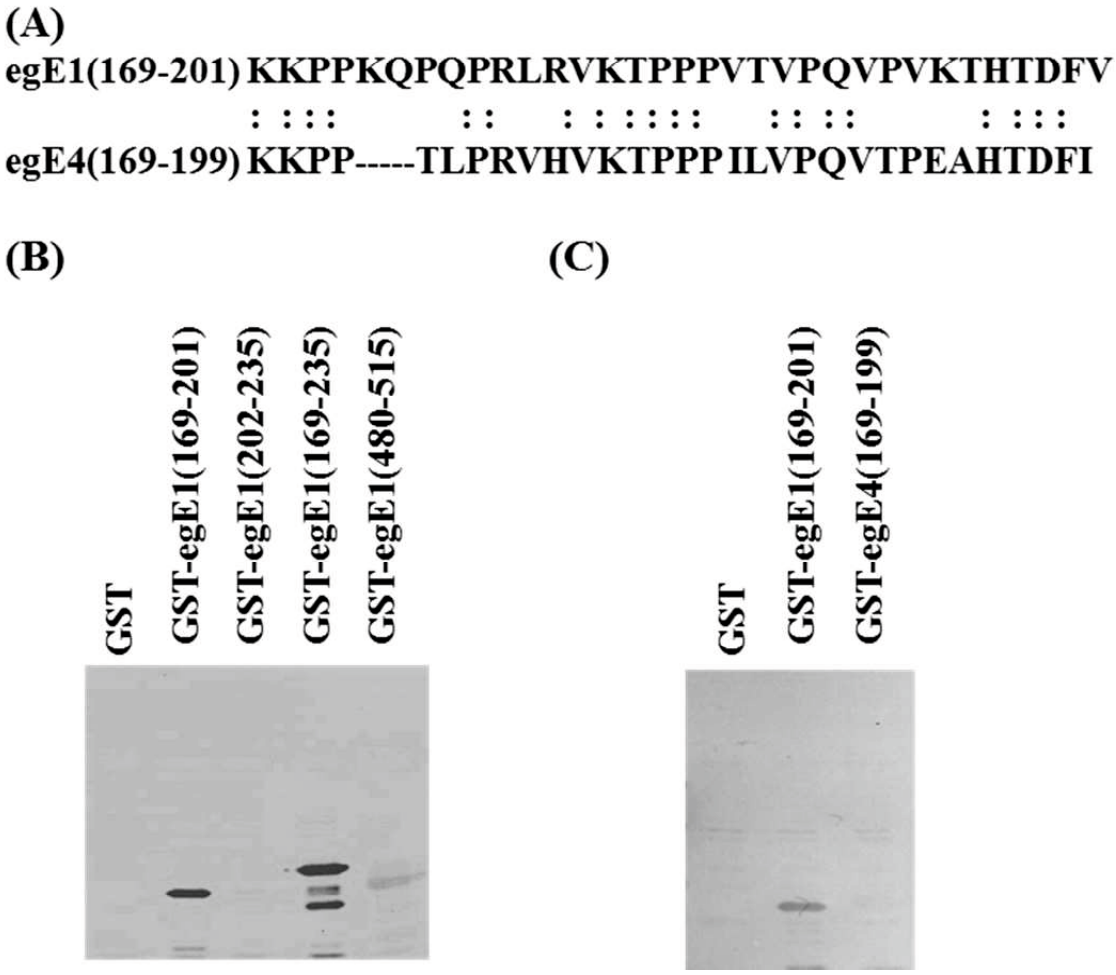

(A)
egE1(169-201) KKPPKQPQPRLRVKTPPPVTVPQVPVKTHTDFV
egE4(169-199) KKPP-----TLPRVHVKTPPP ILVPQVTPEAHTDFI
(B)
GST
GST-egE1(169-201)
GST-egE1(202-235)
GST-egE1(169-235)
GST-egE1(480-515)
(C)
GST
GST-egE1(169-201)
GST-egE4(169-199)

Fig. 4. 3.

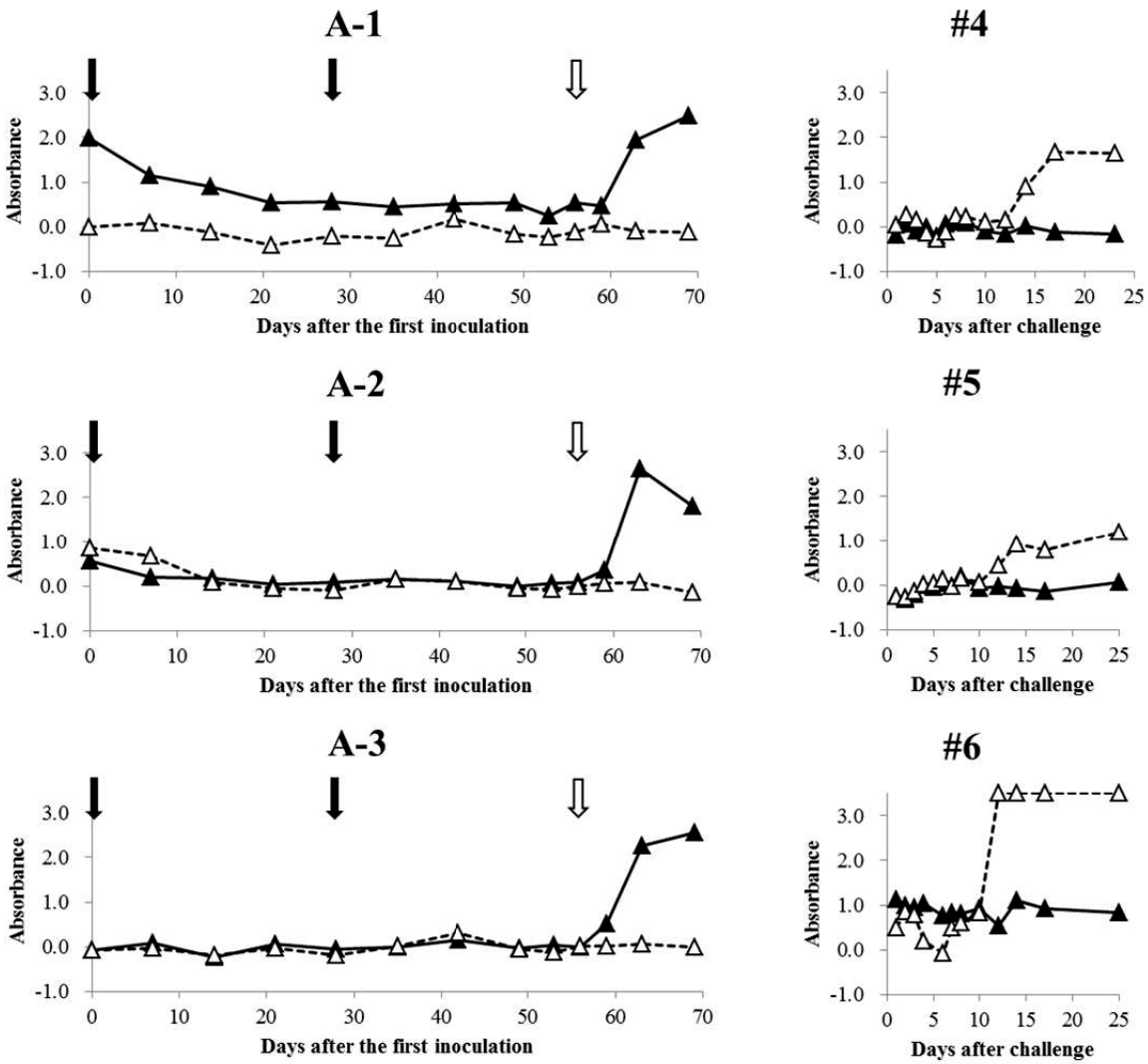

A-1
#4
A-2
#5
A-3
#6
Absorbance
Days after the first inoculation
Days after challenge

Fig. 4. 4.

(A)

egE1(169-188) KKPPKQPQPRLRVKTPPPVT
egE1(176-195) QPRLRVKTPPPVTVPQVPVK
egE1(182-201) KTPPPVTVPQVPVKTHTDFV

egG4(319-330) MKNNPVYSESL

(B)

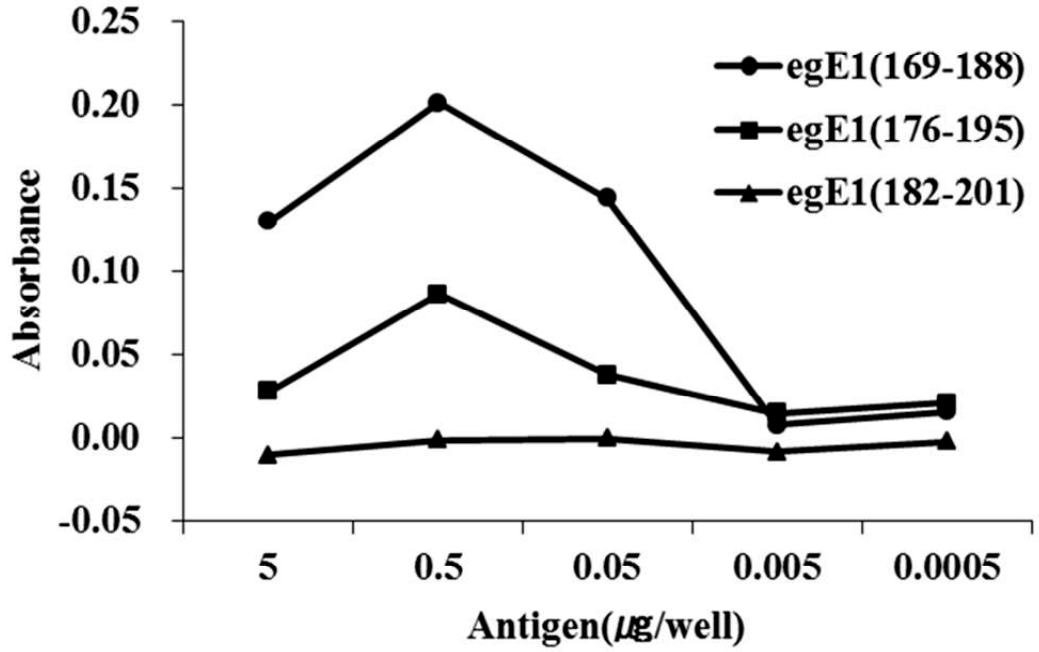
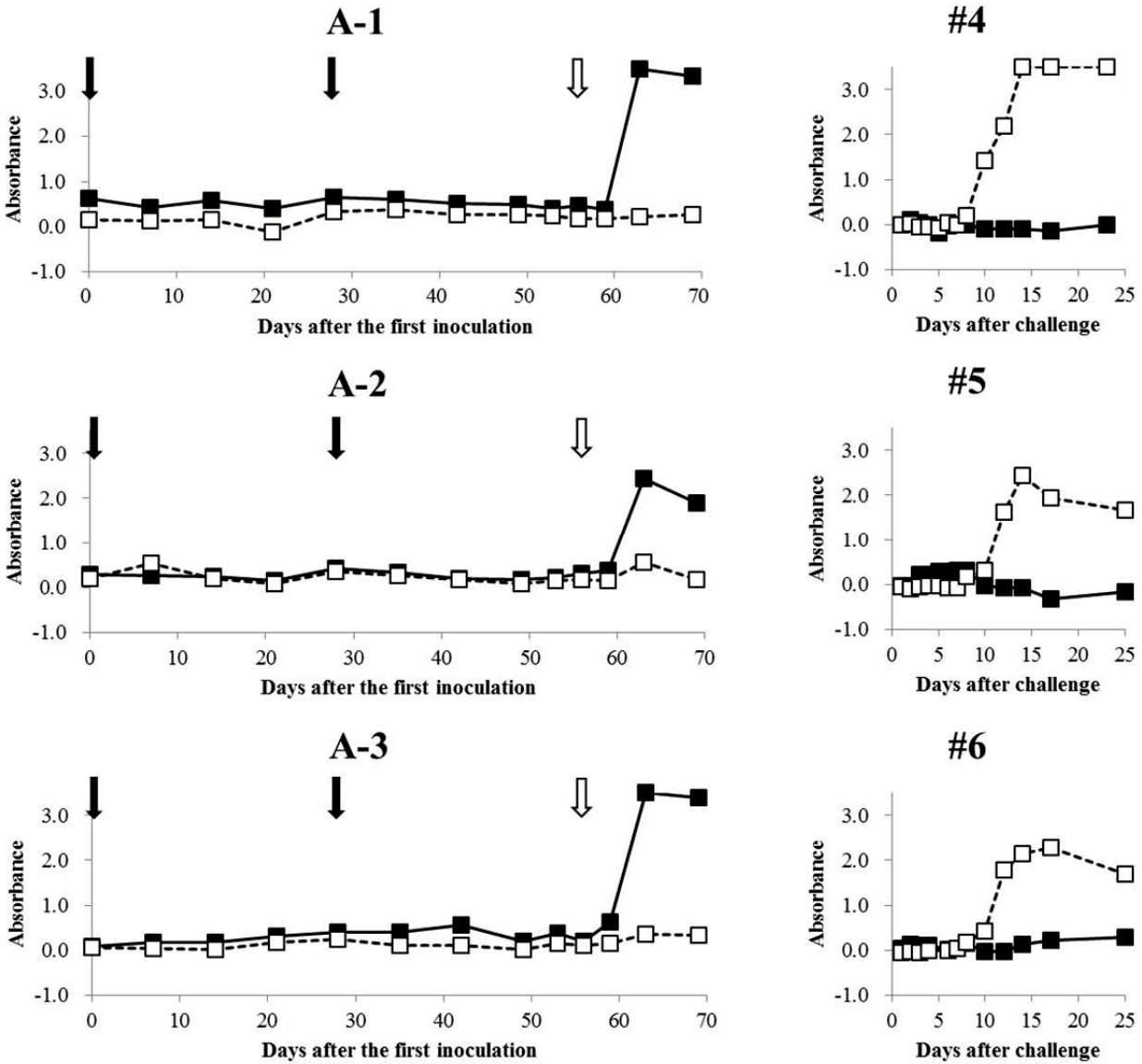


Fig. 4. 5.


A-1
#4
A-2
#5
A-3
#6
Absorbance
Days after the first inoculation
Days after challenge

Chapter 5

General conclusion

In my PhD course, the new equine cell line was established and the haemagglutinin of EHV-1 was identified. In addition, EHV-1 gE specific epitope peptide was identified, resulting in development of the new ELISA using these epitope peptides.

Chapter 2: Establishment of equine cell line "FHK-Tcl3.1"

In Chapter 2, we established new equine derived cell line. As cell line derived from horse were very rare, it was so difficult to isolation, propagation and research of EHV-4. Therefore, comparative analysis between EHV-1 and -4 was difficult to perform. To resolve the problem, we established new cell line, named FHK-Tcl3.1, which was derived from fetal horse kidney and immortalized by expression of SV40 large T antigen. FHK-Tcl3.1 cells could propagate EHV-1, -2 and -4 with clear CPE. Furthermore, another group also reported that FHK-Tcl3.1 cells could propagate many equine viruses (Oguma et al., 2013). These results indicated that FHK-Tcl3.1 cells are useful for the research on EHVs.

Chapter 3: The haemagglutination activity of equine herpesvirus type 1 glycoprotein C

In Chapter 3, we determined the haemagglutinin of EHV-1 using expression plasmids encoding EHV-1 glycoproteins. HA test using 293T cells transfected with the expression plasmids encoding EHV-1 gp2, gB, gC, gD, gE, gG, gI, gM and gN was performed and only 293T cells

expressing gC adsorbed and agglutinated equine RBC. In addition, the HA activity was inhibited by MAbs against EHV-1 gC, suggesting that gC is certainly responsible for the HA activity. Furthermore, the HA activity was not inhibited by addition of heparin, indicating that the EHV-1 gC has different character from gCs of other herpesviruses. Therefore, it is hypothesized that HA activity of EHV-1 might be related to EHV-1-specific pathogenesis.

Chapter 4: Identification of a major immunogenic region of equine herpesvirus type 1 glycoprotein E and its application to enzyme-linked immunosorbent assay

In Chapter 4, we identified EHV-1 gE specific epitope to develop a new diagnosis method for diagnosis of EHVs infection. Various fragments of EHV-1 gE were expressed as fusion proteins with GST in *E. coli* and their antigenicities were compared by immunoblot analysis. gE1 (169–188) was finally identified as the EHV-1-specific epitope. The new ELISA using synthetic peptides is a simple method to distinguish between EHV-1 and EHV-4 infections and could use for the diagnosis in the field. The new ELISA will be suitable as a vaccine marker after introduction of EHV-1 ΔgE into field horses.

Conclusion

In this study, there are some novel findings.

1. The novel cell line derived from horse was established.

2. EHV-1 gC is responsible for the HA activity and the HA activity is not inhibited by heparin.

3. EHV-1 gE specific epitope is located in amino acid position 169 -188 (gE1 (169-188)).

4. New ELISA using epitope peptide gE1 (169-188) is useful for the differentiation between EHV-1 infection and vaccine inoculation.

These findings must be available for the research of EHVs and development of effective vaccine.

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